Universidade de Aveiro Departamento de Biologia
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Artur Jorge da Costa Peixoto Alves

Espécies de *Botryosphaeria* em *Quercus* spp. e outros hospedeiros lenhosos: taxonomia e filogenia

Botryosphaeria species on *Quercus* spp. and other woody hosts: taxonomy and phylogeny

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António Carlos Matias Correia, Professor Associado do Departamento de Biologia da Universidade de Aveiro e do Doutor Alan John Lander Phillips, Investigador Principal da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa.

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Ao meu Pai.
Serás sempre para mim motivo de orgulho e fonte de inspiração.

o júri

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Prof. Dr. Manuel António da Silva Santos
professor auxiliar do Departamento de Biologia da Universidade de Aveiro

Prof. Dr. António Carlos Matias Correia
professor associado do Departamento de Biologia da Universidade de Aveiro

Prof. Dr. Alan John Lander Phillips
investigador principal da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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palavras-chave

Botryosphaeria, *Botryosphaeriaceae*, teleomorfos, anamorfos, taxonomia, sistemática, filogenia

resumo

O género *Botryosphaeria* é bem conhecido devido ás espécies que causam doenças numa grande diversidade de plantas. A taxonomia deste género e seus géneros anamórficos, assim como das espécies tem sido algo confusa. Neste trabalho foram caracterizadas espécies de *Botryosphaeria* associadas a *Quercus* spp. e outros hospedeiros lenhosos, com base em características morfológicas e sequências nucleotídicas de um ou vários genes. A utilização desta abordagem permitiu a clarificação da taxonomia de algumas espécies já descritas, bem como a identificação e descrição de novos taxa.

O género *Dothiorella* foi reintroduzido para acomodar os anamorfos de *Botryosphaeria* que possuem conídios castanhos e 1-septados. Esta decisão baseou-se no estudo das características morfológicas da espécie tipo do género bem como em análises filogenéticas. A análise filogenética de sequências nucleotídicas de vários genes confirmou que os géneros *Diplodia* e *Lasiodiplodia* representam taxa distintos. Demonstrou-se que as estrias longitudinais em conídios não têm valor taxonómico para distinguir entre os dois géneros. O género *Lasiodiplodia* distingue-se do género *Diplodia* pela presença de paráfises.

Os métodos baseados em PCR, ARDRA, MSP-PCR e rep-PCR foram utilizados para discriminar entre espécies do género *Botryosphaeria* e espécies anamórficas relacionadas. O método ARDRA permitiu a diferenciação de 10 espécies, enquanto os métodos MSP-PCR e rep-PCR permitiram a identificação de 24 espécies. Estes métodos representam procedimentos simples e rápidos que podem ser utilizados para a identificação rotineira de isolados de *Botryosphaeria* ao nível da espécie. Os métodos MSP-PCR e rep-PCR também se revelaram úteis para estudar a variabilidade intraespecífica.

Foi realizada uma análise sistemática molecular da família *Botryosphaeriaceae* recorrendo a sequências nucleotídicas de diversos genes. Esta análise mostrou que o género *Botryosphaeria sensu lato* é parafilético, sendo composto por várias linhagens filogenéticas. Estas linhagens representam géneros distintos que são caracterizados essencialmente com base na morfologia das formas anamórficas. O género *Melanops* foi reintroduzido para acomodar uma espécie anteriormente classificada no género *Botryosphaeria*. Não foi possível definir qual a posição da família *Botryosphaeriaceae* na classificação dos Ascomicetos. De acordo com os dados disponíveis prevê-se que esta família deverá ser incluída numa nova ordem distinta das ordens *Pleosporales* e *Dothideales*.

keywords

Botryosphaeria, *Botryosphaeriaceae*, teleomorphs, anamorphs, taxonomy, systematics, phylogeny

abstract

The genus *Botryosphaeria* is well known by the species that cause disease in a wide range of plant hosts. The taxonomy of *Botryosphaeria* and associated anamorph genera as well as of the species has been confused in the past. In this work, *Botryosphaeria* species occurring on *Quercus* spp. and other woody hosts were characterised on the basis of morphology and nucleotide sequence data from single or multiple genes. Using this approach it was possible to clarify taxonomic uncertainties related to some known species and also to identify and describe new species.

Through a study of the type species of *Dothiorella* this genus was reinstated to accommodate anamorphs of *Botryosphaeria* with brown, one-septate conidia. This was further supported by phylogenetic analyses. Phylogenetic analyses of combined multi-gene sequence data supported the genera *Diplodia* and *Lasiodiplodia* as distinct. It was shown that conidial striations are not a taxonomically meaningful character to separate both genera, and that *Lasiodiplodia* is distinguished from *Diplodia* by the presence of paraphyses.

The PCR-based methods ARDRA, MSP-PCR and rep-PCR were used to discriminate between species of the genus *Botryosphaeria* as well as associated anamorphic species. The ARDRA procedure allowed for the differentiation of 10 species while MSP-PCR and rep-PCR allowed a clear identification of 24 species. These methods provide simple and rapid procedures that can be used for routine identification of *Botryosphaeria* isolates at the species level. MSP-PCR and rep-PCR also proved useful to study intraspecific variability.

A molecular systematic analysis of the family *Botryosphaeriaceae* was performed using multi-gene sequence data. The genus *Botryosphaeria sensu lato* was shown to be paraphyletic, and composed of different phylogenetic lineages. These represent distinct genera which are characterised essentially on the basis of morphological features of the anamorphs. The genus *Melanops* was reinstated to accommodate a species previously included in *Botryosphaeria*. The position of the *Botryosphaeriaceae* within the higher classification of Ascomycetes could not be indubitably established. Given the current data it is envisaged that this family should be included in a new order distinct from the *Pleosporales* and *Dothideales*.

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CHAPTER

1

Introduction

The genus *Botryosphaeria*

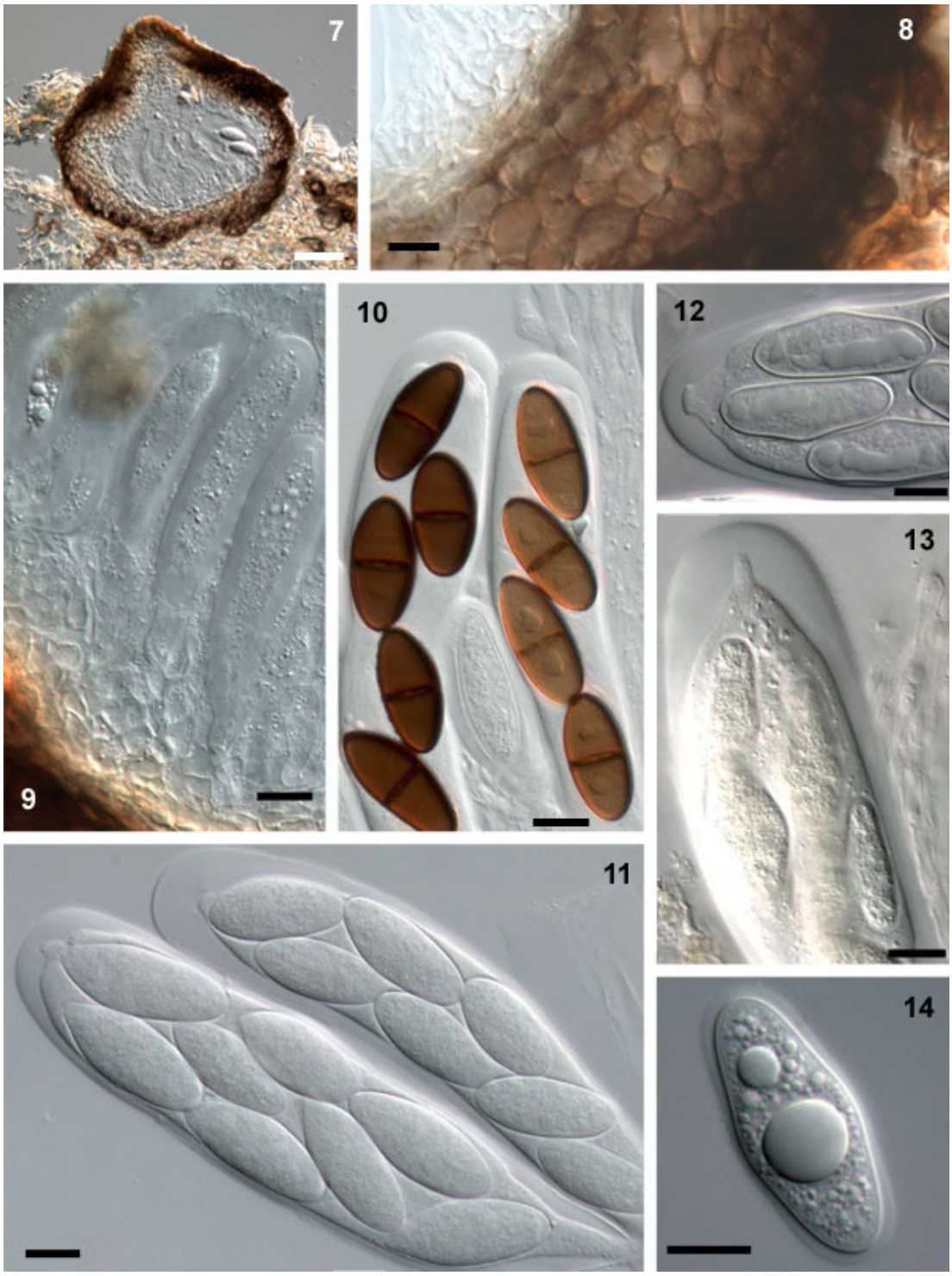
Taxonomic history and characteristics of the genus

The genus *Botryosphaeria* Ces. & De Not. was introduced by Cesati and De Notaris in 1863, who based the description on characters of the teleomorph and anamorph, and included species transferred from *Sphaeria* and *Gibbera*. Saccardo (1877) emended the genus concept of Cesati and De Notaris (1863) to exclude the hypocreaceous species, which he transferred to *Gibberella* and *Lisea*. This concept of the genus has been further cited in several works (von Arx and Müller, 1954, 1975; Barr, 1972; Sivanesan, 1984; Hanlin, 1990; Denman *et al*., 2000).

Taking into account the various versions, the genus can be described as follows: "Ascomata scattered, solitary, botryose to aggregated, stromatic, black, uniloculate or multiloculate, with a thick pseudoparenchymatic wall composed of textura angularis or textura globosa with the outer layer blackened and its cells more thickened, ostiolate, embedded in the substrate and partially erumpent at maturity. Asci clavate or cylindric-clavate, stalked or sessile, bitunicate, ectotunica thin, endotunica rather thick, 3-layered (sensu Eriksson, 1981), with a prominent apical chamber, 8-spored, developing on a broad basal hymenial layer. Ascospores irregularly biseriate, hyaline, thin-walled, ovoid, fusoid, fusoid-ellipsoid, usually widest in the middle, often inequilateral, smooth, one-celled sometimes 1–2-septate, contents granular, guttulate. Ascospores may become brown with age and may possess evanescent hyaline appendages or a thin gelatinous coat. Pseudoparaphyses thin-walled, hyaline, frequently septate, often constricted at the septa, usually disintegrating when the asci mature."

Since Cesati and De Notaris (1863) did not designate a type species, Von Höhnel (1909) suggested *B. berengeriana* De Not. as the type, while Theissen and Sydow (1915) suggested *B. quercuum* (Schwein.) Sacc. The latter species was accepted by von Arx and Müller (1954, 1975) and von Arx (1981). However, neither species was included in the original description of the genus and therefore both are unsuitable as the type. Barr (1972) proposed *B. dothidea* (Moug. : Fr.) Ces. & De Not. as lectotype because it was one of the original species described and conforms with Saccardo's (1877) emendation. Barr's (1972) proposal has been generally accepted (e.g. Slippers *et al*., 2004a).

Figures 1–6. Ascomata of *Botryosphaeria* species erumpent through the host bark (left) and cut through horizontally (right). 1, 2. *B. dothidea*. 3, 4. *B. corticola*. 5, 6. *B. melanops*. Scale bars = 500 µm.



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Closely related genera

A number of closely related genera included in the family *Botryosphaeriaceae* are differentiated from *Botryosphaeria* based on morphological features (Barr, 1987). For example, the ascomata of *Auerswaldia* Sacc., *Auerswaldiella* Theiss. & Syd., and *Homostegia* Fuckel are borne in a pulvinate stroma. The ascospores are multiseptate in *Homostegia*, aseptate and brown in *Auerswaldia* and hyaline and aseptate in *Auerswaldiella* (Barr, 1987).

The genus *Sivanesania* W.H. Hsieh & C.Y. Chen also has pulvinate ascostromata, but is readily distinguished by the unique ascospores that have a cellular, filiform, basal, hyaline, simple appendage (Hsieh and Chen, 1994). As in *Botryosphaeria* the hyaline and aseptate ascospores often turn brown with age (Hsieh and Chen, 1994).

Barr (1987, 1989) separated species of *Dothidotthia* Höhn. from *Botryosphaeria* by their ascospores, which are various shades of brown and 1–3-septate in *Dothidotthia*. However, the distinction between the two genera is tenuous due to the recent description of *Botryosphaeria* species with brown 1-septate ascospores (Chapter 3; Luque *et al*., 2005), and it seems possible that *Dothidotthia* could be a later synonym of *Botryosphaeria*. On the other hand, it is possible that *Botryosphaeria* is paraphyletic and comprises a number of separate genera (Chapter 3; Luque *et al*., 2005). The relationship between *Dothidotthia* and *Botryosphaeria* is further expanded in Chapter 10.

Neodeightonia Booth was used by Barr *et al*. (1986) for *Dothidotthia*. However, *Neodeightonia* had been reduced to synonymy with *Botryosphaeria* by von Arx and Müller (1975). This synonymy was later supported by Barr (1987), although *Neodeightonia* has brown ascospores and an unusual chambered ostiolar neck (Hanlin, 1990).

The genus *Guignardia* Viala & Ravaz was regarded as a synonym of *Botryosphaeria* (Barr, 1972). Later, Barr (1987) placed *Guignardia* in *Discochora* Höhn and separated it from *Botryosphaeria*. Although *Discochora* predates *Guignardia* (Bisset, 1986) this latter name has been conserved. *Guignardia* is easily distinguished from *Botryosphaeria* in having smaller ascospores with persistent mucilaginous caps on the apices, and anamorphs in *Phyllosticta* Pers. (van der Aa, 1973; Sivanesan, 1984, Hanlin, 1990).

Species of *Otthia* Nitschke ex Fuckel have short-stalked, cylindrical, bitunicate asci with a thin endotunica bearing obliquely uniseriate ascospores. Ascospores are hyaline when young but become brown and 1-septate when mature, and slightly constricted at the septum (Dennis, 1981; Sivanesan, 1984). The type species of the genus *Otthia spiraeaea* (Fuckel) Fuckel has been recognized has the teleomorph of *Diplodia*

sarmentorum (Fr.) Fr. (Booth, 1958). Since *Diplodia* species are usually regarded as anamorphs of *Botryosphaeria* Laundon (1973) and Denman *et al*. (2000) expressed doubts about the tenability of *Otthia* and suggested it could be a synonym of *Botryosphaeria*. This possibility was rejected by Eriksson (2000) who states that asci, ascospores and ascomatal walls are different in *Otthia* (this subject is followed up in Chapter 3).

According to Eriksson (2000) *Botryosphaeria* is easily distinguished from other similar genera in their characteristic ascomatal walls of large violet brown cells that turn blackish blue in KOH. Another important feature is the laminated endotunica of the asci (Funk and Shoemaker, 1967; Eriksson, 1981).

Physalospora Niessl is a long-standing name that was misapplied to *Botryosphaeria* species. However, *Physalospora* differs from *Botryosphaeria* in the unitunicate asci and hamathecial tissues composed of paraphyses (Hanlin, 1990) as opposed to the pseudoparaphyses in *Botryosphaeria*. Von Arx and Müller (1954) placed various *Physalospora* species in synonymy with *Botryosphaeria* species and since then several others have been transferred to the genus (Index of Fungi[1]).

Anamorph genera associated with *Botryosphaeria*

Anamorphs of *Botryosphaeria* species have been described in at least 17 Coelomycete form genera, but the most commonly used are *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino, *Macrophomopsis* Petrak, *Sphaeropsis* Sacc. and *Fusicoccum* Corda (Sutton, 1977, 1980; Sivanesan, 1984; Barr, 1987; Denman *et al*., 2000). While *Phyllosticta* was linked to *Botryosphaeria* (Barr, 1972), this genus should be reserved for anamorphs of *Guignardia* (van der Aa, 1973; Sivanesan, 1984; Hanlin, 1990).

The anamorph genera associated with *Botryosphaeria* were not clearly delimited because the morphological features that separate them were poorly defined, or the genera included a heterogeneous assembly of taxa (Crous and Palm, 1999; Denman *et al*., 2000). Considerable progress has been made towards redefining and stabilizing the genera.

Thus, Sutton (1980) examined the type specimen of *Macrophoma* and found it to be a synonym of *Sphaeropsis*. *Macrophomopsis* was separated from *Fusicoccum* on account of its annellidic conidiogenous cells (Sutton, 1980). However, Pennycook and Samuels (1985) showed that this type of conidiogenous cell is also seen in *Fusicoccum*

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

and they considered *Macrophomopsis* to be a synonym of *Fusicoccum*. This was further supported by the observations of Crous and Palm (1999) and more recently by phylogenetic analysis of LSU rDNA sequence data (Crous *et al*., 2006). Crous and Palm (1999) showed that the name *Botryodiplodia* is a *nomen dubium*, since it refers to a valsoid ascomycete and therefore the name cannot be used for a Coelomycete.

The genera *Sphaeropsis, Lasiodiplodia* and *Diplodia* were distinguished essentially on the basis of conidial characters (colouration, septation and wall ornamentation) that develop with age (Sutton, 1980; Denman *et al*., 2000). Percurrent proliferation seen in conidiogenous cells was considered a feature that separated *Sphaeropsis* from *Diplodia* (Sutton, 1980). However, *Diplodia* species also produce percurrent proliferation in conidiogenous cells (Denman *et al*., 2000; Chapter 2). *Lasiodiplodia* was also separated by the presence of paraphyses in conidiomata (Denman *et al*., 2000).

Denman *et al*. (2000) questioned the value of the features used to distinguish between these genera and suggested that *Sphaeropsis* and *Lasiodiplodia* should be regarded as synonyms of *Diplodia*. The synonymy of *Sphaeropsis* under *Diplodia* has been accepted by most authors (e.g. de Wet *et al*., 2003; Burgess *et al*., 2004a, b; Pavlic *et al*., 2004) and is supported by phylogenetic analyses of multiple gene regions like ITS, EF1-α and β-tubulin (e.g. Zhou and Stanosz, 2001b; Slippers *et al*., 2004a; Pavlic *et al*., 2004; Chapters 2,3,7,9). Nevertheless, Xiao and Rogers (2004) recently described a new species in *Sphaeropsis* (*S. pyriputrescens* Xiao & J.D. Rogers) thus preferring to keep *Sphaeropsis* as a separate genus. The genus *Lasiodiplodia* is still retained by some authors due to morphological (presence of paraphyses and conidial striations) and phylogenetic distinctions (Pavlic *et al*., 2004; Burgess *et al*., 2006; see Chapter 9).

The generic concept of *Dothiorella* has been the subject of great confusion, as it appears that the name has been used in different senses (Sutton, 1977; Phillips and Lucas, 1997; Crous and Palm, 1999). According to Crous and Palm (1999) *Dothiorella* should be regarded as an additional synonym of *Diplodia*. This view is not supported by the present work (Chapter 3).

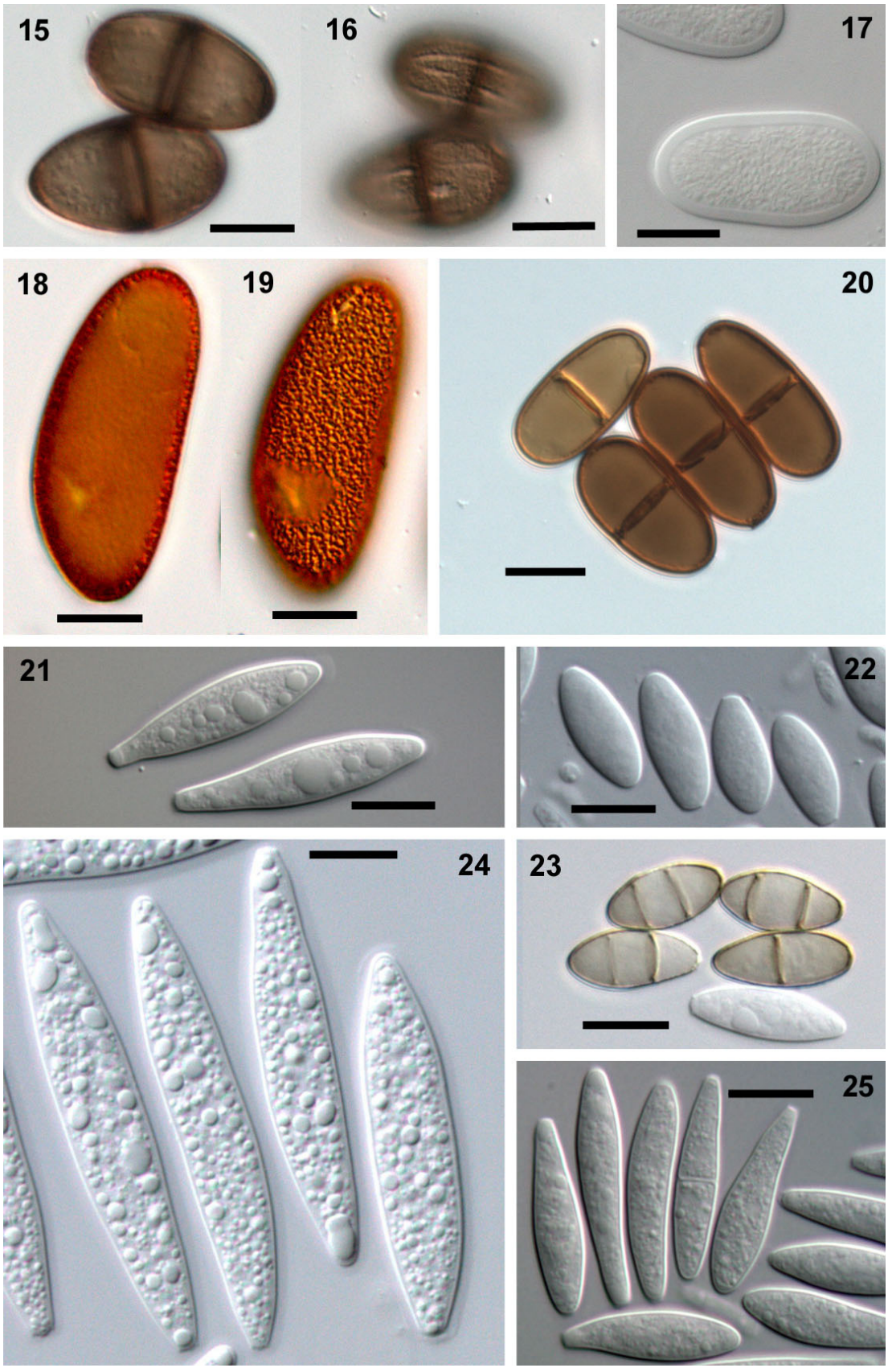
Morphological characteristics of the conidia are the main features that differentiate between the anamorph genera. Thus, *Fusicoccum* has hyaline conidia, sometimes becoming olivaceous or darker with age, thin-walled, smooth, aseptate, occasionally forming one or two septa with age or before germination, with shapes varying from elliptical to fusiform or clavate. Conidia of *Diplodia* are thick-walled, initially hyaline, becoming dark brown after discharge from the pycnidium but sometimes the colouration is delayed or never occurs, occasionally becoming one- or two-euseptate.

Sphaeropsis conidia differ from those of *Diplodia* since they regularly become dark-walled while still within the pycnidium. In *Lasiodiplodia* conidia are hyaline when young, later becoming medianly 1-euseptate, dark brown, thick-walled, ellipsoid, base truncate, with longitudinal striations. *Dothiorella* conidia are initially hyaline, becoming dark brown and one-euseptate within the pycnidial cavity and often while still attached to the conidiogenous cell, thick-walled, externally smooth, internally verruculose (Chapters 2–10).

As shown in recent works on phylogenetic analyses of ITS and EF1-α gene regions (Zhou and Stanosz, 2001b; Zhou *et al*., 2001; Slippers *et al*., 2004a, b; Farr *et al*., 2005; Barber *et al*., 2005; Chapters 2 to 6) species in the genus *Fusicoccum* reside in two sub-clades. On the basis of phylogenetic analysis of the LSU ribosomal DNA gene as well as morphological distinctions, Crous *et al*. (2006) proposed a split of the genus into *Fusicoccum* and *Neofusicoccum* Crous, Slippers & A.J.L. Phillips. and a new genus *Pseudofusicoccum* Mohali, Slippers & M.J. Wingf. was introduced to accommodate some recently described species (Mohali *et al*., 2006). Barber *et al*. (2005) recently showed that some *Fusicoccum* (*Neofusicoccum*) species have *Dichomera* Cooke synanamorphs.

Crous *et al*. (2006) showed that *Macrophomina phaseolina* (Tassi) Goid. and *Neoscytalidium dimidiatum* (Nattrass) Crous & Slippers (synanamorph *Hendersonula toruloidea* Natrass) are phylogenetically related to the genus *Botryosphaeria*. This last species which has a *Fusicoccum*-like coelomycete anamorph had been linked previously to *Botryosphaeria* by Farr *et al*. (2005) who made the new combination *Fusicoccum dimidiatum* (Penz.) D.F. Farr. However, according to Crous *et al*. (2006) this species represents a separate genus, which they described as *Neoscytalidium* Crous & Slippers based on the *Scytalidium*-like anamorph.

FIGURES 15–25. Conidia produced by *Botryosphaeria* anamorphs. 15, 16. Two different levels of focus of *Lasiodiplodia theobromae* mature conidia to show the characteristic longitudinal striations. 17. Hyaline, aseptate and thick-walled conidia of *Diplodia mutila*. 18, 19. Conidia of *Diplodia pinea* (previously *Sphaeropsis*). Two levels of focus showing roughening on the inner face of the wall. 20. Brown, septate and thick-walled conidia typical of *Dothiorella*. 21, 25. Hyaline, thin-walled, smooth, fusiform conidia typical of *Fusicoccum*. 21. *Fusicoccum* anamorph of *B. corticis*. 25. *F. aesculi*. 22. Young, hyaline and aseptate conidia of *Neofusicoccum parvum*. 23. Aged conidia of *N. parvum* with brown walls and one or two septa. 24. Conidia of the *Fusicoccum* anamorph of *B. melanops*. Scale bars = 10 µm (see next page).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Position in Ascomycete systematics

Since it was introduced in 1863, the position of the genus *Botryosphaeria* within the higher classification of ascomycetes has been the subject of many taxonomic rearrangements, as thoroughly explained by Denman *et al*. (2000) and Crous *et al*. (2006). *Botryosphaeria* species have a mixture of *Dothideales* and *Pleosporales* morphological characters (Berbee, 1996), which explains why the ordinal placement of the genus has been the subject of so much controversy.

Luttrel (1955, 1973) acknowledged that *Botryosphaeria* species had a centrum typical of the *Pleosporales*, and so he placed the genus (family *Botryosphaeriaceae* Theiss. & Sydow) in that order. Barr (1972, 1979) initially placed *Botryosphaeria* in the *Dothideales* (family *Dothioraceae* Theiss. & Sydow), but later (Barr, 1987) she supported Luttrell's view and returned it to the *Pleosporales* (family *Botryosphaeriaceae*). Von Arx and Müller (1975) placed all bitunicate ascomycetes, including *Botryosphaeria*, in a single order the *Dothideales*. To complicate this issue further, Sivanesan (1984) placed *Botryosphaeria* in the *Dothideaceae* (*Dothideales*), whereas Hawksworth *et al*. (1995) list *Botryosphaeria* under the *Botryosphaeriaceae* in the *Dothideales*.

Currently it is widely accepted that the genus *Botryosphaeria* belongs in the family *Botryosphaeriaceae* erected by Theissen and Sydow (1918). This family has been discussed in detail by von Arx and Müller (1954). In the most recent classification scheme 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.

A number of studies employing nuclear and mitochondrial rDNA sequences (Berbee, 1996, 2001; Winka *et al*., 1998; Silva-Hanlin and Hanlin, 1999; Liew *et al*., 2000; Tehler *et al*., 2000, 2003) as well as sequences of protein-coding genes like RPB2 (Liu *et al*., 1999; Liu and Hall, 2004; Lutzoni *et al*., 2004) deal with the phylogenetic placement of ascomycetes and consequently of *Botryosphaeria*. However, the ordinal position of *Botryosphaeria* species remains equivocal. In some analyses the genus clusters with the *Dothideales*, while in others it clusters with the *Pleosporales*. In all cases, however, the placement of the genus in each of the orders lacked statistical support. The only exception is the work of Liu and Hall (2004), where the phylogenetic analysis of RPB2 sequences from ascomycetes places *Botryosphaeria* in the *Pleosporales* with statistically significant support. This subject is followed up in Chapter 10 where the placement of *Botryosphaeria* in higher taxonomy of the Ascomycetes is analysed based on SSU and LSU phylogenies.

The species in *Botryosphaeria*

How many species are there?

When Cesati and De Notaris (1863) described the genus *Botryosphaeria* they listed nine species together with another six species that they did not recombine in the genus. De Notaris (1864) described a further four species.

In 1954, von Arx and Müller examined 183 species in several genera and reduced them to 12 species, nine of which they described as new. Although only 31 of the taxa examined by von Arx and Müller (1954) were *Botryosphaeria* species, they made extensive synonymies under *B. quercuum* and *B. dothidea*. Von Arx and Müller (1954) based their work on a study of herbarium specimens of the teleomorph alone. However, it is now clear that morphological variation in teleomorph characters is insufficient to separate species in *Botryosphaeria* and the synonymies proposed by von Arx and Müller (1954) are considered to be too conservative. For example, although they included *B. obtusa* (Schwein.) Shoemaker under *B. quercuum*, this species has been shown to be distinct (Shoemaker, 1964). While Slippers *et al*. (2004) agreed with von Arx and Müller's (1954) decision to include *B. berengeriana* as a synonym of *B. dothidea*, they regarded *B. ribis* Grossenb. & Dugg. as distinct.

Until 2005, the Index of Fungi lists 231 species names for *Botryosphaeria*. Some of these names have been reduced to synonymy, or reclassified in other genera. The validity of most of these names was never confirmed. In view of the morphological conservation of the teleomorphs, the lack of information regarding teleomorph-anamorph connections for many taxa, and the absence of authentic cultures linked to the vast majority of specimens, knowing the exact number of *Botryosphaeria* species cannot be determined with any degree of certainty.

However, few of the described names are in regular use in the mycological and phytopathological literature. TABLE 1 presents a list of names of *Botryosphaeria* species and corresponding anamorphic species most frequently found in the literature and for which there is morphological evidence supporting its status as a species. In most cases the morphological data is further corroborated by molecular data.

TABLE 1. List of *Botryosphaeria* species names commonly used in the literature

Teleomorph	Anamorph	Reference(s)
Botryosphaeria australis	*Fusicoccum australe*	Slippers *et al*. (2004b)
B. lutea	*F. luteum*	Phillips *et al*. (2002)
B. ribis	*F. ribis*	Slippers *et al*. (2004a)
B. parva	*F. parvum*	Slippers *et al*. (2004a)
B. eucalyptorum	*F. eucalyptorum*	Smith *et al*. (2001)
B. eucalypticola	*F. eucalypticola*	Slippers *et al*. (2004c)
B. protearum	*F. protearum*	Denman *et al*. (2003)
Unknown	*F. viticlavatum*	van Niekerk *et al*. (2004)
Unknown	*F. vitifusiforme*	van Niekerk *et al*. (2004)
Unknown	*F. arbuti*	Farr *et al*. (2005)
Unknown	*F. dimidiatum*	Farr *et al*. (2005)
Unknown	*F. mangiferum*	Slippers *et al*. (2005)
Unknown	*F. andinum*	Mohali *et al*. (2006)
Unknown	*F. stromaticum*	Mohali *et al*. (2006)
Unknown	*F. macroclavatum*	Burgess *et al*. (2005)
B. dothidea	*F. aesculi*	Slippers *et al*. (2004a)
B. corticis	*Fusicoccum* sp.	this work (Chapter 6)
B. mamane	*Fusicoccum* sp.	Gardner (1997)
B. melanops	*F. advenum*	Phillips and Pennycook (2005)
B. stevensii	*Diplodia mutila*	this work (Chapter 2)
B. obtusa	*Diplodia sp.* (*Sphaeropsis* sp.)	Shoemaker (1964)
B. corticola	*Di. corticola*	this work (Chapter 2)
B. tsugae	*Diplodia sp.* (*Macrophoma* sp.)	Funk (1964)
Unknown	*Di. pinea*	Punithalingam and Waterston (1970)
Unknown	*Di. scrobiculata*	de Wet *et al*. (2003)
Unknown	*Sphaeropsis pyriputrescens*	Xiao and Rogers (2004)
Unknown	*Di. porosum*	van Niekerk *et al*. (2004)
Unknown	*Di. rosulata*	Gure *et al*. (2005)
Botryosphaeria sp.	*Di. cupressi*	this work (Chapter 7)
B. rhodina	*Lasiodiplodia theobromae*	Punithalingam (1976); von Arx (1981)
Unknown	*L. pseudotheobromae*	this work (Chapter 9)
Unknown	*L. parva*	this work (Chapter 9)
Unknown	*L. gonubiensis*	Pavlic *et al*. (2004)
Unknown	*L. crassispora*	Burgess *et al*. (2006)
Unknown	*L. venezuelensis*	Burgess *et al*. (2006)
Unknown	*L. rubropurpurea*	Burgess *et al*. (2006)
B. sarmentorum	*Dothiorella sarmentorum*	this work (Chapter 3)
B. iberica	*Do. iberica*	this work (Chapter 3)
B. viticola	*Do. viticola*	Luque *et al*. (2005)
B. quercuum	*Diplodia* sp.	Shoemaker (1964)
B. laricis	a form of *Macrophoma sapinea*	Smerlis (1970)
B. pseudotsugae	Unknown	Funk (1975)
B. piceae	Unknown	Funk (1965)
B. pipturi	*Fusicoccum* sp.	Gardner and Hodges (1988)
B. subglobosa	*S. subglobosa*	von Arx and Müller (1975); Punithalingam (1969)
B. visci	*S. visci*	von Arx and Müller (1954); Sivanesan (1984)
B. xantochepala	*F. cajani*	Samuels and Singh (1986)
B. zeae	*Macrophoma zeae*	von Arx and Müller (1954); Sivanesan (1984)
*B. proteae**	*F. proteae*[+]	Crous *et al*. (2004)

* reclassified in the genus *Saccharata* as *S. proteae*.

[+] a Diplodia-like synanamorph was also described.

Taxonomy of the species

Taxonomy of the genus *Botryosphaeria* is problematic at several levels of classification. At the specific level much confusion has occurred regarding the allocation of species to the genus. This is due essentially to ill-defined concepts describing morphological features and insufficient diversity of these features to allow unequivocal identification (Denman, 2002).

Morphological features of the teleomorphs are extremely conserved and do not allow for a clear differentiation of species (Shoemaker, 1964; Laundon, 1973; Jacobs and Rehner, 1998; Denman *et al*., 2000). Moreover, teleomorphs are rarely seen in nature and have seldom been induced in artificial culture (Witcher and Clayton, 1963; Laundon, 1973; Denman *et al*., 2000). Thus, identification of the species relies mainly on morphological characters of the anamorph, which is the most commonly seen and morphologically diverse form (Shoemaker, 1964; Laundon, 1973; Pennycook and Samuels, 1985; Jacobs and Rehner, 1998; Denman *et al*., 2000).

Anamorph characters used for delimitation of species include size, shape, color, septation, wall thickness and wall texture of the conidia. Certain cultural aspects like colony morphology, chromogenicity, and temperature effects on mycelial growth rate have also been used to differentiate species (Shoemaker, 1964; Laundon, 1973; Pennycook and Samuels, 1985; Denman *et al*., 2000; Phillips, 2002).

Some of the characters used to circumscribe species within anamorphic genera associated with *Botryosphaeria* exhibit extensive plasticity. Morphological characteristics of the anamorphs can be strongly influenced by the substrate in which they are produced. The size ranges of conidia of different *Botryosphaeria* species overlap; age and state of maturity affect conidial pigmentation and septation. Moreover, the diversity of hosts colonized by a single species and the occurrence of different species on the same host largely precludes host association as a reliable character for species determination. Taking all these aspects into consideration, species identification can be a difficult task to perform (Pennycook and Samuels, 1985; Jacobs and Rehner, 1998; Denman *et al*., 2000).

The introduction of molecular methods into mycology has led to major developments in the study of fungi. Advances in molecular techniques, like DNA sequencing, and particularly the analysis of nucleotide sequences of ribosomal RNA genes (rDNA) have made a great contribution to fungal taxonomy and systematics (Bruns *et al*., 1991; Berbee and Taylor, 1995, 2001; Seifert *et al*., 1995; Guarro *et al*., 1999; Taylor *et al*., 1999).

Among the variable regions of rDNA, the internal transcribed spacer region (ITS) has been successfully applied to study the genus *Botryosphaeria* (e.g. Jacobs and

Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001b). Significant advances in the taxonomy of this genus resulted from these studies allowing, for instance, the possibility of relating anamorph and teleomorph genera, and the separation of species complexes into distinct species (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001b; Smith and Stanosz, 2001; Smith *et al.*, 2001; Phillips *et al*., 2002). Sequencing of other genomic regions besides the ITS, namely introns from protein coding genes like EF1-α and β-tubulin, have contributed to the taxonomy of *Botryosphaeria* by providing means to resolve cryptic species complexes into their component parts (de Wet *et al.*, 2003; Slippers *et al*., 2004a, b, c; van Niekerk *et al*., 2004; Burgess *et al.*, 2005; Chapters 3, 7 and 9).

Phylogeny of the species

The phylogenetic analysis of nuclear ribosomal genes, specifically of the more variable ITS region, has made a significant contribution to *Botryosphaeria* phylogenetics. Jacobs and Rehner (1998) were the first to use ITS rDNA sequences in phylogenetic analysis of *Botryosphaeria* and showed that there was some congruence between morphological and cultural characters and ITS sequences, at least for some taxa. These authors combined morphological characters with nucleotide sequence data and proved that *B. dothidea* was paraphyletic. Later, this paraphyletic group was shown to represent the species *B. dothidea* and *B. ribis* (Zhou and Stanosz, 2001b; Smith and Stanosz, 2001; Smith *et al.*, 2001a). Since then many studies have addressed the phylogenetic relationships between these *Botryosphaeria* species with *Fusicoccum* anamorphs (Smith *et al.*, 2001a; Smith *et al.*, 2001b; Phillips *et al*., 2002; Denman *et al*., 2003; Slippers *et al*., 2004a, b, c; van Niekerk *et al*., 2004; Burgess *et al*., 2005, Farr *et al.*, 2005; Slippers *et al*., 2005a).

Denman *et al.* (2000) reviewed the anamorph genera associated with *Botryosphaeria* and on the basis of ITS phylogenies recognised that the species studied separated into two main clades corresponding to the anamorph genera *Fusicoccum* and *Diplodia*. According to these authors the separation was correlated with morphological features of the anamorphs which they recognized as the only conidial states of *Botryosphaeria* species. Thus *Fusicoccum* species have thin-walled, hyaline conidia while *Diplodia* species have thick-walled, brown conidia (Denman *et al*., 2000). This was later supported by Zhou and Stanosz (2001b) who divided the genus *Botryosphaeria* into sections *Hyala* (species with narrow, hyaline conidia of the *Fusicoccum* type) and *Brunnea* (species with wide, brown conidia of the *Diplodia* type). Phillips (2002) supported the retention of only these two genera as anamorphs of

Botryosphaeria species. However, because conidia of some *Diplodia* species can remain hyaline and in *Fusicoccum* they can become brown he stated that they were better defined by cell wall thickness and width of conidia. Thus in *Diplodia* the conidia are more than 10 µm wide with a relatively thick-wall, while in *Fusicoccum* the conidia are less than 10 µm wide and thin-walled (Phillips, 2002).

This separation of the species into only two anamorph genera was later proven to be too simplistic when a larger number of *Botryosphaeria* species representative of the full diversity of the genus were analysed, and phylogenetic analyses have shown that *Botryosphaeria* species reside in other anamorph genera besides *Diplodia* and *Fusicoccum* (see Chapter 3; van Niekerk *et al*., 2004).

Zhou and Stanosz (2001a) used the mitochondrial ribosomal small subunit (mtSSU rDNA) to address phylogenetic questions in *Botryosphaeria*. The phylogenies retrieved from these analyses were not in agreement with ITS phylogenies. Thus the split between *Diplodia* and *Fusicoccum* was not supported by mtSSU rDNA phylogenies and some *Botryosphaeria* species with *Fusicoccum* anamorphs, namely *B. dothidea* and *B. corticis*, grouped within the *Diplodia* clade. Nevertheless, mtSSU rDNA phylogenies clearly separated *Botryosphaeria* from the closely related genus *Guignardia* (Zhou and Stanosz, 2001a).

Despite the profound impact that nucleotide sequence data from the ITS region has had on *Botryosphaeria* species taxonomy and phylogeny, the use of this single gene can underestimate the true diversity especially among closely related or cryptic species (Taylor *et al*., 2000). In fact, the ITS region alone proved unsuitable to discriminate between closely related *Botryosphaeria* species like *B. ribis* and *B. parva* Pennycook & Samuels (Slippers *et al.*, 2004a) or *D. pinea* (Desm.) J. Kickx f. and *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. (de Wet *et al*., 2003). Combining nucleotide sequence data of the ITS gene with other gene regions like EF1-α and β-tubulin has been applied successfully to discriminate between cryptic species and elucidate species-level phylogenetic relationships in *Botryosphaeria* and associated anamorphs (de Wet *et al.*, 2003; Slippers *et al*., 2004a, b, c; van Niekerk *et al*., 2004; Burgess *et al.*, 2005; Chapters 3, 7 and 9).

Botryosphaeria as a pathogen

Botryosphaeria is a species-rich genus with a cosmopolitan distribution (von Arx and Müller, 1954; Barr, 1972, 1987; Sivanesan, 1984). Although most of the species (e.g. *B. dothidea*, *B. parva*, and *B. obtusa*) are distributed worldwide others are restricted to certain geographic areas. For example, the species *Botryosphaeria rhodina* (Berkeley & M.A. Curtis) Arx (anamorph *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.) is apparently restricted to warmer climates and occurs mainly in the tropics and subtropics (Punithalingham, 1976, 1980; von Arx, 1981), while *Botryosphaeria corticis* has been reported in the USA only (Demaree and Wilcox, 1942; Taylor, 1958; Chapter 6). *Botryosphaeria mamane* D.E. Gardner originally described on *Sophora chrysophylla* in Hawaii (Gardner, 1997) was regarded as an endemic species. However, it was recently reported on *Eucalyptus urophylla* in Venezuela (see Crous *et al*., 2006).

Botryosphaeria species occur in a wide range of plant hosts (angiosperms and gymnosperms), on woody branches, herbaceous leaves, stems and haulms of grasses, on twigs and on the thalli of lichens. The species in the genus can be saprophytes on dead or dying stems, branches, stalks, culms or leaves of plants, but they are best known as pathogens of woody hosts causing dieback, cankers, shoot blights, leaf spots, fruit and seed rots, witches' brooms, and other symptoms in a wide range of plant hosts of agricultural, forestry, ecological and economic importance (Barr, 1972, 1987; Sivanesan, 1984; von Arx, 1987).

Not only is there some controversy regarding the taxonomy of the genus *Botryosphaeria*, pathogenicity of *Botryosphaeria* species has also been the subject of some confusion. Contradictory reports exist regarding the pathogenic nature of some species (see Phillips 2002). Also, variation in virulence of species as well as of isolates within the same species has been reported (Phillips, 1998, 2002; Brown-Rytlewski and McManus, 2000). Some of this confusion is probably related to the fact that species were not properly defined resulting in misidentifications. Phillips (1998) reported differences in virulence between *B. dothidea* isolates from grapevines. However, the strains of *B. dothidea* were later shown to be a complex of species that included *B. dothidea*, *B. parva* and *B. lutea* A.J.L. Phillips (Phillips, 2000, 2002; Phillips *et al.*, 2002) and the differences in virulence were in fact attributed to different species.

Botryosphaeria species are frequently referred to as secondary or opportunistic pathogens usually wound and stress related. However, the infection process may also occur through lenticels, stomata or other natural openings (Demaree and Wilcox, 1942; Taylor, 1958; Witcher and Clayton, 1963; Michailides, 1991; Brown-Rytlewski and McManus, 2000).

There are also a number of reports of *Botryosphaeria* species occurring as endophytes or latent pathogens on several hosts (Smith, 1995; Smith *et al*., 1996, 2001b; Ragazzi *et al*., 2001; Denman, 2002; Pavlic *et al*., 2004; Mohali *et al*., 2005; Stanosz *et al*., 1997, 2005). Apparently, these fungi can survive in an asymptomatic endophytic stage until stress caused to the host triggers the development of disease (Michailides and Morgan, 1992; Smith, 1995; Ragazzi *et al*., 1999; Ma *et al*., 2001; Denman, 2002).

The species in *Botryosphaeria* as well as associated anamorphic genera are common and widespread pathogens of many plant hosts of agricultural, forestry, and ecological importance (Table 2). Diseases caused by these fungi are the source of serious and in some cases devastating losses.

The most important limiting factor in production of blueberries in the USA is the stem canker disease, caused by *B. corticis* (Demaree and Wilcox, 1942; Taylor, 1958). This same crop is also affected by *B. dothidea* which causes stem-blight disease. Although occurrence and severity of the disease is variable it was reported to affect up to 9% of the plants (Witcher and Clayton, 1963).

Botryosphaeria panicle and shoot blight of pistachio caused by *B. dothidea* is a common disease in orchards in California. The fungus attacks and kills fruit clusters, and yield losses of 40 to 100 % have been reported (Michailides and Morgan, 1992; Ma *et al*., 2001).

Excoriose is an important disease of grapevines that can cause losses of up to 50% of the normal yield. Several *Botryosphaeria* species have been shown to play an important role on the disease (Phillips, 1998, 2000).

Quercus canker caused by *B. stevensii* (*B. corticola* see Chapter 2) has been described as the most frequent disease associated with *Quercus* spp., especially *Q. suber* (cork oak) in the Mediterranean region. In fact, trunk cankering caused by *B. stevensii* (*B. corticola* see Chapter 2) is considered to be the most common disease causing death of *Q. suber* in Spain (Luque and Girbal, 1989; Luque *et al.*, 2000; Sánchez *et al*., 2003).

Some well studied species like *B. dothidea*, *B. parva*, *B. lutea*, *B. obtusa*, and *B. rhodina* have an extremely wide host range (Punithalingham, 1980; Pennycook and Samuels, 1985; Phillips *et al*., 2002; Slippers *et al*., 2004a). For instance, Smith (1995) listed more than 100 hosts for the plurivorous species *B. dothidea*, and *L. theobromae* has been associated with approximately 500 host plants (Punithalingam, 1980). However, many of the host associations reported for *B. dothidea*, have not been confirmed and indeed may be due to other species because of the broad species

concept that was applied to *B. dothidea* before phylogenetic studies clarified the status of that species. The same may as well be true for *L. theobromae*.

In contrast, many other species appear to be specialized on a particular host or group of hosts. Thus, *D. pinea* and *D. scrobiculata* have been reported as pathogens of *Pinus* species and other conifers (de Wet *et al*., 2002, 2003); *B. tsugae* A. Funk has been isolated from *Tsuga heterophylla* only (Funk, 1964), while *B. eucalyptorum* Crous, H. Smith & M.J. Wingfield and *B. eucalypticola* Slippers, Crous & M.J. Wingfield apparently occur only on *Eucalyptus* species (Smith *et al*., 2001b; Slippers *et al*., 2004c) and *B. protearum* Denman & Crous on Proteacae (Denman *et al*., 2003). Also, *B. corticis* appears to be restricted to *Vaccinium* species (Demaree and Wilcox, 1942; Chapter 6) while *B. corticola* was reported from *Quercus* species only (Chapter 2). *Botryosphaeria mamane* was thought to be restricted to *Sophora chrysophylla* (1997), but it was recently reported on *Eucalyptus urophylla* (see Crous *et al*., 2006). Most of these species were described recently and it is possible that their host ranges have not been fully explored.

Although the reports are scarce some *Botryosphaeria* species, namely *L. theobromae* and *B. subglobosa* (C. Booth) Arx & E. Müller (anamorph *Sphaeropsis subglobosa* Cooke), have been reported to occasionally cause phaeohyphomycosis and keratomycosis in humans (Rebell and Forster, 1976; Kirkness *et al.*, 1991; Summerbell *et al*., 2004).

TABLE 2. List of some plant hosts reported for *Botryosphaeria* species

Plant hosts	***Botryosphaeria* species**	References
Grapevines	*Botryosphaeria dothidea*	Phillips (1998)
	B. stevensii	Phillips (2002)
	B. obtusa	van Niekerk *et al*. (2004)
	B. lutea	Taylor *et al*. (2005)
	B. australis	Luque *et al*. (2005)
	B. parva	
	B. rhodina	
	Fusicoccum viticlavatum	
	F. vitifusiforme	
	Diplodia porosum	
	B. viticola	
Oaks	*B. corticola*	Sánchez *et al*. (2003)
	B. dothidea	Chapters 2 and 3
	B. iberica	Shoemaker (1964)
	B. quercuum	Phillips and Pennycook (2005)
	B. melanops	
Olives	*B. dothidea*	Chapter 4
Eucalypts	*B. eucalyptorum*	Smith *et al*. (2001b)
	B. eucalypticola	Slippers *et al*. (2004c)
	B. dothidea	Burgess *et al*. (2005)
	B. australis	Mohali *et al*. (2006)
	B. ribis	
	B. parva	
	F. macroclavatum	
	F. andinum	
	F. stromaticum	
	Dichomera eucalypti	
	Dc. saubinetii	
	Dc. versiformis	
Pines and other conifers	*B. tsugae*	Funk (1964)
	B. piceae	Funk (1965)
	B. pseudotsugae	Funk (1975)
	Di. pinea	de Wet *et al.* (2002)
	Di. scrobiculata	de Wet *et al.* (2003)
	B. parva	Slippers *et al*. (2005b)
	B. australis	
Pistachio	*B. dothidea*	Michailides and Morgan (1992)
		Ma *et al*. (2001)
Blueberry	*B. corticis*	Demaree and Wilcox (1942)
	B. dothidea	Taylor (1958)
		Witcher and Clayton (1963)
		Chapter 6
Proteaceae flowers	*B. dothidea*	Denman *et al*. (2003)
	B. lutea	
	B. ribis	
	B. obtusa	
	B. rhodina	
	B. proteae	
	B. protearum	
Pyrus communis	*Sphaeropsis pyriputrescens*	Xiao and Rogers (2004)
Sophora chrysophylla	*B. mamane*	Gardner (1997)
Arbutus menziesii	*F. arbuti*	Farr *et al*. (2005)
Prunus africana	*Di. rosulata*	Gure *et al*. (2005)
Mangifera indica	*B. dothidea*	Slippers *et al*. (2005a)
	F. mangiferum	
Cistus ladanifer	*B. dothidea*	Sánchez-Hérnandez *et al*. (2002)
Blackberry	*B. dothidea*	Maas and Uecker (1984)
Actinidia deliciosa	*B. dothidea*	Pennycook and Samuels (1985)
	B. parva	
	F. luteum	
Apple trees	*B. dothidea*	Brown-Rytlewski and McManus (2000)
	B. obtusa	
Cupressus sempervirens	*Di. cupressi*	Chapter 7
Juniperus spp.		

Aims of the work

The basis of any disease investigation is a thorough understanding and correct identification of the pathogenic agent. The genus *Botryosphaeria* and associated anamorphic genera have been in taxonomic disarray. Despite the recent advances in the taxonomy and systematics of botryosphaeriaceous fungi, many aspects concerning the generic and species concept are still unresolved as well as the position of the genus in the higher classification of the Ascomycetes.

This work was undertaken with the overall objective of contributing to the clarification of the taxonomy and phylogeny of the genus *Botryosphaeria* and the species occurring on woody hosts. A particular emphasis is given to species occurring on *Quercus suber* (and other *Quercus* spp.) since these plants have great economic and ecological importance in Portugal.

The work presented here had the following aims:

1. Collect and study the species of *Botryosphaeria* that occur in association with cankers and dieback of *Quercus* species and other woody hosts

2. Reassess the species concept applied to *Botryosphaeria* species and amend them in terms of morphological and molecular characters

3. Assess different molecular techniques to be used in establishing a good taxonomy of the genus and species

4. Determine suitable molecular techniques to differentiate species and assess genetic diversity within species

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CHAPTER

2

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Botryosphaeria corticola sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*

ABSTRACT

Botryosphaeria stevensii has frequently been associated with dieback and canker diseases of oaks, mainly in the Western Mediterranean area but more rarely in other regions. The species concept of *B. stevensii* has been unclear, and it is possible that some collections were incorrectly identified. A collection of fungal strains isolated from diseased oak trees and initially identified as *B. stevensii* was characterised on the basis of morphology and ITS nucleotide sequences. Morphology was compared with the type specimens of *Physalospora mutila* (= *B. stevensii*) and its anamorph *Diplodia mutila*. It was concluded that the isolates from oaks differed from *B. stevensii* in having larger ascospores and conidia as well as different spore shapes, and represented an as yet undescribed species, which is described here as *B. corticola*. Moreover, ITS sequence data separated *B. corticola* from all other known species of *Botryosphaeria*. Amended descriptions of *B. stevensii* and its anamorph are provided to differentiate *B. stevensii* from *B. corticola* and to clarify some of the earlier taxonomic uncertainties.

***Key words*:** Ascomycetes, *Botryosphaeriaceae*, ITS, oak decline, phylogeny, ribosomal DNA, systematics

INTRODUCTION

Since the early 1980's a serious decline of oak trees has been recognised in the Iberian peninsula and the Mediterranean basin (Brasier, 1992, 1996; Brasier *et al*., 1993; Muñoz *et al*., 1996; Robin *et al*., 1998; Ragazzi *et al*., 2000). Several factors have been implicated in the decline including drought, severe summer flooding, changes in traditional agronomic practices, attacks by wood-boring insects and fungal diseases (Brasier, 1996). While root rot caused by *Phytophthora cinnamomi* Rands has been regarded as a major factor in the decline of Iberian oaks (Brasier, 1992; Brasier *et al*., 1993; Gallego *et al*., 1999), several fungi that cause cankers and diebacks are also considered to be important contributing factors (Luque *et al*., 2001). The most common and important canker and dieback pathogen is considered to be *Botryosphaeria stevensii* Shoemaker (anamorph: *Diplodia mutila* Fr.). Thus, Oliva and Molinas (1986) reported a *Diplodia* species associated with diseased cork oaks (*Quercus suber* L.) in NE Spain, while Vajna (1986) reported that *D. mutila* caused a

branch canker and dieback of sessile oak [*Q. petraea* (Matt.) Liebl.] in Hungary. Later, Luque and Girbal (1989) reported that after cork removal, *B. stevensii* invades the exposed trunks and causes wilting and mortality of *Q. suber* in NE Spain.

As with most *Botryosphaeria* species, *B. stevensii* is normally encountered as its anamorph. The taxonomic history of *D. mutila* was explained by Stevens (1933) and by Sutton (1980), but some controversy surrounds the characters that define this fungus. 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, 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 but becoming dark brown and one-septate when mature. In his illustration of this species he depicts a predominance of dark conidia. The only species of *Botryosphaeria* with mostly hyaline, aseptate, thick walled conidia of the *Diplodia* type are *B. stevensii* and *B. quercuum* (Schwein.) Sacc. Shoemaker (1964) separated the two species on the basis of conidium shape as defined by their length/width (L/W) ratios. Thus, in *B. quercuum*, the conidia are subglobose with a L/W ratio of about 1.5, while in *B. stevensii* they are oblong with a L/W ratio of about 2.3. The general consensus is that conidia of *B. stevensii* are (20–)25–27 × 10–12 µm (Stevens, 1933; Shoemaker, 1964; Laundon, 1973; Sivanesan, 1984), but Sutton (1980) considered they can be up to 31 µm long.

The history of *B. stevensii* is also somewhat confused. Stevens (1936) 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. Since this was published after 1 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 name, *Botryosphaeria stevensii* Shoemaker typified by the type of *P. mutila* N. E. Stevens ex Shoemaker.

Because the concepts of *B. stevensii* and *D. mutila* are not entirely clear, it is possible that some collections have been misidentified. When Luque and Girbal (1989) reported *B. stevensii* from *Q. 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. More recently, Zhou and Stanosz (2001a, b) suggested that the name *B. stevensii* might have been applied to more than one species, thus raising the possibility that the fungus reported on oaks is in fact not *B. stevensii*.

Analysis of nucleotide sequences of ribosomal genes has contributed greatly to resolving phylogenetic relationships of fungi (Bruns *et al*., 1991; Berbee and Taylor, 1992; O'Donnell *et al*., 1997). Comparison of the nucleotide sequences of the internally transcribed spacers (ITS) regions of the nuclear ribosomal DNA has been used to clarify the taxonomy and phylogenetic relationships of *Botryosphaeria* species (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001b). Analysis of ITS sequence data has also been used to help distinguish morphologically similar species in this genus (Smith *et al*., 2001; Phillips *et al*., 2002).

Since 1989, several *Botryosphaeria* isolates morphologically similar to *B. stevensii* were obtained from oaks in Spain, Portugal and Italy. The aim of the present study was, therefore, to determine if these strains were distinguishable from *B. stevensii* on the basis of morphological characters and the nucleotide sequence of the 5.8S ribosomal gene and its flanking ITS regions. These data were compared with strains previously identified as *B. stevensii* and also morphologically with the type specimens of *B. stevensii* and *D. mutila.*

MATERIALS AND METHODS

Isolates

Material from diseased branches and trunks was collected from *Quercus ilex* L. or *Q. suber* in several regions in Spain and one region in each of Portugal and Italy. Diseased material was also collected from *Vitis vinifera* L. and *Pyrus communis* L. Isolations were made from single ascospores, conidia or by directly plating out pieces of diseased tissue after surface sterilization (3 min in 70 % ethanol).

Ascomata or conidiomata were cut through horizontally and the contents transferred to a drop of sterile water on a flamed microscope slide. A portion of this was taken and spread over a few square cm of a plate of Difco potato dextrose agar (PDA). The water on the slide was allowed to evaporate at room temperature before a drop of 100 % lactic acid was applied and covered with a coverslip. The Petri dish bearing the spores was incubated at 25 ºC overnight. The following day individual germinating spores were transferred to fresh plates of PDA and checked by microscopy to ensure that a single spore had been transferred. In this way, single ascospore and single conidium isolates could be linked to the semi-permanent

preparation and their identity verified by microscopy. Strains isolated by M.E. Sánchez and A. Trapero (University of Córdoba, Spain) were also included in the study (TABLE 1). Fungi isolated in our study were deposited at Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands) and they are referred to throughout this paper by their CBS accession numbers (TABLE 1). Specimens were lodged with the herbarium of Estação Agronómica Nacional, Oeiras, Portugal (LISE). In the phylogenetic analysis, nucleotide sequences of the 5.8S ribosomal DNA gene and the flanking ITS regions of various *Botryosphaeria* species were taken from GenBank (TABLE 1).

Morphology and cultural characters

Cultures were maintained on half-strength PDA or oatmeal agar (Anonymous, 1968). To induce sporulation, a 4 cm length of autoclaved poplar twig was added to each Petri dish. Colony characteristics were recorded from cultures grown on full strength PDA at room temperature (ca. 20–25 ºC) and exposed to indirect sunlight. Growth rates were determined on full strength PDA incubated in darkness at 5-degree intervals between 5 ºC and 35 ºC.

Observations on micromorphological features were made with a Leica DMR HC microscope with bright field or Nomarski differential interference contrast illumination. Digital images were recorded with an Olympus C-3030 camera. Measurements were made with the UTHSCSA *ImageTool* version 3. At least 50 ascospores or conidia of each isolate were measured on images taken with the ×100 objective lens. Data for spore measurements are presented as the lower and upper 95 % confidence limits, with the minimum and maximum dimensions in parentheses. Dimensions of other fungal structures are given as the range of at least 20 measurements where possible.

DNA extraction and PCR amplification

Fungal isolates were grown in Czapek Dox broth or potato dextrose broth for 5 d at approximately 23 ºC. Genomic DNA was isolated from fresh mycelium following an adaptation of the method of Pitcher *et al*. (1989). The mycelium was ground in liquid nitrogen, transferred to a 2 mL Eppendorf tube and mixed with 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the contents divided into two Eppendorf tubes.

TABLE 1. Identity and origin of isolates studied.

Teleomorph	Anamorph[a]	Collection or culture number[b]	Host	Collector	Location	GenBank[c]
Botryosphaeria corticola	*Diplodia corticola*	CBS 112545	*Quercus suber*	M.E. Sánchez, A. Trapero	Cádiz, Spain	AY259089
Botryosphaeria corticola	*Diplodia corticola*	CBS 112546	*Quercus ilex*	M.E. Sánchez, A. Trapero	Huelva, Spain	AY259090
Botryosphaeria corticola	*Diplodia corticola*	CBS 112547	*Quercus ilex*	M.E. Sánchez, A. Trapero	Córdoba, Spain	AY259110
Botryosphaeria corticola	*Diplodia corticola*	CBS 112548	*Quercus suber*	A. Alves	Aveiro, Portugal	AY259099
Botryosphaeria corticola	*Diplodia corticola*	**CBS 112549**[d]	*Quercus suber*	A. Alves	Aveiro, Portugal	AY259100
Botryosphaeria corticola	*Diplodia corticola*	CBS 112550	*Quercus suber*	A. Alves	Aveiro, Portugal	AY259097
Botryosphaeria corticola	*Diplodia corticola*	CBS 112551	*Quercus suber*	A. Alves	Aveiro, Portugal	AY259101
Botryosphaeria corticola	*Diplodia corticola*	CBS 112552	*Quercus suber*	A. Alves	Aveiro, Portugal	AY259102
Botryosphaeria corticola	*Diplodia corticola*	CBS 112070	*Quercus suber*	J. Luque	Barcelona, Spain	AY259105
Botryosphaeria corticola	*Diplodia corticola*	CBS 112077	*Quercus suber*	J. Luque	Girona, Spain	AY259106
Botryosphaeria corticola	*Diplodia corticola*	CBS 112071	*Quercus ilex*	J. Luque	Girona, Spain	AY259107
Botryosphaeria corticola	*Diplodia corticola*	CBS 112072	*Quercus ilex*	J. Luque	Badajoz, Spain	AY259108
Botryosphaeria corticola	*Diplodia corticola*	CBS 112073	*Quercus suber*	J. Luque	Badajoz, Spain	AY268420
Botryosphaeria corticola	*Diplodia corticola*	CBS 112076	*Quercus suber*	J. Luque	Cádiz, Spain	AY259109
Botryosphaeria corticola	*Diplodia corticola*	CBS 112074	*Quercus suber*	J. Luque	Tempio Pausania, Italy	AY268421
Botryosphaeria obtusa	*Diplodia* sp	KJ 93.56	Hardwood shrub	G.J. Samuels	New York, USA	*AF027759*
Botryosphaeria obtusa	*Diplodia* sp	CBS 112555	*Vitis vinifera*	A.J.L. Phillips	Montemor-o-Novo, Portugal	AY259094
Botryosphaeria obtusa	*Diplodia* sp	CBS 112556	*Pyrus communis*	A.J.L. Phillips	Monte da Caparica, Portugal	AY259096
Botryosphaeria obtusa	*Diplodia* sp	ATCC 60851	*Prunus persica*	K. Britton	Georgia, USA	*AF243408*
Botryosphaeria obtusa	*Diplodia* sp	Sano 1	*Malus domestica*	T. Sano	USA	*AB034812*
Botryosphaeria quercuum	Unnamed	CBS 177.89	*Quercus cerris*	A. Vannini	Italy	*AF243399*
Botryosphaeria rhodina	*Lasiodiplodia theobromae*	CBS 356.59	*Theobroma cacao*	E. Müller	Agalawatta, Sri Lanka	*AF243400*
Botryosphaeria rhodina	*Lasiodiplodia theobromae*	ZS 96-112	*Pinus radiata*	W. Swart	South Africa	*AF243401*
Botryosphaeria rhodina	*Lasiodiplodia theobromae*	KJ 93.40	*Pistacia* sp.	T.J. Michailides	California, USA	*AF027760*
Botryosphaeria stevensii	*Diplodia mutila*	CBS 678.88	*Quercus suber*	J. Luque	Girona, Spain	AY259104
Botryosphaeria stevensii	*Diplodia mutila*	KJ 93.35	*Quercus suber*	K.A. Jacobs	Spain	*AF027754*

Botryosphaeria stevensii	*Diplodia mutila*	ATCC 60259	*Malus pumila*	H.J. Boesewinkel	Unknown	*AF243406*
Botryosphaeria stevensii	*Diplodia mutila*	CBS 112553	*Vitis vinifera*	A.J.L. Phillips	Montemor-o-Novo, Portugal	AY259093
Botryosphaeria stevensii	*Diplodia mutila*	ZS 94-3	*Juniperus* sp.	N. Tisserat	USA	*AF243403*
Botryosphaeria tsugae	*Sphaeropsis* sp.	CBS 418.64	*Tsuga heterophylla*	A. Funk	British Columbia, Canada	*AF243405*
Botryosphaeria sp.	*Diplodia quercina*	KJ 93.29	*Quercus* sp.	E. Hecht-Poinar	USA	*AF207753*
Botryosphaeria sp.	Unnamed	KJ 93.58	*Tsuga* sp.	G.J. Samuels	North Carolina, USA	*AF027755*
Unknown	*Sphaeropsis sapinea* f.sp. *cupressi*	ZS 94-158	*Cupressus sempervirens*	W. Swart	Israel	*AF243402*
Unknown	*Sphaeropsis sapinea*	KJ 93.31	*Pinus* sp.	M. de Kam	Netherlands	*AF027756*
Unknown	*Diplodia* sp	M 17-3-98	*Pinus sylvestris*	S. Schroeder	Lower Saxony, Germany	*AJ292761*
Unknown	*Sphaeropsis sapinea*	ZS 92-43	*Pinus sylvestris*	G. R. Stanosz	Wisconsin, USA	*AF243409*
Botryosphaeria corticis	*Fusicoccum* sp.	ATCC 22927	*Vaccinium* sp.	R.D. Millholland	North Carolina, USA	*AF243397*
Botryosphaeria dothidea	*Fusicoccum aesculi*	KJ 93.09[e]	*Cercis canadensis*	K.A. Jacobs	District of Columbia, USA	*AF027752*
Botryosphaeria dothidea	*Fusicoccum aesculi*	ZS 97-23	*Liquidambar styraciflua*	K. Britton	South Carolina, USA	*AF241174*
Botryosphaeria dothidea	*Fusicoccum aesculi*	KJ 94.23	*Malus sylvestris*	P.L. Pusey	Georgia, USA	*AF027747*
Botryosphaeria dothidea	*Fusicoccum aesculi*	KJ 94.26	*Prunus persica*	P.L. Pusey	Japan	*AF027749*
Botryosphaeria dothidea	*Fusicoccum aesculi*	CBS 110302	*Vitis vinifera*	A.J.L. Phillips	Montemor-o-Novo, Portugal	AY259092
Botryosphaeria lutea	*Fusicoccum luteum*	**CBS 110299**	*Vitis vinifera*	A.J.L. Phillips	Oeiras, Portugal	AY259091
Botryosphaeria lutea	*Fusicoccum luteum*	ATCC 58194	*Actinidia chinensis*	G.J. Samuels	New Zealand	*AF243396*
Botryosphaeria mamane	*Fusicoccum* sp.	ZS 97-58	*Sophora chrysophylla*	D.E. Gardner	Hawaii	*AF246929*
Botryosphaeria parva	*Fusicoccum parvum*	CBS 110301	*Vitis vinifera*	A.J.L. Phillips	Palmela, Portugal	AY259098
Botryosphaeria parva	*Fusicoccum parvum*	ATCC 58189	*Malus sylvestris*	G.J. Samuels	New Zealand	*AF243395*
Botryosphaeria ribis	*Fusicoccum* sp.	KJ 94.09	*Melaleuca quinquenervia*	M.B. Rayachhetry	Florida, USA	*AF027743*
Guignardia bidwellii	*Phyllosticta* sp.	ZS 97-105	*Vitis* sp.	A.B. Baudoin	New York, USA	*AF216533*
Mycosphaerella africana	Unknown	CMW 3025	*Eucalyptus viminalis*	P. W. Crous	Stellenbosch, South Africa	*AF283690*

[a]Fungus names are those used by the collector in the original publication in which they were mentioned, or in GenBank.
[b]Designation of isolates and collection numbers: CBS = Centraalbureau voor Schimmelcultures; ATCC = American Type Culture Collection; KJ = Jacobs and Rehner (1998); ZS = Zhou and Stanosz (2001b).
[c]ITS sequences represented in italics were obtained from GenBank, the other sequences were determined in the present study.
[d]Isolates in bold are ex-type.
[e]KJ 93.09 was referred to as *B. obtusa* by Jacbos & Rehner (1998) but as *B. dothidea* in the GenBank accession data. In the present paper, ITS sequence data identified it as *B. corticola*.

To each tube 500 μL of GES buffer (5 M guanidine thiocyanate, 100 mM EDTA, pH 8.0; 0.5 % Sarkosyl) were added, mixed by inversion and placed on ice for 5 min. Then 250 μL of cold 10 M ammonium acetate was added and the tubes were placed on ice for another 5 min. The aqueous phase was extracted with 1 volume of CIA (chloroform:isoamyl alcohol; 24:1 v:v) and nucleic acids were precipitated with 1 volume of cold isopropanol. After centrifugation the pellet was washed with 70 % ethanol and dissolved in 500 μL of TE buffer containing RNase (50 μg/mL) followed by incubation at 37 ºC for 30 min. The aqueous phase was again extracted with CIA and nucleic acids were precipitated with 1/10 volume of cold 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold 100 % ethanol. DNA was recovered by centrifugation and the pellet washed with 70 % ethanol. DNA was dissolved in 100 μL TE buffer and stored at -20 ºC. DNA concentrations were estimated by spectrophotometry (Cary 50 Bio, Varian, Mulgrave, Australia).

PCR amplification of the nuclear 5.8S ribosomal RNA gene and its flanking ITS regions was performed on a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA). The PCR primers ITS1 and ITS4 (White *et al*., 1990) were supplied by MWG Biotech AG (Ebersberg, Germany). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania). The PCR reaction mixtures contained 1 × PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with $(NH_4)_2SO_4$; 1:1 v:v), 3 mM $MgCl_2$, 200 μM of each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50–100 ng of template DNA. Each reaction volume was made up to 50 μl with sterile HPLC-grade water. Negative controls with sterile water instead of the template DNA were used in every PCR reaction. The amplification conditions were as follows: initial denaturation of 7 min at 95 ºC, followed by 25 cycles of 1 min at 94 ºC, 1 min at 50 ºC, and 1 min at 72 ºC, and a final extension period of 10 min at 72 ºC.

After amplification, 5 μL of each PCR product were separated by electrophoresis in 1 % agarose gels in 1 × TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, and visualized on a UV transilluminator to assess PCR amplification. The amplified PCR fragments were purified with the CONCERT Rapid PCR Purification System (Gibco BRL, Eggenstein, Germany) prior to DNA sequencing.

DNA sequencing

Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler.

Cycle sequencing reactions (20 μL) contained 30–90 ng DNA template, 3.2 pmol of each primer, 4 μL BigDye™ Terminator and sterile HPLC-grade water. The cycle sequencing program was as follows: 25 cycles of 96 ºC for 10 s, 50 ºC for 5 s, and 60 ºC for 4 min. To remove excess dye terminator the entire content of each extension reaction was mixed thoroughly with 2 μL of 3 M sodium acetate (pH 4.6) and 50 μL of 100 % ethanol. Extension products were precipitated for 20 min at room temperature and centrifuged for 20 min at 16000 × *g*. The supernatant was discarded and 250 μL of 70 % ethanol was added. The tubes were vortexed, centrifuged for 5 min at 16000 × *g* and the supernatant discarded. Finally the tubes were dried in a heat block at 90 ºC for 1 min.

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The complete sequences of the ITS region (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database.

Phylogenetic analysis

DNA sequences from this study and those retrieved from GenBank were aligned with MegAlign (DNASTAR Inc, Madison, Wisconsin). The alignments were checked visually and improved manually where necessary. In the analyses, alignment gaps were treated as missing data. Sequences of *Guignardia bidwellii* (Ellis) Viala & Ravaz and *Mycosphaerella africana* Crous & M. J. Wingf. were used as outgroup. Phylogenetic analyses were performed with PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2000). Trees were produced using both neighbor-joining (NJ) and maximum parsimony (MP) analyses for the ITS sequence dataset. The kimura-2-parameter distance calculation was used for NJ analysis. For MP analysis, the heuristic search option with 1000 random addition sequences and TBR branch-swapping options were used. Stability of clades was assessed with 1000 bootstrap replications in a heuristic search. Other measures used were tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI).

RESULTS

Morphology and cultural characteristics

All isolates from oak, irrespective of whether they were derived from single ascospores or conidia, were morphologically similar. Colonies on PDA formed abundant aerial mycelium that was initially white, but turned dark-olivaceous after 5–6 d at 25º C. The reverse side was almost black in older cultures. Pycnidia were usually aggregated and appeared after 20–25 d in cultures incubated under black light with a 12 h photoperiod. Cultures of the isolate from *Vitis* (CBS 112553) that was tentatively identified as *D. mutila* were similar, except that pycnidia were produced after 14–21 d under the same conditions.

Ascospores of the strains from oak were (28.2–)33.6–34.7(–40.6) × (12.0–)14.6–15.3(–18.8) µm. In contrast, ascospores of the specimen of *B. stevensii* we studied (BPI599153) were (24.8–)30.8–32.1(–36.2) × (9.5–)11.2–11.7(–13.4) µm. Conidia of the isolates from oak were (23.7–)29.6–30.3(–46.1) × (9.1–)13.4–13.8(–20.5) µm, which is considerably larger than was found in *D. mutila* K(M)99664, which has conidia (23.5–)25.1–25.7(–27.4) × (12.4–)13.2–13.5(–14.3) µm. Conidium dimensions of the strain of *D. mutila* isolated in this work (CBS 112553) were similar to K(M)99664. Conidiomata and ascomata of the collections from oak were mostly multilocular but conidiomata of *B. stevensii* were mostly unilocular. The teleomorph of *B. stevensii* was not found in this work.

Phylogenetic analysis

ITS sequences of 37 strains of *Botryosphaeria* species, either sequenced in this study or retrieved from GenBank, were included in the phylogenetic analysis. The sequence alignment is available from TreeBase (SN1445). Of the 549 characters in the ITS data set, 338 were constant, 53 variable characters were parsimony-uninformative, and 158 were parsimony informative. The 26 equally parsimonious trees generated from the heuristic search exhibited low levels of homoplasy as indicated by a consistency index (CI) of 0.848, a retention index (RI) of 0.940 and a homoplasy index (HI) of 0.152. The topology of the trees differed from one another only in the positions of isolates within terminal groupings. Tree topologies resulting from maximum parsimony and neighbor joining analyses were similar and only the former is shown (FIG. 1).

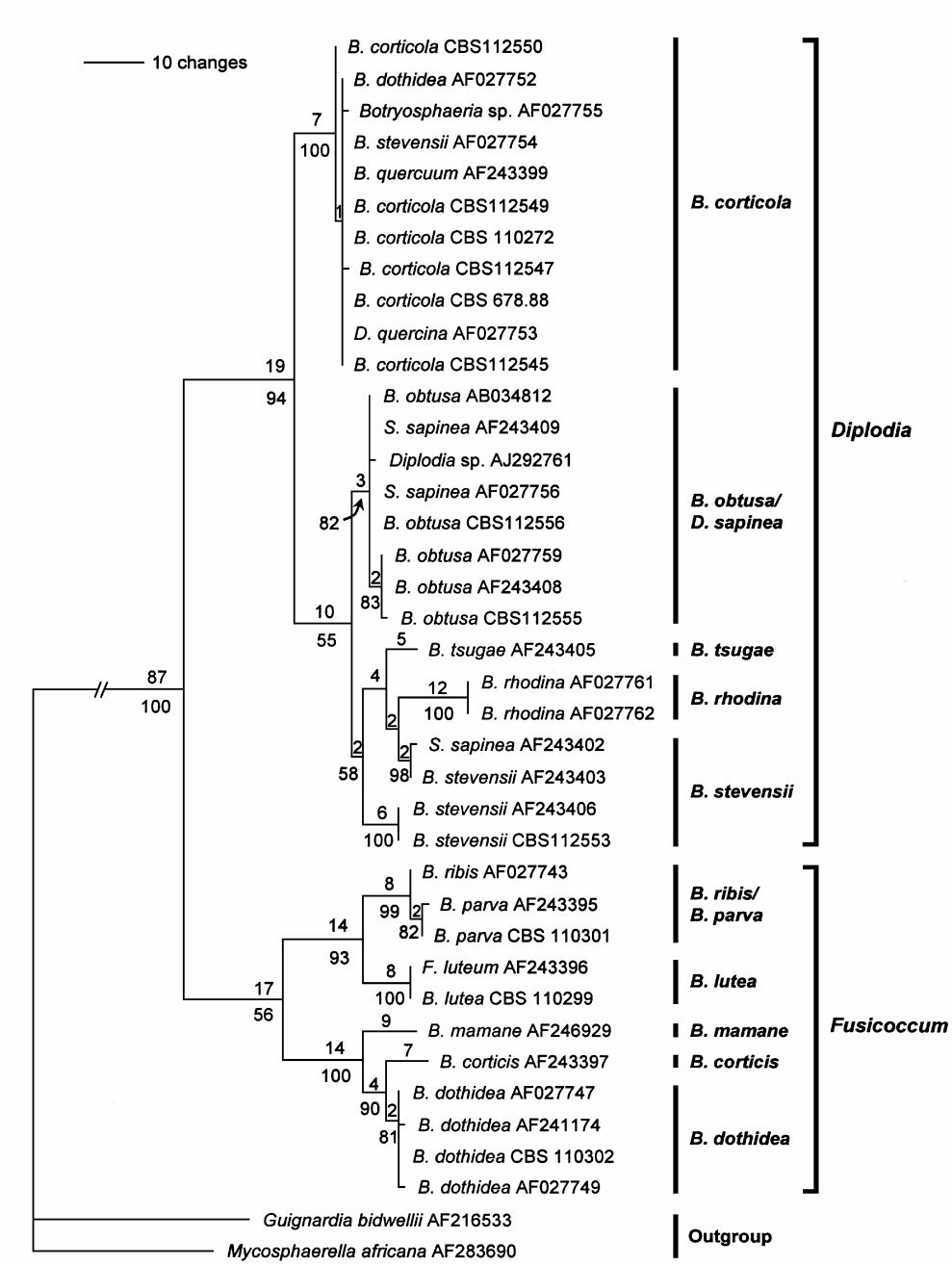


FIGURE 1. Phylogenetic relationships amongst *Botryosphaeria corticola* and other *Botryosphaeria* species based on maximum parsimony analysis of combined ITS1, 5.8S and ITS2 sequence data. Bootstrap percentages are given below the branches (1000 replicates) and branch lengths proportional to the number of changes are given above the branches. GenBank accession numbers are listed for isolates not sequenced in this study.

Two major clades were resolved corresponding to *Botryosphaeria* species with *Fusicoccum* or *Diplodia* anamorphs. Within the *Fusicoccum* clade, five groups were resolved and these corresponded to five *Botryosphaeria* species that are generally accepted to have *Fusicoccum* anamorphs. Five groups were also resolved within the *Diplodia* clade, and four of these corresponded to known species of *Botryosphaeria*. The strains that were isolated from oaks, which we describe here as *B. corticola*, clustered with isolates from oaks retrieved from GenBank, namely AF027754 (KJ 93.35 as *B. stevensii*), AF027753 (KJ 93.29 as *Diplodia quercina* Westend.) and AF243399 (CBS 177.89 as *B. quercuum*). Two isolates from hosts other than *Quercus* also lay within this sub-clade, namely, AF027755 (KJ 93.58 as *Botryosphaeria* sp. from *Tsuga* sp.) and AF027752 (KJ 93.09 as *B. dothidea* (Moug. : Fr.) Ces. et De Not. from *Cercis canadensis* L.). This sub-clade was supported by a high bootstrap value of 100 %, and was distinct from the sub-clade containing isolates considered to be *B. stevensii*. Isolates previously identified as *B. stevensii* were distributed in two separate sub-clades, with the isolates from apple and grapevine (AF243406 and CBS 112553) grouping together while an isolate from *Tsuga* sp. grouped in a separate sub-clade with *Sphaeropsis sapinea* f. sp *cupressi* from *Cupressus sempervirens* L. These groups were supported by high bootstrap values (> 98 %).

Other major sub-clades in the *Diplodia* clade corresponded to *B. obtusa* (Schwein.) Shoemaker, *B. rhodina* (Cooke) Arx, and *B. tsugae* Funk. The *B. obtusa* clade contained isolates that had been identified as *B. obtusa* and others identified as *Diplodia pinea* (Desm.) J. Kickx f. (=*Sphaeropsis sapinea* (Fr. : Fr.) Dyko & Sutton).

Based on morphological features as well as DNA phylogeny, we consider the isolates from oaks to represent a new species. We describe it here as *Botryosphaeria corticola*.

TAXONOMY

Botryosphaeria corticola A.J.L. Phillips, Alves et Luque, sp. nov. FIGS. 2–18

Anamorph: ***Diplodia corticola*** A.J.L. Phillips, Alves et Luque, sp. nov.

Ascostromata in contextu hospitis inclusa, usque 1 mm diametro, erumpescentia, solitaria, stromatiformis, multilocularis, atrobrunnea vel nigra, cum ostiolis centralibus. Asci clavati, inter pseudoparaphyses filiformes interspersi, 160–250 × 30–33 µm, octosporati, bitunicati cum loculo apicali bene evoluto. Ascosporae irregulariter biseriatae, hyalinae, unicellulares, (28.2–)33.6–34.7(–40.6) × (12.0–)14.6–15.3(–18.8) µm, fusoides vel rhomboides, medio latissimae, fundis obtusis, apicibus obtusis vel subobtusis. Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia,

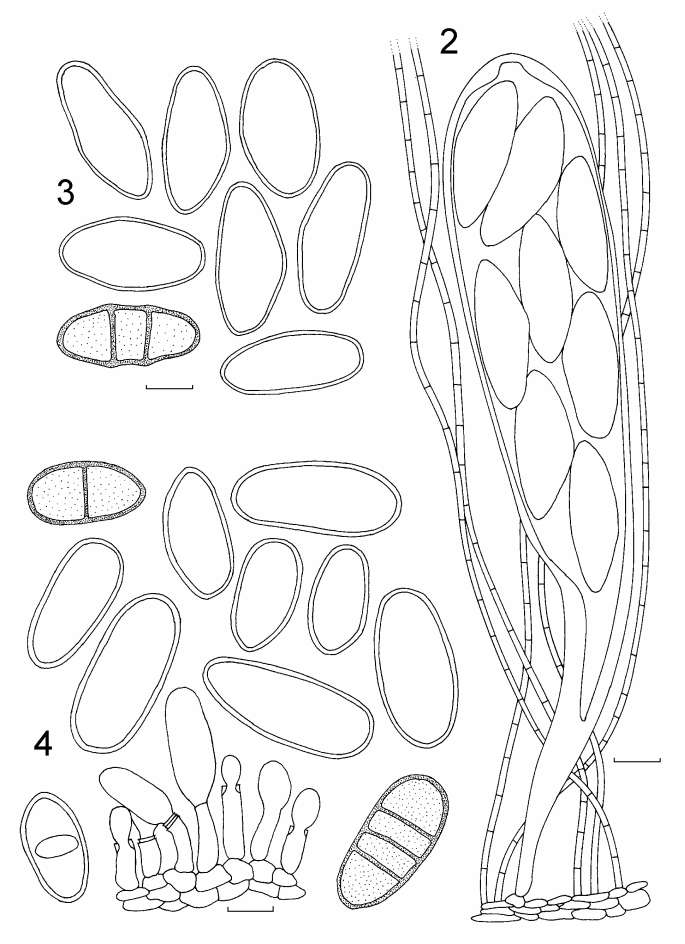
globosa, usque 1 mm diametro. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 12–19(–24) × 4–6 µm, percurrenter cum 2–3 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia hyalinae, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, (23.7–)29.6–30.3(–46.1) × (9.1–)13.4–13.8(–20.5) µm.

Pseudothecia stromatic, immersed, partially erumpent when mature, dark brown to black, more or less circular, up to 1 mm diam, multiloculate, individual locules 200–300 µm diam, thick-walled, wall composed of outer layers of thick-walled, dark brown *textura angularis*, inner layers of thin-walled, hyaline *textura angularis*. Ostiole circular, central, papillate, periphysate. Pseudoparaphyses hyaline, branched, septate, 2–3 µm wide. Asci 160–250 × 30–33 µm (including stipe), clavate, stipitate, bitunicate, containing eight, biseriate ascospores. Ascospores (28.2–)33.6–34.7(–40.6) × (12.0–)14.6–15.3(–18.8) µm (mean ± S.D. of 90 ascospores = 34.1 ± 2.5 × 14.9 ± 1.6 µm), L/W ratio of 2.3 ± 0.2, broadly fusiform to rhomboid, widest in the middle, both ends obtuse, hyaline, moderately thick-walled (ca. 1 µm), smooth-walled, aseptate, rarely becoming light brown and 1- or 2-septate with age. Conidiomata eustromatic, immersed, partially erumpent when mature, dark brown to black, more or less circular, up to 1 mm diam, multiloculate, individual locules 200–300 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, and an inner layer of thin-walled hyaline cells. Ostiole central, circular, papillate. Conidiophores absent. Conidiogenous cells 12–19(–24) × 4–6 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 annelations. Conidia (23.7–)29.6–30.3(–46.1) × (9.1–)13.4–13.8(–20.5) µm, mean and standard deviation of 250 conidia = 29.9 ± 2.6 × 13.6 ± 1.4 µm, length/width ratio (average of 250 conidia) = 2.2 ± 0.3, hyaline, aseptate, eguttulate or sometimes with a large central guttule, contents granular, smooth, thick-walled, oblong to cylindrical, straight, both ends broadly rounded, rarely becoming brown and septate when aged. Colonies 36–44 mm diam on PDA after 4 d in the dark at 25 ºC. Cardinal temperatures for growth were min 5 ºC, max below 35 ºC, opt 20–25 ºC.

Etymology: Derived from the Latin root for the word for bark in reference to its habitat on its main host, the cork oak (*Quercus suber*).

Hosts: *Quercus* species and possibly other hosts.

Distribution: Iberian Peninsula, Italy, N. America.



FIGURES 2–4. *Botryosphaeria corticola* and its anamorph *Diplodia corticola*. 2. Ascus and pseudoparaphyses of LISE 94840. 3. Ascospores of LISE 94840. 4. Conidia and conidigenous cells of CBS 112549 on oatmeal agar. Scale bars = 10 µm. (drawings by Dr. Alan J.L. Phillips).

HOLOTYPE of *B. corticola*. PORTUGAL. BEIRA LITORAL: Requeixo near Aveiro, on dead branches of *Quercus suber* L., February 2002, *A. Alves* (LISE 94840, culture ex type CBS 112549). HOLOTYPE of *D. corticola*. PORTUGAL. BEIRA LITORAL: Requeixo near Aveiro, on dead branches of *Quercus suber* L., February 2002, *A. Alves*, (LISE 94839, culture ex type CBS112549).

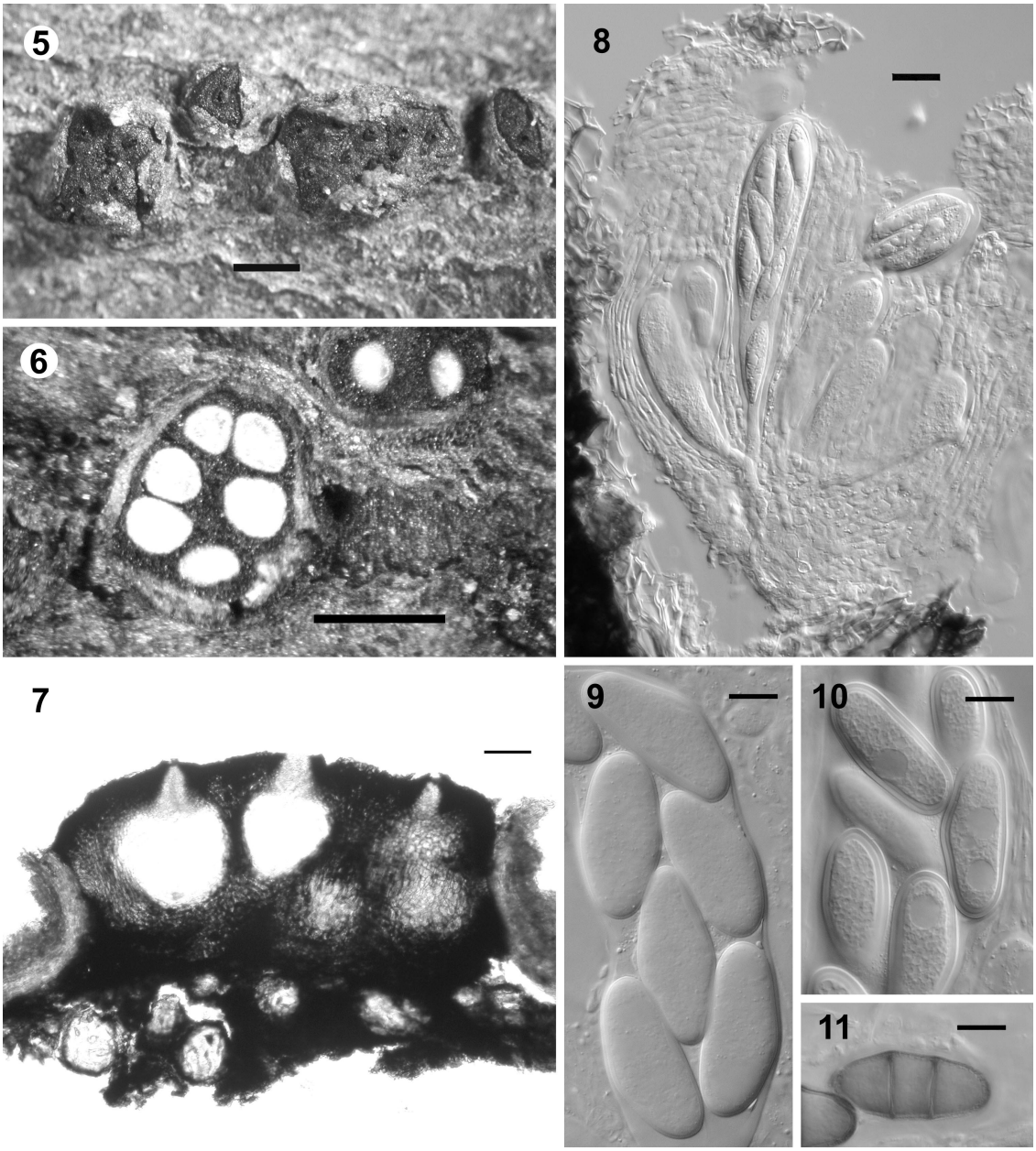
Additional cultures examined: ITALY. SARDINIA: Tempio Pausania, on trunk of *Q. suber*, February 2001, *J. Luque & A. Franceschini*, (CBS 112074). PORTUGAL. BEIRA LITORAL. Requeixo near Aveiro, on dead branch of *Quercus suber* L., February 2002, *A. Alves*, (CBS 112550, CBS 112551, CBS 112548, CBS 112552). SPAIN. ANDALUSIA: Cádiz, cankered branch of *Q. ilex*, July 2000, *M.E. Sanchez & A. Trapero*, (CBS 112545); Huelva, branch of *Q. ilex*, August 2000, *M.E. Sanchez & A. Trapero*, (CBS 112546); Córdoba, branch of *Q. ilex*, August 2000, *M.E. Sanchez & A. Trapero*, (CBS 112547). CATALONIA: Girona, Vidreres, trunk bark of *Q. suber*, March 1989, *J. Luque*

(CBS 678.88), May 1994, *J. Luque* (CBS 112077), Girona, Sant Feliu De Buixalleu, dead branch of *Quercus ilex* L., May 1995, *J. Luque* (CBS 112071); Barcelona, Vallgorguina, trunk bark of *Quercus suber*, January 1992, *J. Luque* (CBS 112070), trunk canker of *Q. suber*, December 2000, *J. Luque* (CBS 112076). EXTREMADURA: Badajoz, Valdebotoa, dead branch of *Q. ilex*, August 2000, *J. Luque*, (CBS 112072), Villar Del Rey, trunk canker of *Q. suber*, August 2000, *J. Luque* (CBS 112073).

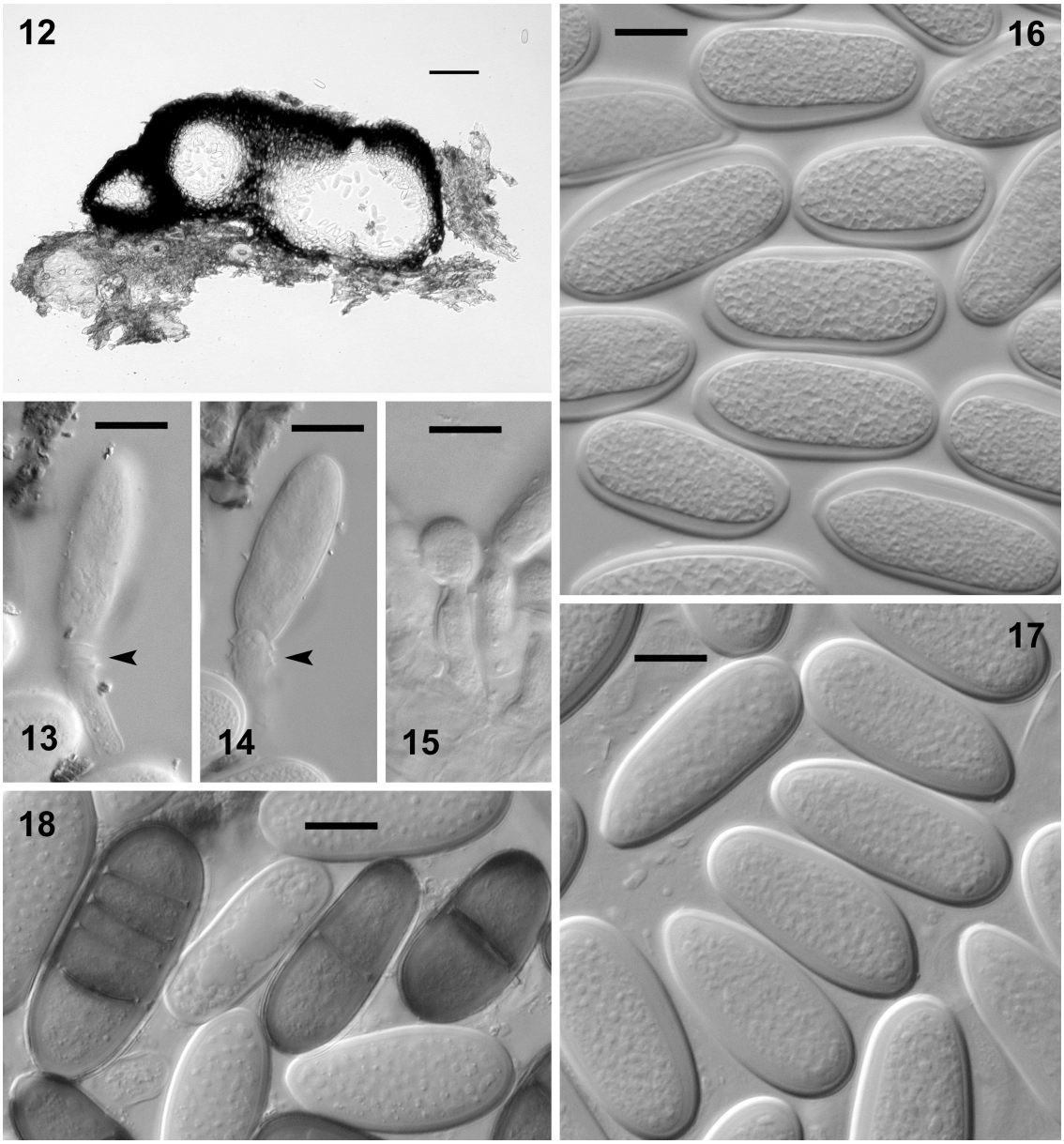
Because of the similarity of *B. corticola* to *B. stevensii*, and to clarify some taxonomic uncertainties in the latter species and its anamorph (*D. mutila*) we studied material filed under *B. stevensii* in BPI, and *D. mutila* in STR and K.

Fries (1823) described *Sphaeria mutila* and distributed two exsiccati under that name as *Scler. Suec.* 164 and 385. We examined material of these two exsiccati in STR and found both to be devoid of spores. Stevens (1933) and Sutton (1980) also reported that these two exsiccati in BPI and K had no spores. Sutton (1980) reported that 164 was an ascomycete of the *Botryosphaeria* type and pointed out that *Sphaeria mutila* should be adopted for the ascomycetous element it represents. Montagne sent Fries a fungus which 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), it is typified by his material and the correct citation is *Diplodia mutila* Fr. in Montagne (1834).

Montagne distributed this fungus in his exsiccatus Nº 498. According to Françoise Deluzarche of the Institut de Botanique, Strasbourg, France, no material of this could be found in STR. However, Montagne's specimen of *D. mutila* in Kew, K(M)99664 (isotype), was examined and agreed in all aspects with Stevens (1933) account of Montagne's exs. 498 but differed from the description given by Sutton (1980). While Sutton (1980) referred to the conidia as initially hyaline with a large central guttule, later becoming dark brown and medianly 1 eusepate, we found that the vast majority of conidia in K(M)99664 were hyaline and aseptate, although pale brown and one- or two-septate conidia were rarely seen. The conidia usually had a large central guttule. Furthermore, the dimensions that Sutton (1980) reported (27–31 × 12–13.5) are somewhat larger than we found (23.5–27.5 × 12–14). Stevens (1933) gave the conidia as (20–)25–27 × 10–12(–16) µm. In these respects, our findings from this specimen correspond more closely to Stevens' (1933) description of this species than to Sutton's (1980) account.



Figures 5–11. *Botryosphaeria corticola* LISE 94840. 5. Ascomata partially erumpent through the host bark. 6. Multilocular ascoma cut through horizontally showing the white contents typical of *Botryosphaeria* of six loculi. 7. Vertical section through an ascostroma showing the thick wall and three loculi opening through periphysate ostioles. 8. A mature ascus containing ascospores, several immature asci and pseudoparaphyses. 9, 10. Ascospores. 11. A brown, 2-septate ascospore. Scale bars: 5 = 1 mm, 6 = 0.5 mm, 7 = 100 µm, 8 = 20 µm, 9–11 = 10 µm.



FIGURES 12–18. *Diplodia corticola*. 12. Sectioned conidiomata of LISE 94839. 13, 14. Percurrently proliferating conidiogenous cell (CBS 112549) in surface view (13) and optical section (14) with annelations arrowed. 15. Phialide of CBS 112549. 16. Conidia (CBS 112549). 17. Conidia (CBS 112077). 18. Brown and septate conidia (CBS 112077). Scale bars: 12 = 100 µm, 13–18 = 10 µm.

When Stevens (1936) described *Physalospora mutila* he referred to a specimen on a cut twig of *Fraxinus excelsior* L. collected from Saltash, Cornwall, England as the type specimen. A specimen fitting this description (BPI 599151) was examined. Unfortunately, no ascomycete could be found, but the anamorphic fungus on this specimen corresponded in all ways with *Diplodia mutila* K(M)99664. There was, however, ample material of the teleomorph on BPI 599153, which is a specimen on apple collected by Stevens from the same locality, and at the same time as he collected BPI 599151. This specimen (BPI 599153) is designated here as lectotype of *P. mutila*.

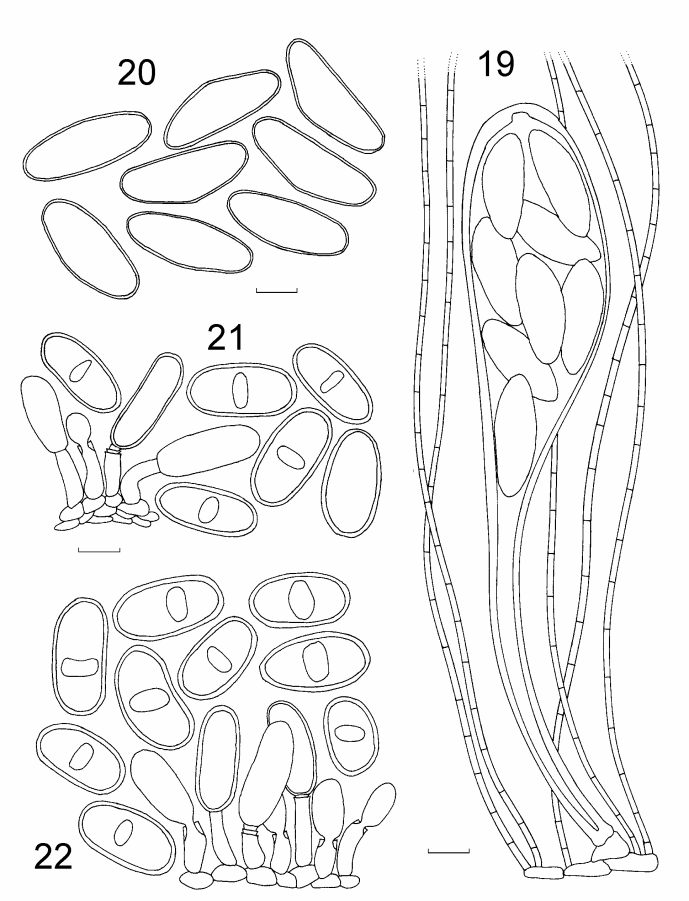
Botryosphaeria stevensii Shoemaker, Can. J. Bot. 42: 1299. 1964. FIGS. 19–38
= *Physalospora mutila* N. E Stevens, Mycologia 28: 333. 1936, as *Physalospora mutila* (Fries) N. E. Stevens comb. nov.
Anamorph: ***Diplodia mutila*** Fries in Montagne, *Ann. Sci. nat.*, sér. 2, 1: 302 (1834).
≡ *Sphaeria mutila* Fries, Syst. Mycol. 2: 424–425. 1823.

Synonyms of *D. mutila* are given by Stevens (1933). The following description is based on BPI 599153 (*P. mutila*) and K(M)99664 (*D. mutila*).

Ascomata unilocular, solitary or clustered, immersed, partially erumpent when mature, globose, up to 300 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, papillate, periphysate. Pseudoparaphyses hyaline, branched, septate, 2–3 µm wide. Asci clavate, stipitate, bitunicate, 100–160 × 14–22 µm (including stipe), containing eight, biseriate ascospores. Ascospores (24.8–)30.8–32.1(–36.2) × (9.5–)11.2–11.7(–13.4) µm (mean ± S.D. of 50 ascospores = 31.5 ± 2.3 × 11.4 ± 0.9 µm) with length width ratio of 2.8 ± 0.3, fusiform, widest in the middle, both ends obtuse, hyaline, thin-walled, smooth, aseptate, rarely becoming light brown and 1- or 2-septate with age.

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–)12–15 × 4–5 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 annelations. Conidia hyaline, aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded,

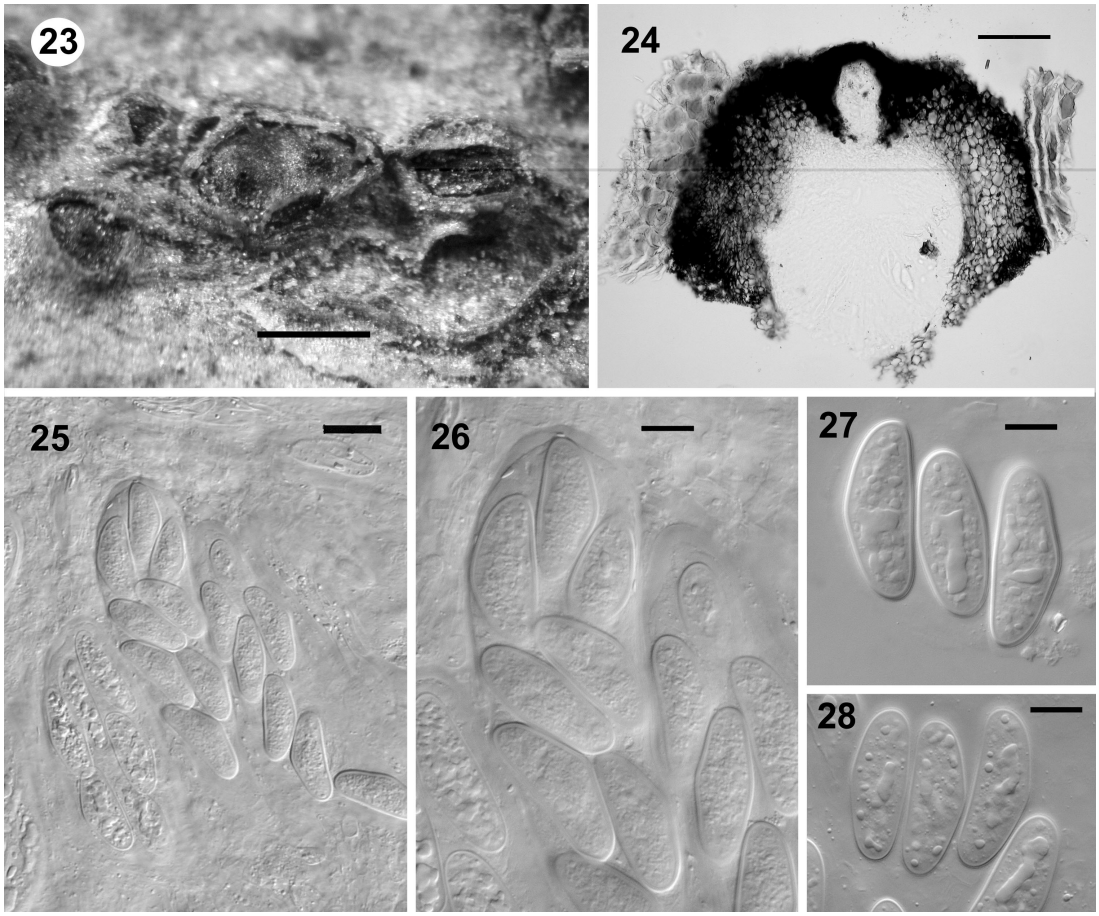
(23.5–)25.1–25.7(–27.4) × (12.4–)13.2–13.5(–14.3) µm, mean and standard deviation of 50 conidia = 25.4 ± 1.0 × 13.4 ± 0.5 µm, length/width ratio (average of 50 conidia) = 1.9 ± 0.1, rarely becoming pale brown and septate when aged.



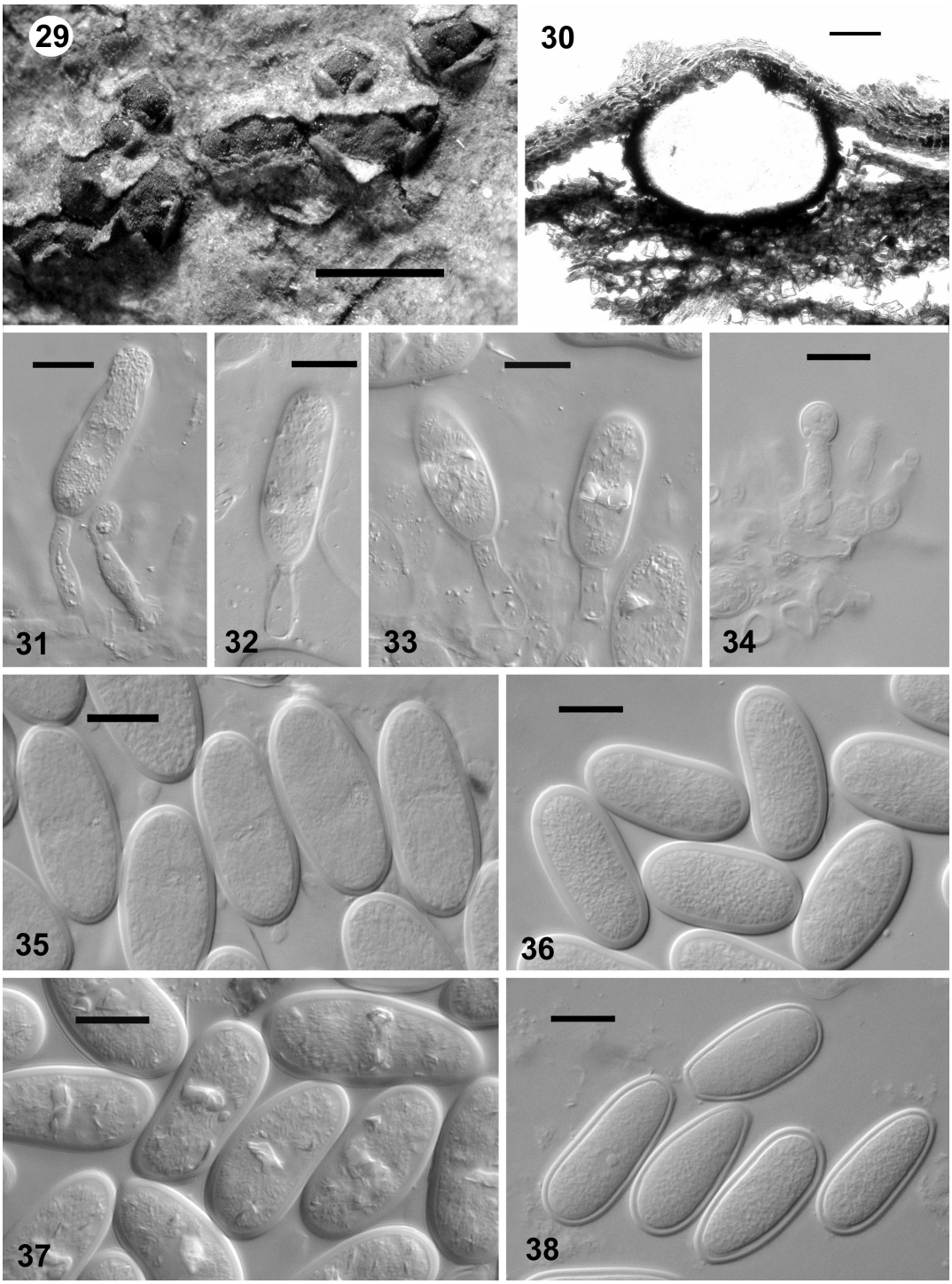
FIGURES 19–22. *Botryosphaeria stevensii* and its anamorph *Diplodia mutila*. 19. Ascus and pseudoparaphyses (BPI 599153). 20. Ascospores (BPI 599153). 21. Conidia and conidiogenous cells (BPI 599153). 22. Conidia and conidiogenous cells (K(M)99664). Scale bars = 10 µm. (drawings by Dr. Alan J.L. Phillips).

Culture examined: PORTUGAL. ALENTEJO. Montemor-o-Novo, *Vitis vinifera*, 1996, *A. J. L. Phillips* (CBS 112553).

Specimens examined: *Physalospora mutila*. ENGLAND. CORNWALL: Saltash, on bark of *Fraxinus excelsior*, 22 August 1935, *N.E. Stevens* (BPI 599151); Saltash, on bark of *Malus* sp., 22 August 1935, *N.E. Stevens* (BPI 599153, LECTOTYPE designated herein). SURREY: Ranmore Common, on *Fraxinus* sp., 19 April 1957, *C. Booth* (BPI 599150 ex IMI 69064). *Diplodia mutila*: FRANCE. ARDENNE: Sedan, on bark of *Populus nigra*, date not known, *Montagne* (K(M)99664 ISOTYPE). *Sphaeria mutila*: Scler. Suec. 164 (STR); Scler. Suec. 385 (STR).



Figures 23–28. *Botryosphaeria stevensii* BPI 599153. 23. Ascomata partially erumpent through the host bark. 24. Sectioned ascoma. 25. Ascus with ascospores. 26. Ascus tip. 27, 28. Ascospores. Scale bars: 23 = 0.5 mm, 24 = 100 µm, 25 = 20 µm, 26–28 = 10 µm.



Figures 29–38. *Diplodia mutila*. 29. Conidiomata partially erumpent through the host bark (K(M)99664). 30. Sectioned conidioma (K(M)99664). 31–34. Conidiogenous cells: 31. K(M) 99664, 32. BPI 599151, 33. BPI 599153, 34. CBS 112553. 35–38. Conidia: 35. K(M)99664, 36. BPI 599151, 37. BPI 599153, 38. CBS 112553.

DISCUSSION

Based on morphological characters and nrDNA ITS sequences we conclude that the *Botryosphaeria* that occurs mainly on oaks is different from *B. stevensii* and that it represents a previously undescribed species, namely *B. corticola*. It closely resembles both *B. stevensii* and *B. quercuum*, two other *Botryosphaeria* species with mostly hyaline, aseptate, and thick walled conidia, but differs in the larger ascospores and conidia, and in the shapes of the spores. Additionally, ITS sequence data clearly separate *B. corticola* from all other known species of *Botryosphaeria* and place it within the group with *Diplodia* anamorphs. It is probable that *B. corticola* has previously been identified under other names. This is reflected in the GenBank sequences accessed in this study in which strains originally identified as *B. dothidea*, *B. quercuum*, *B. stevensii* or *D. quercina* (TABLE I) had ITS sequences that grouped them with *B. corticola*. However, we were unable to confirm the identity of the species described on *Q. petraea* in Hungary by Vajna (1986), nor the *Diplodia* species that Oliva and Molinas (1986) isolated from *Q. suber* in NE Spain. Therefore it is possible that *B. stevensii* and *B. quercuum* also occur on oaks.

Differences in conidium dimensions between *B. corticola* and *B. stevensii* are most apparent in the 95 % confidence intervals and means calculated from the corresponding measurements. Furthermore, conidia of *B. stevensii* rarely exceed 30 µm in length, while those of *B. corticola* are commonly longer, and can reach 40 µm long. Differences between the two species are also seen in the teleomorph. Asci and ascospores of *B. corticola* are larger and the ascospores have a different shape from those of *B. stevensii*, but this feature may be less reliable for species differentiation than conidium characters. Although Stevens (1936) gave the ascospores of *P. mutila* (=*B. stevensii*) as 30–36(–39) × (12–)13–14(–16) µm, we found that in BPI 599153 (which Stevens regarded as *P. mutila*) they were somewhat smaller, measuring (24–)28–35(–36) × (9.5–)10–13(–13.5) µm. Ascomata and conidiomata of *B. corticola* in nature are mainly multilocular, while in *B. stevensii* they are mostly unilocular. Again, this feature may have minor taxonomic significance because the shape and form of ascomata and conidiomata varies within a species according to the substrate on which they are formed (Stevens, 1933; Witcher and Clayton, 1963; Rayachhetry *et al*., 1996; Phillips *et al*., 2002).

The fungus described here as *B. corticola* is clearly different from *B. quercuum*. Arx and Müller (1954) quoted an extensive synonymy under *B. quercuum* including *B. stevensii* (as *Physalospora mutila*) and some other species with *Fusicoccum* anamorphs. But Arx and Müller (1954) based their assessment of the genus on a study of herbarium material of the teleomorph and took no account of anamorph

characters. It has since been recognised that although teleomorphs of *Botryosphaeria* species are morphologically similar, they can be differentiated on characters of the anamorphs (Shoemaker, 1964; Laundon, 1973; Pennycook and Samuels, 1985). Thus, when Shoemaker (1964) redescribed *B. quercuum* he distinguished it from *B. stevensii* on the shape of the conidia. In *B. quercuum* the conidia are subglobose (18–)21–24(–25) × (12–)15–16(–17), L/W=1.5, but in *B. stevensii* they are oblong (L/W=1.9). This distinction was followed by Sivanesan (1984) and it is generally regarded as the character that separates the two species. The subglobose conidia of *B. quercuum* are also distinctly different from the oblong conidia of *B. corticola* (L/W=2.2).

Conidia of *D. mutila* have often been referred to as becoming brown and septate with age (Shoemaker, 1964; Laundon, 1973), but Sutton (1980) inferred that mature conidia are dark brown and septate. However, we consider that conidia of this species are hyaline, even when mature. Conidia of both *B. stevensii* and *B. corticola* can germinate when hyaline and aseptate, thus indicating that in this state they are fully mature. The pale brown and septate conidia that are occasionally seen should be regarded as atypical since they are seen only in old cultures or on material from the field whose age cannot be determined accurately. Conidia in the specimen of *D. mutila* in K(M)99664 were clearly hyaline. Sutton (1980) considered this an isotype of *D. mutila* and this was accepted here.

Denman *et al*. (2000) suggested that the name *Diplodia* should be adopted to accommodate the dark-spored anamorphs of *Botryosphaeria* species. Zhou and Stanosz (2001b) developed this idea and referred to the two main clades in the genus *Botryosphaeria* as sections *Brunnea* (species with wide, brown conidia of the Diplodia type) and section *Hyala* (species with narrow, hyaline, Fusicoccum-type conidia). However, because *B. corticola*, *B. stevensii* and *B. quercuum* have mainly hyaline conidia, the distinction between the two sections on the basis of conidium coloration is tenuous. The main difference is that in sect. *Brunnea* the conidia are more than 10 µm wide with a relatively thick-wall, while in sect. *Hyala* the conidia are less than 10 µm wide and thin-walled. These characters comply with the genera *Diplodia* and *Fusicoccum* that are already linked to these anamorphs, and at present there does not seem to be any reason to replace these genus names with section names. Furthermore, some strains of species in the *B. dothidea* complex with *Fusicoccum* anamorphs develop a brown pigment when aged. The name *Diplodia* should not be restricted to anamorphs with brown conidia, but can also apply to species with hyaline conidia, as reflected in the amended genus concept for *Diplodia* (Denman *et al*., 2000).

Percurrent proliferation in conidiogenous cells has been regarded as more typical of *Sphaeropsis* than of *Diplodia* (Sutton, 1980). Although Denman *et al*. (2000) stated that isolates of *Diplodia* produce percurrent proliferations, they did not refer to any published source for this information. However, Phillips (2002) showed annellate conidiogenous cells in the *Diplodia* anamorphs of *B. stevensii* and *B. obtusa*. In the present study we confirm that percurrent proliferation occurs in conidiogenous cells of *D. mutila* and *D. corticola*. Furthermore, proliferation at the same level resulting in periclinal thickenings was seen in both species. Therefore, the description of *Diplodia* given by Denman *et al*. (2000) should be further amended to include phialides *sensu* Sutton (1980).

The data generated from analysis of the ITS sequences of the strains isolated in this study and from sequences retrieved from GenBank revealed that *B. stevensii* is not a homogeneous species, confirming the suggestion by Zhou and Stanosz (2001a, b) that the name has been applied to more than one species. This species appears to be a complex that needs to be resolved by examining more isolates and collections. Thus, there appears to be a distinction between strains of *B. stevensii* isolated from apples and grapevines and those from Gymnosperms. This distinction suggests that these two groups may in fact represent different taxa. However, before any firm conclusions can be drawn on this, more strains from these and other hosts will have to be studied.

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CHAPTER

3

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Two new species of *Botryosphaeria* with brown, one-septate ascospores and *Dothiorella* anamorphs

ABSTRACT

Botryosphaeria sarmentorum sp. nov. and *B. iberica* sp. nov. are described and illustrated. These two species are unusual in this genus on account of their brown, one-septate ascospores. Phylogenetic analysis based on ITS and EF1-α sequences place them within the clade containing species with *Fusicoccum* anamorphs. The brown, one-septate conidia, however, do not conform to *Fusicoccum*. Therefore, phylogenetically and morphologically the anamorphs of these two species belong in a genus distinct from any of the currently accepted anamorph genera assigned to *Botryosphaeria*. Through a study of the type species of *Dothiorella* this genus is resurrected to accommodate anamorphs of *Botryosphaeria* with brown, one-septate conidia. *Botryosphaeria sarmentorum* is shown to be the teleomorph of *Diplodia sarmentorum*, which in turn is transferred to *Dothiorella*. *Otthia quercus* is transferred to *Botryosphaeria* as *B. quercicola* nom. nov.

Key words: *Botryosphaeriaceae*, *Diplodia*, translation elongation factor EF1-α, *Fusicoccum*, ITS, molecular phylogenetics, *Otthia*.

INTRODUCTION

The genus *Botryosphaeria* Ces. & De Not. was first described by Cesati and De Notaris (1863) and later emended by Saccardo (1877). Since Cesati and De Notaris (1863) did not designate a type species, Von Höhnel (1909) suggested *B. berengeriana* De Not. as lectotype, while Theissen and Sydow (1915) suggested *B. quercuum* (Schwein.) Sacc. Both proposals were rejected because these species were not included in the original description. Barr (1972) proposed *B. dothidea* (Moug. : Fr.) Ces. & De Not. since it was one of the original species described and it conforms with Saccardo's (1877) emendation. This proposal has been generally accepted and Slippers *et al.* (2004a) provided a revised description of this species based on the type specimen and fresh collections.

Botryosphaeria is well-defined morphologically and descriptions have been given by von Arx and Müller (1954), Eriksson (1981) and Sivanesan (1984). Characters of the genus are: pseudothecia thick-walled, multilocular or unilocular sometimes forming botryose clusters. They are often united with conidiomata on a common basal stroma and are embedded in the host, becoming partially erumpent at maturity. Septate pseudoparaphyses, often constricted at the septum, are frequently present in the

centrum of immature pseudothecia, but they gradually disappear as the asci develop and mature. Asci are bitunicate with a thick endotunica, stalked or sessile, clavate with a well developed apical chamber, and they develop on a broad basal hymenial layer. Ascospores are hyaline, thin-walled, aseptate and vary from fusoid to ellipsoid to ovoid and are irregularly biseriate in the ascus. Ascospores sometimes become brown and 1- or 2-septate with age (Shoemaker, 1964; Sivanesan, 1984; Denman *et al.* 2000; Alves *et al.*, 2004).

Species in *Botryosphaeria* were described largely on the basis of form of their ascomata and host association, and this led to a proliferation of names. Von Arx and Müller (1954) examined 183 taxa and reduced them to 11 species, with extensive synonymies under *B. dothidea* and *B. quercuum*, and nine new combinations. However, since von Arx and Müller (1954) did not take into account the characters of the anamorphs, and because species of *Botryosphaeria* are difficult to separate on the basis of teleomorph characters, these synonymies have not always been accepted (Shoemaker, 1964; Sivanesan, 1984; Slippers *et al.*, 2004a).

While morphology of the teleomorphs of *Botryosphaeria* species differ little between species, a wide range of morphologies are seen in their anamorphs. According to Denman *et al.* (2000) anamorphs of *Botryosphaeria* species have been assigned to 18 coelomycete genera, among which the most common are *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Corda, *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino and *Sphaeropsis* Sacc. (Sutton, 1980; Pennycook and Samuels, 1985; Samuels and Singh, 1986; Morgan-Jones and White, 1987; Slippers *et al.*, 2004a). These genera were not clearly delimited and several have been reduced to synonyms. Thus, Sutton (1980) synonymized *Macrophoma* with *Sphaeropsis* and Denman *et al.* (2000) suggested that *Sphaeropsis* and *Lasiodiplodia* are synonyms of *Diplodia*. Crous and Palm (1999) showed that *Botryodiplodia* is a *nomen dubium* and that *Dothiorella* should be regarded as an additional synonym of *Diplodia*.

Recent studies on the taxonomy of *Botryosphaeria* have employed molecular methods to reveal phylogenetic relationships between species (Jacobs and Rehner 1998) and to help resolve species complexes (Smith *et al.*, 2001; Phillips *et al.,* 2002; Denman, *et al.* 2003; Alves *et al.*, 2004; Slippers *et al.*, 2004a). In these respects, nucleotide sequences of the 5.8S nuclear ribosomal DNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) have been widely used. Phylogenies resulting from ITS sequence analyses reveal two major clades that correspond to species with *Fusicoccum* or *Diplodia* anamorphs (Jacobs and Rehner, 1998; Zhou and Stanosz, 2001). This has been interpreted as evidence for a major split in the genus

into species with hyaline, fusoid, thin-walled, usually aseptate conidia (*Fusicoccum*) and those with hyaline or colored, thick-walled, aseptate or one-septate, ellipsoid conidia (*Diplodia*) (Denman *et al.,* 2000; Zhou and Stanosz, 2001). Within these two clades, several species can be distinguished on their morphology. The *Fusicoccum* clade has been studied in detail and at least 11 phylogenetic species can be recognised (Smith *et al.,* 2001; Denman *et al.,* 2003; Slippers *et al*., 2004a, b).

In contrast, the *Diplodia* clade has received less attention and several taxonomic problems remain to be resolved. At present it is possible to recognize at least four phylogenetic species in this clade (Zhou and Stanosz, 2001; Alves *et al.,* 2004). Recently, van Niekerk *et al.* (2004) described *D. porosum* Niekerk & Crous, which they regarded as occupying a position intermediate between *Fusicoccum* and *Diplodia*.

In this paper we describe two new species of *Botryosphaeria* that are unusual in having predominantly dark brown, one-septate ascospores. Conidia of their anamorphs are thick-walled, brown and one-septate and in this respect they resembled *Diplodia sarmentorum* (Fr.) Fr. (Wollenweber, 1941; Laundon, 1973). Since the teleomorph of *D. sarmentorum* is considered to be *Otthia spiraeae* (Fuckel) Fuckel (Booth, 1958), the relationship of the two new species to *Otthia* was determined through a study of type specimens and their relationship to other *Botryosphaeria* species was determined through a study of their morphology and ITS and EF1-α sequence analysis.

MATERIALS AND METHODS

Isolates and type material

Isolations were made either by directly plating out pieces of diseased tissue after surface sterilization in 70 % ethanol for 5 mins, or by isolation from single conidia or, in one collection, from ascospores. Isolates were cultured on oatmeal agar (OA) prepared according to Anonymous (1968) and incubated at 25 ºC under daylight fluorescent tubes. Sporulation was enhanced by placing autoclaved twigs of *Populus nigra* L. on the agar surface. Growth rates were determined on Difco potato dextrose agar (PDA) plates incubated in the dark at temperatures ranging from 5–35 ºC in 5 ºC intervals.

TABLE 1. Isolates included in this study

Accession no.[1]	Species	Type of conidia	Reference	Host	Collector	Locality	Genbank	
							ITS	EF1-α
CBS 112877	*Botryosphaeria australis*	Hyaline, aseptate	Slippers *et al.* (2004b)	*Vitis vinifera*	F. Halleen	South Africa	[2]*AY343385*	*AY343346*
CBS 112872	*B. australis*	Hyaline, aseptate	Slippers *et al.* (2004b)	*V. vinifera*	F. Halleen	South Africa	*AY343388*	*AY343347*
CBS 112549[3]	*B. corticola*	Hyaline, aseptate	Alves *et al.* (2004)	*Quercus suber*	A. Alves	Portugal, Aveiro	*AY259100*	AY573227
CBS 112545	*B. corticola*	Hyaline, aseptate	Alves *et al.* (2004)	*Q. suber*	M.E. Sánchez	Spain, Cádiz	*AY259089*	AY573226
CMW 8000	*B. dothidea*	Hyaline, aseptate	Slippers *et al.* (2004a)	*Prunus* sp.	B. Slippers	Switzerland, Crocifisso	*AY236949*	*AY236898*
CBS 110302	*B. dothidea*	Hyaline, aseptate	Slippers *et al.* (2004a)	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY259092*	AY573218
CBS 110300	*B. dothidea*	Hyaline, aseptate	Slippers *et al.* (2004a)	*Populus nigra*	A.J.L. Phillips	Portugal, Braga	AY640253	AY640256
CAP 125	*B. dothidea*	Hyaline, aseptate	Slippers *et al.* (2004a)	*Olea europea*	I. Rumbos	Greece, Thessalia	AY640254	AY640257
CMW 10125	*B. eucalyptorum*	Hyaline, aseptate	Smith *et al.* (2001)	*Eucalyptus grandis*	H. Smith	South Africa, Mpumalanga	*AF283686*	*AY236891*
CMW 10126	*B. eucalyptorum*	Hyaline, aseptate	Smith *et al.* (2001)	*E. grandis*	H. Smith	South Africa, Mpumalanga	*AF283687*	*AY236892*
CBS 115041	*B. iberica*	Brown, 1-septate	This study	*Q. ilex*	J. Luque	Spain, Aragon	AY573202	AY573222
JL 384	*B. iberica*	Brown, 1-septate	This study	*Malus pumila*	J. Luque	Spain, Catalonia	AY573211	AY573231
CAP 146	*B. iberica*	Brown, 1-septate	This study	*Q. ilex*	J. Luque	Spain, Aragon	AY573203	
JL 361	*B. iberica*	Brown, 1-septate	This study	*Q. ilex*	J. Luque	Spain, Aragon	AY573205	
CBS 110299	*B. lutea*	Hyaline, aseptate	Pennycook and Samuels (1985)	*V. vinifera*	A.J.L. Phillips	Portugal, Oeiras	*AY259091*	AY573217
CMW 9076	*B. lutea*	Hyaline, aseptate	Pennycook and Samuels (1985)	*Malus* x *domestica*	S.R. Pennycook	New Zealand	*AY236946*	*AY236893*
CMW 7775	*B. obtusa*	Brown*, aseptate*	Shoemaker (1964)	*Ribes* sp.	B. Slippers, G. Hudler	USA, New York	*AY236954*	*AY236903*
CBS 112555	*B. obtusa*	Brown, aseptate	Shoemaker (1964)	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY259094*	AY573220
CMW 9080	*B. parva*	Hyaline, aseptate	Pennycook and Samuels (1985)	*P. nigra*	G.J. Samuels	New Zealand	*AY236942*	*AY236887*
CBS 110301	*B. parva*	Hyaline, aseptate	Pennycook and Samuels (1985)	*V. vinifera*	A.J.L. Phillips	Portugal, Palmela	*AY259098*	AY573221
CBS 164.96	*B. rhodina*	Brown, 1-septate	Punithalingam (1976)	Fruit on coral reef coast	A. Aptroot	Papua New Guinea, Madang	AY640255	AY640258
CMW 9074	*B. rhodina*	Brown, 1-septate	Punithalingam (1976)	*Pinus* sp.	T. Burgess	Mexico	*AY236952*	*AY236901*
CMW 10130	*B. rhodina*	Brown, 1-septate	Punithalingam (1976)	*Vitex donniana*	J. Roux	Uganda	*AY236951*	*AY236900*
CMW 7772	*B. ribis*	Hyaline, aseptate	Slippers *et al.* (2004a)	*Ribes* sp.	B. Slippers, G. Hudler	USA, New York	*AY236935*	*AY236877*

CMW 7773	*B. ribis*	Hyaline, aseptate	Slippers *et al.* (2004a)	*Ribes* sp.	B. Slippers, G. Hudler	USA, New York	*AY236936*	*AY236878*
IMI 63581b	*B. sarmentorum* (*Otthia spiraeae*)	Brown, 1-septate	Booth (1958), this study	*Ulmus* sp.	E.A. Ellis	England, Warwickshire	AY573212	AY573235
CBS 431.82	*B. stevensii*	Hyaline, aseptate	Alves *et al.* (2004)	*Fraxinus excelsior*	H.A. van der Aa	Netherlands, Maarseveen	*AY236955*	*AY236904*
CBS 112553	*B. stevensii*	Hyaline, aseptate	Alves *et al.* (2004)	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY259093*	AY573219
CBS 110496	*Diplodia porosum*	Brown, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	J.M. van Niekerk	South Africa	*AY343379*	*AY343340*
CBS 110574	*Di. porosum*	Brown, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	J.M. van Niekerk	South Africa	*AY343378*	*AY343339*
CBS 165.33	*Do. sarmentorum* (*Di. pruni*)	Brown, 1-septate	Wollenweber (1941)	*Prunus armeniaca*	R.M. Nattrass	Unknown	AY573208	AY573225
CBS 120.41	*Do. sarmentorum* (*Di.sarmentorum*)	Brown, 1-septate	Wollenweber (1941)	*Pyrus communis*	H.W. Wollenweber	Norway	AY573207	AY573224
CBS 115035	*Dothiorella iberica*	Brown, 1-septate	This study	*Q. ilex*	N. Ibarra	Spain, Aragon	AY573213	AY573228
JL 376	*Do. iberica*	Brown, 1-septate	This study	*M. pumila*	J. Luque	Spain, Catalonia	AY573204	
CBS 115039	*Do. iberica*	Brown, 1-septate	This study	*Quercus* sp.	A. Mazzaglia	Italy, Lazio	AY573210	AY573234
JL 366	*Do. iberica*	Brown, 1-septate	This study	*Quercus* sp.	I. Librandi	Italy, Lazio	AY573209	
CBS 113189	*Do. iberica*	Brown, 1-septate	This study	*Q. ilex*	M.E. Sánchez	Spain, Andalucía	AY573199	AY573230
DE 27	*Do. iberica*	Brown, 1-septate	This study	*Q. ilex*	M.E. Sánchez	Spain, Andalucía	AY573200	
JL 220	*Do. iberica*	Brown, 1-septate	This study	*Q. ilex*	N. Ibarra	Spain, Aragon	AY573215	
CBS 115040	*Do. iberica*	Brown, 1-septate	This study	*Q. ilex*	J. Luque	Spain, Catalonia	AY573214	AY573232
CBS 113188	*Do. iberica*	Brown, 1-septate	This study	*Q. suber*	M.E. Sánchez	Spain, Andalucía	AY573198	
CBS 115037	*Do. iberica*	Brown, 1-septate	This study	*Q. suber*	M.E. Sánchez	Spain, Andalucía	AY573201	AY573229
CBS 115036	*Do. iberica*	Brown, 1-septate	This study	*Q. suber*	J. Luque	Spain, Extremadura	AY573216	AY573233
CBS 115038	*Do. sarmentorum*	Brown, 1-septate	This study	*M. pumila*	A.J.L. Phillips	Netherlands, Delft	AY573206	AY573223
CBS 112878	*Fusicoccum viticlavatum*	Hyaline, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	F. Halleen	South Africa	*AY343380*	*AY343341*
CBS 112977	*F. viticlavatum*	Hyaline, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	F. Halleen	South Africa	*AY343381*	*AY343342*
CBS 110887	*F. vitifusiforme*	Hyaline, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	J.M. van Niekerk	South Africa	*AY343383*	*AY343343*
CBS 110880	*F. vitifusiforme*	Hyaline, aseptate	van Niekerk *et al.* (2004)	*V. vinifera*	J.M. van Niekerk	South Africa	*AY343382*	*AY343344*
CBS 447.68	*Guignardia philoprina*			*Taxus baccata*	H.A. van der Aa	Netherlands	*AY236956*	*AY236905*
CBS 119.25	*Cercospora apii*			*Apium graveolens*	L.J. Klotz	Unknown	*AY179949*	*AY179915*

[1]Acronyms of culture collections: CAP – A.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; DE – M.E. Sánchez, University of Córdoba, Spain; IMI – CABI Bioscience, Egham, U.K.; JL – J. Luque, IRTA, Barcelona, Spain. [2]Sequence numbers in italics were retrieved from the GenBank public database. All others were obtained in this study.
[3]Isolate accession numbers in bold signify cultures ex-type, or from samples that have been linked morphologically to the type material.

Representative isolates were deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Specimens were lodged with the herbarium of Estação Agronómica Nacional (LISE), Oeiras, Portugal.

Type material as well as other representative specimens of the studied taxa were obtained from several herbaria, including G, IMI, K, NYS, and UPS. Conidia and other structures were mounted in 100 % lactic acid and digital images recorded with a Leica DFC320 digital camera on a Leica DMR HC microscope fitted with Nomarski differential interference contrast optics. Measurements were made with the Leica IM500 measurement module. From measurements of 50 conidia and ascospores the mean, standard deviation and 95 % confidence intervals were calculated. Dimensions are given as the 95 % confidence limits with minimum and maximum dimensions in parentheses. Dimensions of other fungal structures are given as the range of at least 20 measurements.

Phylogenetic analysis

The procedures described by Alves *et al.* (2004) were used to extract genomic DNA from fungal mycelium and to amplify part of the nuclear rRNA operon using the primers ITS1 and ITS4 (White *et al.,* 1990). 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. 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). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 55 ºC, and 1½ min at 72 ºC, and a final extension period of 10 min at 72 ºC. In some cases where amplification of the EF1-α region was not accomplished, a second PCR was performed using as template 1 μl of the first PCR amplification.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described elsewhere (Alves *et al.* 2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The sequences of the ITS (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) and partial EF1-α regions were read and edited with Chromas 1.45

(http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for both DNA regions of additional *Botryosphaeria* species were taken from GenBank (TABLE 1).

The ITS and EF1-α sequences were aligned with MegAlign (DNASTAR Inc, Madison, Wisconsin) and manual adjustments were made where necessary. Phylogenetic analyses of sequence data were done using PAUP* version 4.0b10 (Swofford, 2003). All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. The ITS and EF1-α sequences were combined and a partition homogeneity test was conducted in PAUP (Swofford, 2003) to examine the possibility of a joint analysis of the two data sets. 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. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

RESULTS

Phylogenetic analysis

The ITS and EF1-α datasets contained 11 isolates with brown, one-septate conidia and a further 29 isolates, either determined in this study or retrieved from GenBank, and representing 13 other *Botryosphaeria* species (TABLE 1). The result of a partition homogeneity test (P=0.370) indicated that the ITS and EF1-α trees reflect the same underlying phylogeny, therefore the two datasets were combined in a single analysis. Sequence alignments are available from TreeBASE (SN1805) and new sequences were deposited in GenBank (TABLE 1). The combined dataset contained 873 characters, of which 304 were constant, 196 variable characters were parsimony-uninformative and 373 were parsimony-informative. Maximum parsimony analysis resulted in eight trees, one of which is shown in FIG. 1. The eight trees differed only in the positions of the isolates in the terminal clades and exhibited low levels of homoplasy as indicated by a consistency index of 0.700, a retention index of 0.888 and a homoplasy index of 0.300.

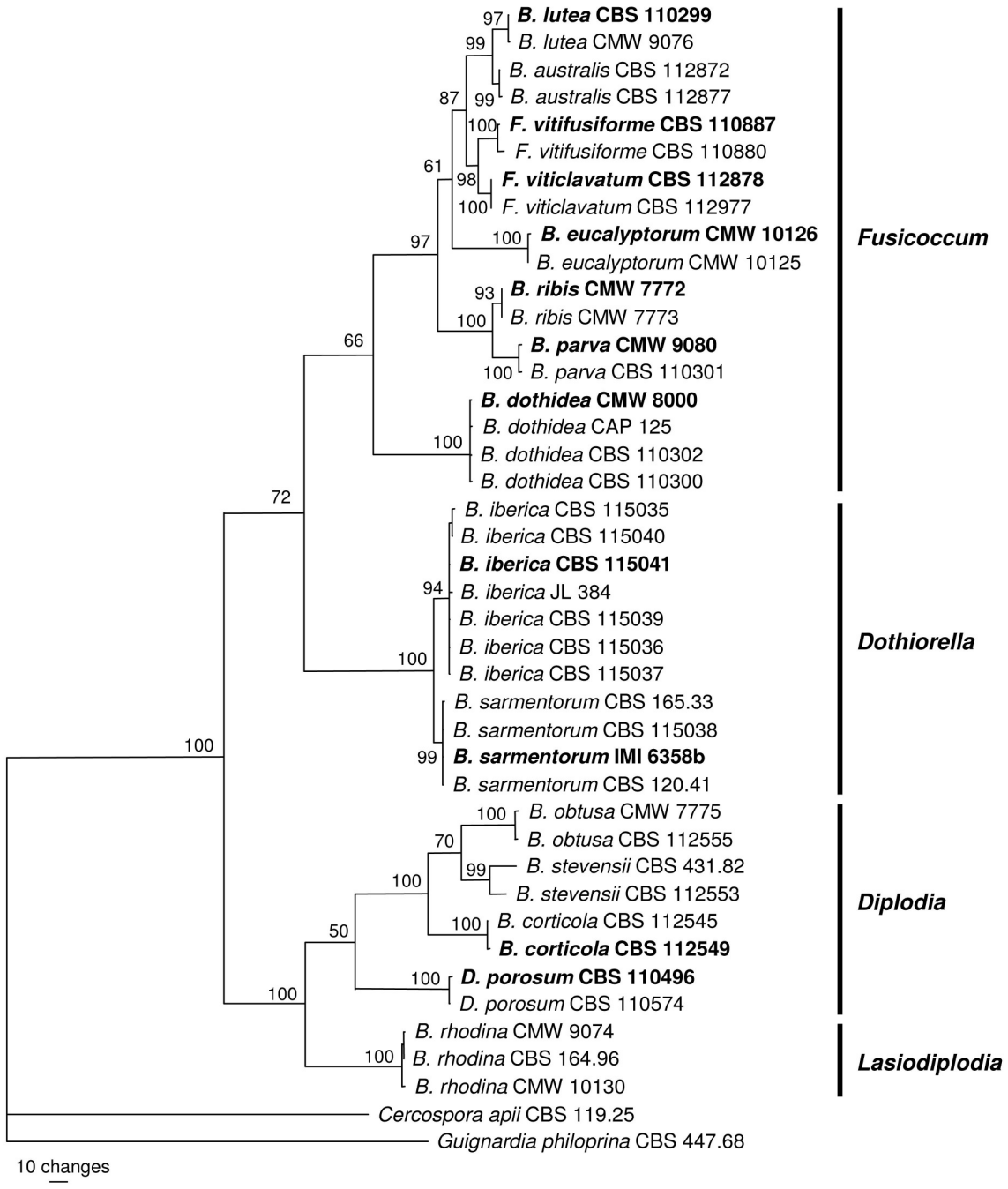


FIGURE 1. One of eight most parsimonious trees obtained from combined ITS and EF1-α sequence data. Bootstrap support values from 1000 replications are shown at the nodes. The tree was rooted to *Cercospora apii* CBS 119.25 and *Guignardia philoprina* CBS 447.68. The bar represents 10 changes.

Two major clades were resolved. One (100 % bootstrap support) corresponded to species with *Diplodia* and *Lasiodiplodia* anamorphs and this was further resolved into four *Botryosphaeria* species that are recognized in this group together with *Diplodia porosum*, which is known only from its anamorph (Van Niekerk *et al.*, 2004). The second major clade (72 % bootstrap support) was resolved into ten groups, eight of which corresponded to *Botryosphaeria* species with *Fusicoccum* anamorphs. In addition, two new species (*Botryosphaeria sarmentorum* A.J.L. Phillips, Alves and Luque and *B. iberica* A.J.L. Phillips, Alves and Luque) with anamorphs morphologically similar to *D. sarmentorum* fell within this clade. Isolates determined to be the anamorph of *B. sarmentorum* and originally recorded under the names *O. spiraeae* (IMI 63581b), *Diplodia pruni* Fuckel (CBS 165.33) and *D. sarmentorum* (CBS 120.41) grouped with an isolate from *Malus pumila* Mill. from The Netherlands (CBS 115038), while all the isolates from oaks clustered together with an isolate from *M. pumila* from the Eastern Pyrenees (JL 384). These two groups were supported by high bootstrap values of ≥ 94 % and differed by three base pairs in the ITS dataset and two substitutions and 13 insertions/deletions in the EF1-α dataset.

Morphological characterisation

A total of 19 strains with brown, one-septate conidia were isolated from *Quercus* spp. or *Malus pumila* (TABLE 1). Further strains were obtained from the CABI or CBS culture collections (TABLE 1). All strains, except CBS 120.41 and CBS 165.33 sporulated in culture. Conidiomata formed on *Populus* twigs on OA cultures were stromatic, uniloculate with a well-defined ostiole. Conidiogenesis was holoblastic with proliferation at the same level giving rise to periclinal thickenings. Very rarely they were seen to proliferate percurrently to form one or two indistinct annellations. Conidia in all strains were brown, thick-walled and one-septate with both ends rounded or occasionally with a truncate base (FIGS. 3–4, 12–13, 15, 24–27). These characters fit closely with those reported for *Diplodia sarmentorum* (Stevens, 1936; Wollenweber, 1941; Booth, 1958; Laundon, 1973). Conidia of the isolates from oaks tended to be larger (23–26 × 9–12.5 µm) than the conidia of the two isolates from *Ulmus* and *Malus* (19–23.5 × 8.5–10.5 µm). The type specimen of *Sphaeria sarmentorum* Fr. in UPS (Fries Scl. Suec. Nº 18) and an epitype (K104852) were examined. Conidia in these two specimens were brown, thick-walled, one-septate and measured 20–23 × 9–11 µm. In this respect they closely resembled the isolates from hosts other than oaks. We also examined the type of *Dothiorella pyrenophora* Sacc. (K 54912). Pycnidia of this specimen were not fully mature, but we confirm the findings

of Crous and Palm (1999) that the conidia become brown and one-septate at an early stage.

Of the 19 strains examined, two (JL 384 and CAP 146) were isolated from the centrum of immature ascomata, and one (CBS 115041) was isolated from a single ascospore. Asci of this latter collection (LISE 94944) were clavate and bitunicate with a thick endotunica (FIGS. 14, 19). Ascospores were brown, one-septate, ovoid with a broadly rounded apex and they tapered to a rounded base (FIG. 20). Pseudoparaphyses were broad and frequently septate (FIG. 18).

Since the anamorphs considered in this work were morphologically similar to *D. sarmentorum*, and the teleomorph of this is considered to be *Otthia spiraeae* (Booth 1958) we examined the lectotype species of this genus (K104853, Fuckel, Fungi Rhenani No. 975). This specimen has long, cylindrical, bitunicate asci with a thin endotunica and each ascus bears eight, obliquely uniseriate ascospores (FIG. 37). The ascospores are brown and one-septate, oval, with both ends rounded (FIG. 39) and the pseudoparaphyses are narrow and infrequently septate (FIG. 40). These morphological features differ markedly from those of LISE 94944 as described above and so the name *O. spiraeae* cannot be applied. We also examined IMI 63581b, the specimen from which Booth (1958) isolated *D. sarmentorum*. The clavate asci with a thick endotunica (FIGS. 2, 9, 10), and broad, frequently septate pseudoparaphyses (FIG. 8) of the ascomycete in IMI 63581b confirm that morphologically this specimen is not a species of *Otthia*. It is, however, similar to LISE 94944 in the brown, one-septate ascospores, but the size and shape of the ascospores separate these two collections. Thus, in IMI 63581b the ascospores are oval and do not taper to the base as in LISE 94944. Furthermore, asci of IMI 63581b contain between four and eight ascospores, while in LISE 94944 the asci are mostly eight-spored. Phylogenetically these two fungi belong in *Botryosphaeria*, and morphologically they are distinct from one another and other known species in this genus. Therefore they are described here as *B. sarmentorum* and *B. iberica*.

DISCUSSION

Botryosphaeria sarmentorum and *B. iberica* are unusual in their brown, one-septate ascospores. While ascospores in other *Botryosphaeria* species are known to occasionally become brown and one- or two-septate with age (Shoemaker, 1964; Sivanesan, 1984; Alves *et al.,* 2004), the majority remain hyaline and aseptate. In contrast, nearly all the ascospores of *B. sarmentorum* and *B. iberica* are brown and one-septate, while hyaline, aseptate ascospores were rarely seen. Furthermore,

ascospore coloration of the two new species described here was more intense than is seen in other *Botryosphaeria* species. This suggests that ascospore coloration and septation are not useful characters at the generic level.

The brown and one-septate ascospores of the two species described here could place them in *Dothidotthia* Höhn. The only differences that separates these two genera are the ascospores, which are brown and one- or two-septate in *Dothidotthia* but hyaline and aseptate in *Botryosphaeria* (Barr, 1987, 1989). The phylogenetic analysis placed both of the new species within *Botryosphaeria*, thus raising the question of the validity of *Dothidotthia*. Morphology of IMI 63581b correlates closely with the description of *Dothidotthia ramulicola* (Peck) Barr as given by Barr (1987, 1989). We examined the type specimen of *Sphaeria ramulicola* Peck (basionym of *D. ramulicola*) in NYS. The brown, phragmosporous ascospores of this specimen confirmed it to be a species of *Leptosphaeria* Ces. & De Not. Therefore the name of *L. ramulicola* (Peck) Sacc. applies and the name *D. ramulicola* could not be applied to IMI 63581b or LISE 94944.

The anamorphs of the two species of *Botryosphaeria* described in this paper are also unusual in that their conidia become brown and one-septate at an early stage in their development, often before they are released from the conidiogenous cell. Apart from *Lasiodiplodia*, all other *Botryosphaeria* species thus far subjected to phylogenetic analysis have conidia that are aseptate but sometimes they become brown and one-septate after a period of aging. In the phylogenetic analysis, the two species described in this paper clustered within the *Fusicoccum* clade. However, they are morphologically distinct from *Fusicoccum* (Crous and Palm, 1999) and cannot be accommodated in this genus. Phylogenetically they do not belong in *Diplodia*. Clearly, another anamorph genus is needed to accommodate them.

When Crous and Palm (1999) studied the type specimen of *Dothiorella pyrenophora*, which is the type species of the genus (Sutton, 1977), they reported brown and one-septate conidia of the type that have been associated with *Diplodia* (Fuckel, 1870; Wollenweber, 1941). For that reason they regarded *Dothiorella* as a synonym of *Diplodia*. We examined the type of *Do. pyrenophora* (K54912) and confirm the observations of Crous and Palm (1999). However, *Do. pyrenophora* has several morphological features that are not consistent with the concept of *Diplodia* as typified by *D. mutila* Fr. Thus, in *Diplodia* spp. the conidia are initially hyaline and aseptate but they can become brown and develop one or two eusepta some time after they are formed (Alves *et al.* 2004). This typical form is seen in the anamorphs of *B. tsugae* A. Funk (a *Macrophoma* sp.), *B. stevensii* Shoemaker (*Diplodia mutila* Fr.), *B. quercuum* and *B. corticola* A.J.L. Phillips *et al.* (*D. corticola* A.J.L. Phillips *et al.*), (Funk, 1964;

Shoemaker, 1964; Alves *et al.,* 2004). In contrast, conidia of *Do. pyrenophora* become brown and one-euseptate while they are still inside the pycnidial cavity, and often while attached to the conidiogenous cell (Crous and Palm, 1999; present observations). In this respect the anamorphs of the two species described in this paper correlate most closely with *Dothiorella* as typified by *Do. pyrenophora*. Furthermore, phylogenetically these anamorphs do not belong in *Diplodia*. For these reasons we consider *Dothiorella* a suitable genus to accommodate anamorphs of *Botryosphaeria* species with conidia that become brown and one-septate soon after they are formed. Since the teleomorphs of the two species described as new in this paper are rare, we feel justified in giving names to their anamorphs, which we describe here as *Dothiorella sarmentorum* and *Dothiorella iberica*. To clarify the differences between *Diplodia* and *Dothiorella* we provide amended descriptions of both genera to include new information that separates them morphologically.

Although *Dothiorella* appears to be a suitable genus for these anamorphs, species determinations are difficult. In contrast to other *Botryosphaeria* species, morphological differences are more apparent in the teleomorphs while the anamorphs are morphologically similar and dimensions of the conidia overlap considerably. However, by taking into consideration average lengths of conidia and 95 % confidence limits, the two species can be separated. Thus, conidia of *B. iberica* are (17.2–)23.0–23.4(–28.6) × (8.1–)10.8–11.0(–16) µm and averaged 23.2 × 10.9 µm (n=400) while in *B. sarmentorum* they are (17.6–)21.4–21.9(–24.6) × (7.9–)9.7–9.9(–11.6) µm and averaged 21.6 × 9.8 µm (n=150). The range of dimensions reported by Crous and Palm (1999) for *Do. pyrenophora* [(12–)25–30(–35) × (7–)11–14(–16) µm] cover the entire range of the isolates we studied but the mean dimensions were 26.5±1.56 × 11.83±1.11 µm which is larger than both *B. iberica* and *B. sarmentorum*.

The genus *Dothiorella* has been the source of much confusion in the past and the name has been used in more than one sense. This genus has been used for anamorphs with hyaline aseptate conidia of the type normally associated with *Fusicoccum*. Presumably this confusion started when Petrak (1922) transferred *F. aesculi* Corda to *Dothiorella* citing the species as the conidial state of *B. berengeriana* (Sutton, 1980). In later years, *Dothiorella* has been used for *Fusicoccum*-like anamorphs with multiloculate conidiomata (Grossenbacher and Duggar, 1911; Barr, 1987; Rayachhetry, 1996). Sivanesan (1984) confused matters further by placing *Dothiorella pyrenophora* in synonymy with *Dothichiza sorbi* Lib., which has small, hyaline, aseptate conidia and is the anamorph of *Dothiora pyrenophora* (Fr.) Fr. However, he was referring to *Dothiorella pyrenophora* Sacc., 1884, which is a later homonym of *Dothiorella pyrenophora* Sacc., 1880 (Sutton, 1977). The taxonomic

history of *Dothiorella* has been explained by Sutton (1977) and Crous and Palm (1999).

A further matter that developed from this study concerned the relationship between *Otthia spiraeae* and *Diplodia sarmentorum*. Booth (1958) connected these two fungi through isolations made from a collection of *O. spiraea* on *Ulmus*. This resulted in speculation about the taxonomic position of *Otthia* and led to suggestions that it should be regarded as a synonym of *Botryosphaeria* (Laundon, 1973; Denman *et al.*, 2000). However, *Otthia*, typified by *O. spiraeae*, is morphologically distinct from *Botryosphaeria*. Thus, in *Otthia*, the asci are cylindrical with a thin endotunica while in *Botryosphaeria* they are clavate with a thick endotunica. Pseudoparaphyses in *Otthia* are narrow and sinuous, infrequently septate and not constricted at the septa compared to the broader, frequently septate ones in *Botryosphaeria*. The specimen that Booth (1958) studied is morphologically distinct from *Otthia* and we consider it to be a species of *Botryosphaeria*, which we describe here as *B. sarmentorum*. To clarify this aspect we provide a description of *O. spiraeae*, the lectotype species of *Otthia*. Finally, we transfer *O. quercus* to *Botryosphaeria* as *B. quercicola* nom. nov.

TAXONOMY

Diplodia Fr. in Mont., Ann. Sci. Nat. Bot., sér. 2, 1: 302. 1834.

= *Sphaeropsis* Sacc., *nom. cons.*, Michelia 2: 105. 1880.

= *Macrophoma* (Sacc.) Berl. & Voglino, Atti. Soc. Venet.-Trent. Sci Nat. 10: 4. 1886, and Sacc., Syll. Fung. Addit. 1–4: 306. 1886.

≡ *Phoma* Westend. subgen. *Macrophoma* Sacc., Syll. Fung. 3: 65. 1884.

Type species: *Diplodia mutila* Fr. in Mont., Ann. Sci. Nat. Bot., sér. 2, 1: 302. 1834.

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 but sometimes the coloration is delayed or never occurs, occasionally becoming one-euseptate.

Notes: The concept of *Diplodia* has changed over the years and has been regarded as including species with dark brown, one-septate conidia. However, the genus is typified by *D. mutila*, which has hyaline, aseptate conidia that can become brown and septate with age. Although Denman *et al.* (2000) regarded *Lasiodiplodia* as a synonym of *Diplodia*, and this was accepted by Slippers *et al.* (2004a), there are grounds for regarding them as separate genera. Thus, paraphyses and the longitudinal striations seen on the conidium wall are characteristic features of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., the type species of *Lasiodiplodia*, that are not found in any species of *Diplodia*. Although paraphyses and striate conidia are seen in *D. gossypina* Cooke, Punithalingam (1976) regarded this species as a synonym of *L. theobromae*.

Dothiorella Sacc., Michelia 2: 5. 1880.
Type species: *Dothiorella pyrenophora* Sacc., Michelia 2: 5. 1880.

Mycelium immersed or superficial, branched, septate, melanized, dark brown. *Conidiomata* pycnidial, ostiolate, individual or in loose clusters of up to 10 pycnidia, immersed, breaking through the bark when mature. *Ostiole* circular, central, non-papillate. *Paraphyses* absent. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth-walled, cylindrical and slightly swollen at the base, determinate or indeterminate and proliferating at the same level to form periclinal thickenings, very rarely proliferating percurrently to produce two or three indistinct annellations, borne directly on the cells lining the pycnidial cavity. *Conidia* initially hyaline, becoming dark brown and one-euseptate within the pycnidial cavity and often while still attached to the conidiogenous cell, thick-walled, externally smooth, internally verruculose.

Notes: This genus differs from *Diplodia* in the conidia, which in *Dothiorella* become brown and one-septate at an early stage of their development and often before they are released from the conidiogenous cell. Conidia in *Dothiorella* are brown and one-septate before they are discharged from the pycnidia, while in *Diplodia* they can become septate and the wall sometimes darkens only some time after discharge from the pycnidium. Furthermore, percurrent proliferation of conidiogenous cells is extremely rare in *Dothiorella* but common in *Diplodia*.

Botryosphaeria sarmentorum A.J.L. Phillips, Alves & Luque, sp. nov. FIGS. 2–13

Anamorph: ***Dothiorella sarmentorum*** (Fr.) A.J.L. Phillips, Alves & Luque, comb. nov.

= *Diplodia sarmentorum* (Fr.) Fr., Summ. veg. Scand. (Sweden) 2: 417. 1849.

≡ *Sphaeria sarmentorum* Fr., Syst. mycol. 2: 498. 1823.

= *Diplodia pruni* Fuckel, Jahrb. Nassauischen Vereins Naturk., 23–24: 169. 1870 [1869].

Additional synonyms for *Do. sarmentorum* according to Wollenweber (1941).

Ascostromata in hospitis inclusa, 350–400 µm diametro, erumpescentia, stromatiformis, atrobrunnea vel nigra, cum ostiolis centralibus, papillatis. Pseudoparaphyses filiformis, septatis. Asci 140–210 × 17–24 µm, stipitati, cylindrici-clavati, quadro- ad octospori, bitunicati cum loculo apicali bene evoluto. Ascosporae irregulariter biseriatae, brunnea, uniseptatae, (21–)22.5–28(–30) × (10–)11–12.5(–14) µm, fusiformis oblongis. Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa, usque 450 µm diametro. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 7–15 × 3–7 µm, proliferatione percurrenti limitata, ut videtur 2–3 annellationibus paucis, vel inplano eodem periclinaliter incrassate. Conidia brunnea, uniseptata, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo truncata, (17.5–)19–23.5(–24.5) × (8–)8.5–10.5(–11.5) µm. Cellulae spermatiogenae 7–10 × 2–3 µm, cylindricae, hyalinae, laevia, holoblasticae, phialidibus typicus periclinaliter spissescentibus. Spermatia hyalina, laevia, unicellulares, 4–5.5 × 2 µm.

Ascomata dark brown to black, globose pseudothecial, 350–400 µm diam, submerged in the substrate, partially erumpent at maturity, ostiolate; ostiole circular, central, papillate; wall 50–75 µm thick, composed of dark brown thick-walled *textura angularis*, cells 10–17 × 6–9 µm, lined with thinner-walled, hyaline, *textura angularis*. *Pseudoparaphyses* thin-walled, hyaline, frequently septate, often constricted at the septa, 3–4 µm wide. *Asci* 140–210 × 17–24 µm, stipitate, arising from the base of the ascoma, cylindric-clavate, bitunicate, endotunica thick-walled, with a well-developed apical chamber, 4–6(–8) spored, obliquely uniseriate or irregularly biseriate. *Ascospores* (21.2–)24.4–25.5(–30.5) × (9.9–)11.8–12.3(–14.0) µm, mean ± S.D. of 50 ascospores = 25.0 ± 2.0 × 12.1 ± 0.9 µm, oblong to ovate, widest in the middle part, straight, (0–)1-septate, slightly constricted at the septum, dark brown, moderately thick-walled, surface smooth, finely verruculose on the inner surface. *Conidiomata* solitary, stromatic, globose, up to 450 µm wide, wall 5–8 cell layers thick, composed of dark brown thick-walled *textura angularis*, becoming thin-walled

and hyaline towards the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the pycnidial cavity, holoblastic, hyaline, subcylindrical, 7–15 × 3–7 µm, proliferating at the same level giving rise to periclinal thickenings, or rarely proliferating percurrently to form one or two close, indistinct annellations. *Conidia* (17.6–)21.4–21.9(–24.6) × (7.9–)9.7–9.9(–11.6) µm, mean ± S.D. of 150 conidia = 21.6 ± 1.5 × 9.8 ± 0.9 µm, L/W ratio = 2.2, brown walled, one-septate, slightly constricted at the septum, ovoid with a broadly rounded apex and truncate base. *Spermatiogenous* cells discrete or integrated, hyaline, smooth, cylindrical, holoblastic or proliferating via phialides with periclinal thickenings, 7–10 × 2–3 µm. *Spermatia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 4–5.5 × 2 µm.

*Cardinal temperatures for growth***:** min 5 ºC, opt 20–25 ºC, max below 35 ºC.

Known hosts: Plurivorous including *Malus*, *Menispermum*, *Prunus*, *Pyrus*, *Ulmus*.

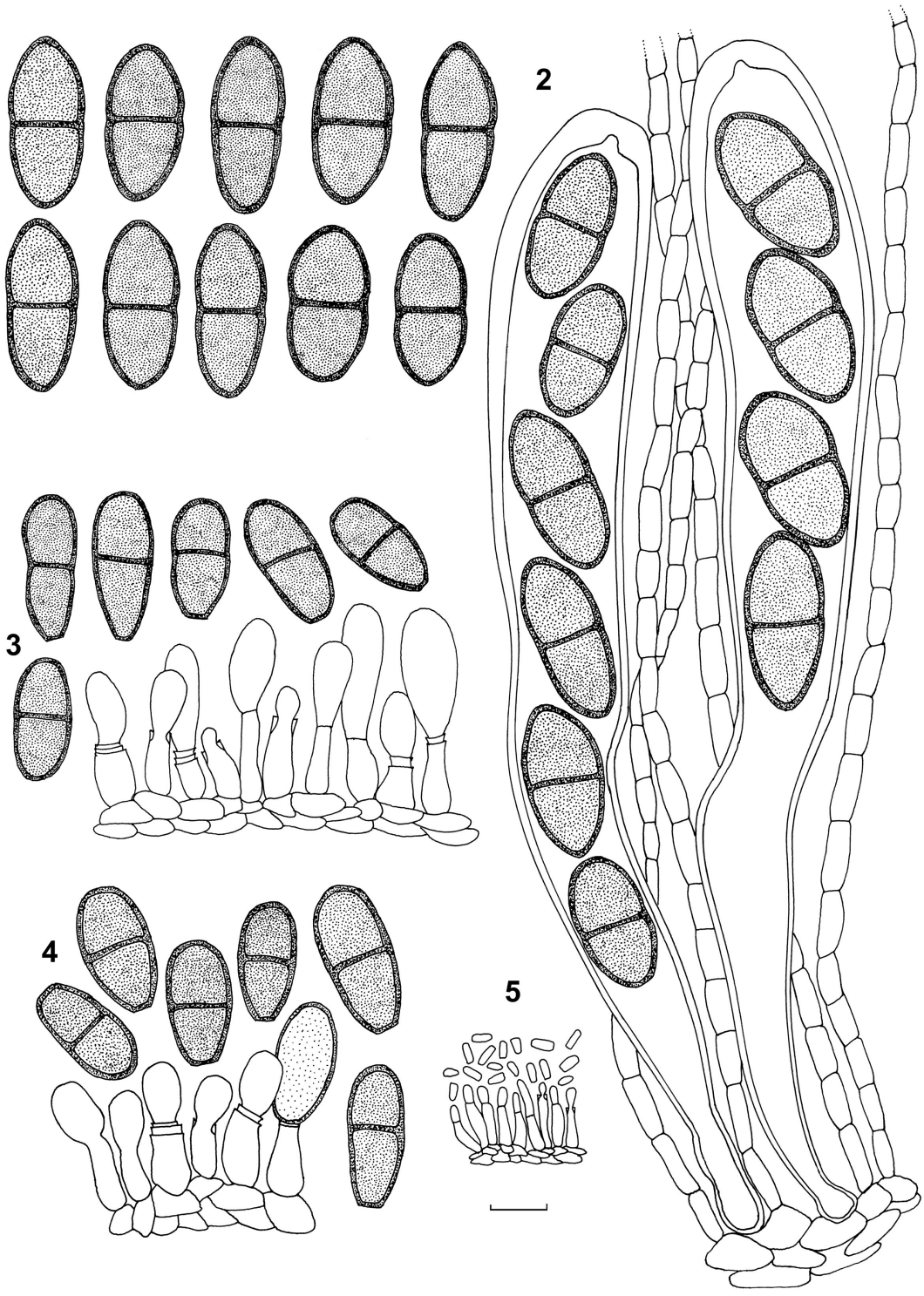
Known geographical range: England, The Netherlands, Norway, Sweden, possibly worldwide.

HOLOTYPE of *B. sarmentorum*: ENGLAND. WARWICKSHIRE. On *Ulmus* sp., Aug. 1956, *E.A. Ellis* (IMI 63581b, as *Otthia spiraeae*).

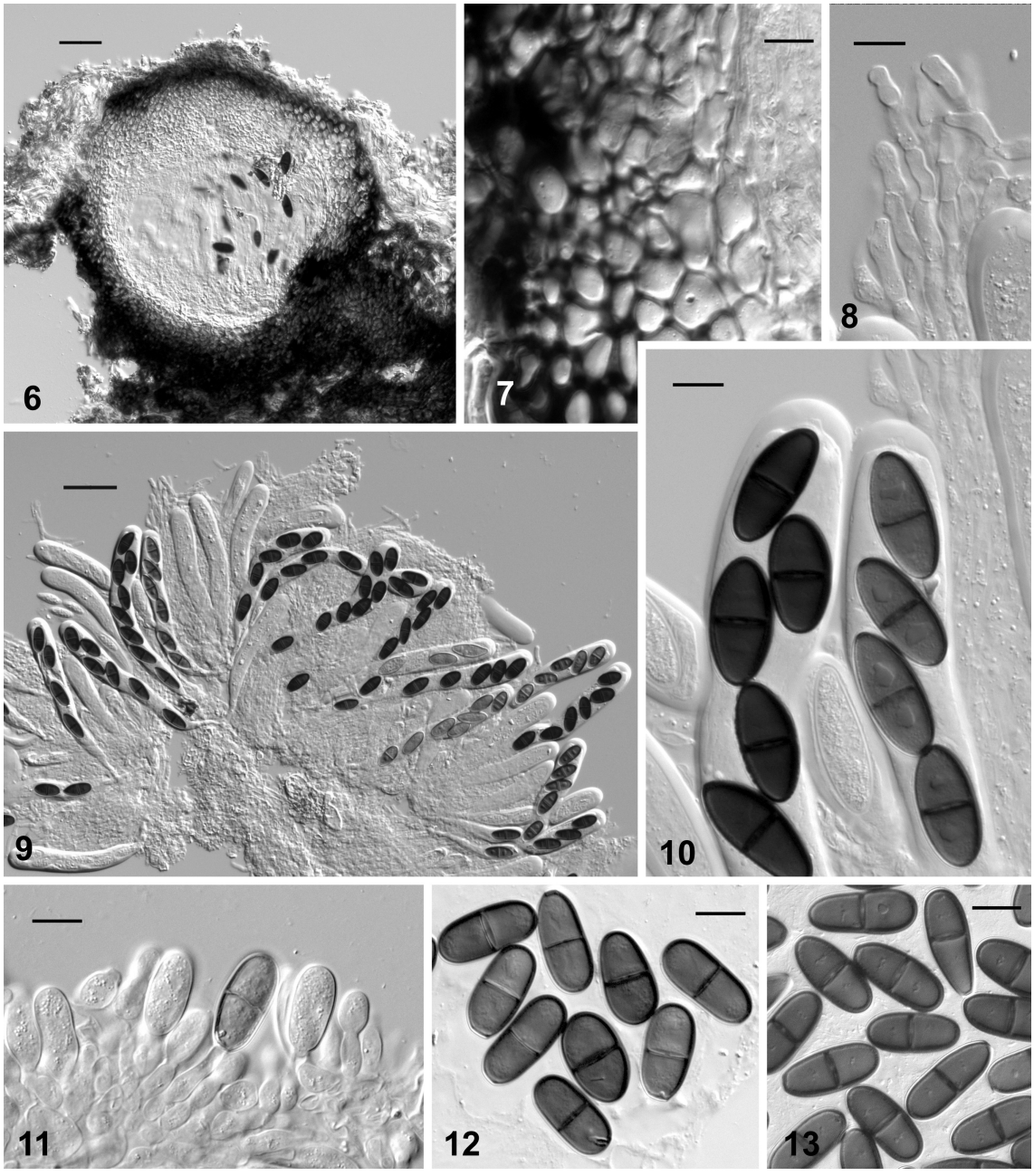
Specimens examined: *Sphaeria sarmentorum*; SWEDEN: Lund, Botanical Garden. On *Menispermum canadense*, 1818, *E.M. Fries Scleromyc. Suec. 18* (HOLOTYPE for the anamorph UPS-FRIES; ISOTYPE for the anamorph K(M)104852). *Diplodia pruni*; GERMANY. On *Prunus armeniaca*, collection date unknown, *K.W.G.L. Fuckel, Fung. Rhen. No. 1710* (HOLOTYPE in G).

Cultures examined: *Dothiorella sarmentorum*; LOCATION UNKNOWN. On *Prunus armeniaca*, Jan 1933, *R.M. Nattrass* (CBS 165.33, as *Diplodia pruni*). NORWAY: On *Pyrus communis*, Aug 1941, *H.W. Wollenweber* (CBS 120.41, as *Diplodia sarmentorum*). ENGLAND. WARWICKSHIRE. On *Ulmus* sp., Aug 1956, *E.A. Ellis* (IMI 63581b, as *Diplodia sarmentorum*, culture ex type of *B. sarmentorum*). THE NETHERLANDS: Delft. On *Malus pumila*, Apr 2003, *A.J.L. Phillips* (CBS 115038).

Notes: In proposing 145 species as synonyms of *Do. sarmentorum*, Wollenweber (1941) reported a wide range of dimensions for the conidia, namely, (15–)20–24(–35) × (7–)7.4–11.5(–15) µm. As shown in the present study, some species in *Dothiorella* are separated by minor differences in conidium dimensions. It is therefore possible that some of Wollenweber's synonyms are in fact distinct species.



Figures 2–5. *Botryosphaeria sarmentorum* and its anamorph *Dothiorella sarmentorum*. 2. Asci, pseudoparaphyses and ascospores of IMI 63581b. 3. Conidia and conidiogenous cells of *Sphaeria sarmentorum* Scl. Suec. 18 in UPS-FRIES. 4. Conidia and conidiogenous cells of IMI 63581b. 5. Spermatiophores and spermatia of CBS 115038 in culture. Scale bars = 10 µm. (drawings by Dr. Alan J.L. Phillips).



Figures 6–13. *Botryosphaeria sarmentorum* and its anamorph *Dothiorella sarmentorum*. 6–12. IMI 63581b. 6. Vertical section through an ascoma. 7. Details of the ascoma wall. 8. Broad, frequently septate pseudoparaphyses. 9. Cylindrical to clavate asci bearing four to eight dark brown, broadly fusiform ascospores. 10. Details of ascus tip and ascospores. 11. Conidiogenous cells from culture. 12. Conidia from culture. 13. Conidia of *Sphaeria sarmentorum* Scl. Suec. 18 in UPS-FRIES. Scale bars: 6 & 9 = 50 µm, 7 & 8, 10–13 = 10 µm.

Botryosphaeria iberica A.J.L. Phillips, Luque & Alves, sp. nov. FIGS. 14–27
Anamorph: ***Dothiorella iberica*** A.J.L. Phillips, Luque & Alves, sp. nov.

Ascostromata in hospitis inclusa, usque ad 350 µm diametro, erumpescentia, stromatiformis, atrobrunnea vel nigra, cum ostiolis centralibus, papillatis. Pseudoparaphyses filiformis, septatis. Asci 100–125 × 18–25 µm, stipitati, cylindrici-clavati, quadro- ad octospori, bitunicati cum loculo apicali bene evoluto. Ascosporae irregulariter biseriatae, brunneae, uniseptatae, (18.0–)19.5–25.5(–29) × (8.5–)9.0–11.5(–12.5) µm, fusiformis oblongis vel subclavatis, apicibus obtusis, in fundo subacutis. Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa, usque 450 µm diametro. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 8–15 × 3–5(–6.5) µm, proliferatione percurrenti limitata, ut videtur 2–3 annellationibus paucis, vel inplano eodem periclinaliter incrassate. Conidia brunnea, uniseptata, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo truncata, (21.5–)23–26(–27.5) × (8.5–)9–12.5(–13.5) µm. Spermatia non visa.

Ascomata dark brown to black, globose pseudothecial, up to 350 µm diam, submerged in the substrate, partly erumpent at maturity, ostiole circular, central, papillate; wall up to 50 µm thick, composed of dark brown thick-walled *textura angularis*, cells 8–17 × 6–10 µm and lined with thinner-walled, hyaline, *textura angularis*. *Pseudoparaphyses* thin walled, hyaline, frequently septate, slightly constricted at the septum, 2.5–3.5(–4) µm wide. *Asci* 100–125 × 18–25 µm, stipitate, arising from the base of the ascoma, clavate, thick-walled, bitunicate with a well-developed apical chamber, stipitate, (4–)8-spored, irregularly biseriate. *Ascospores* (17.8–)22.5–23.7(–29.2) × (8.7–)10.0–10.4(–12.3) µm, mean ± S.D. of 50 ascospores = 23.1 ± 2.1 × 10.2 ± 0.8 µm, oblong, ovate to sub-clavate, (0–)1-septate, slightly constricted at the septum, dark brown, moderately thick-walled, finely verruculose on the inner surface, straight or inequilateral, widest in the lower 1/3 to middle of the apical cell, basal cell tapering towards the rounded end. *Conidiomata* solitary, stromatic, globose, up to 450 µm wide, thick walled, composed of dark brown thick-walled *textura angularis*, becoming thin-walled and hyaline towards the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the cavity, holoblastic, hyaline, subcylindrical, 8–15 × 3–5(–6.5) µm, proliferating at the same level giving rise to periclinal thickenings, or rarely proliferating percurrently forming one or two indistinct annellations. *Conidia* (17.2–)23.0–23.4(–28.6) × (8.1–)10.8–11.0(–16.0) µm, mean ±S.D. of 400 conidia 23.2 ± 1.9 × 10.9 ± 1.2 µm, L/W ratio = 2.2, brown walled, one septate, slightly constricted

at the septum, ovoid with a broadly rounded apex and truncate base. *Spermatia* not seen.

Cardinal temperatures for growth: min 5 ºC, opt 20–25 ºC, max below 35 ºC.

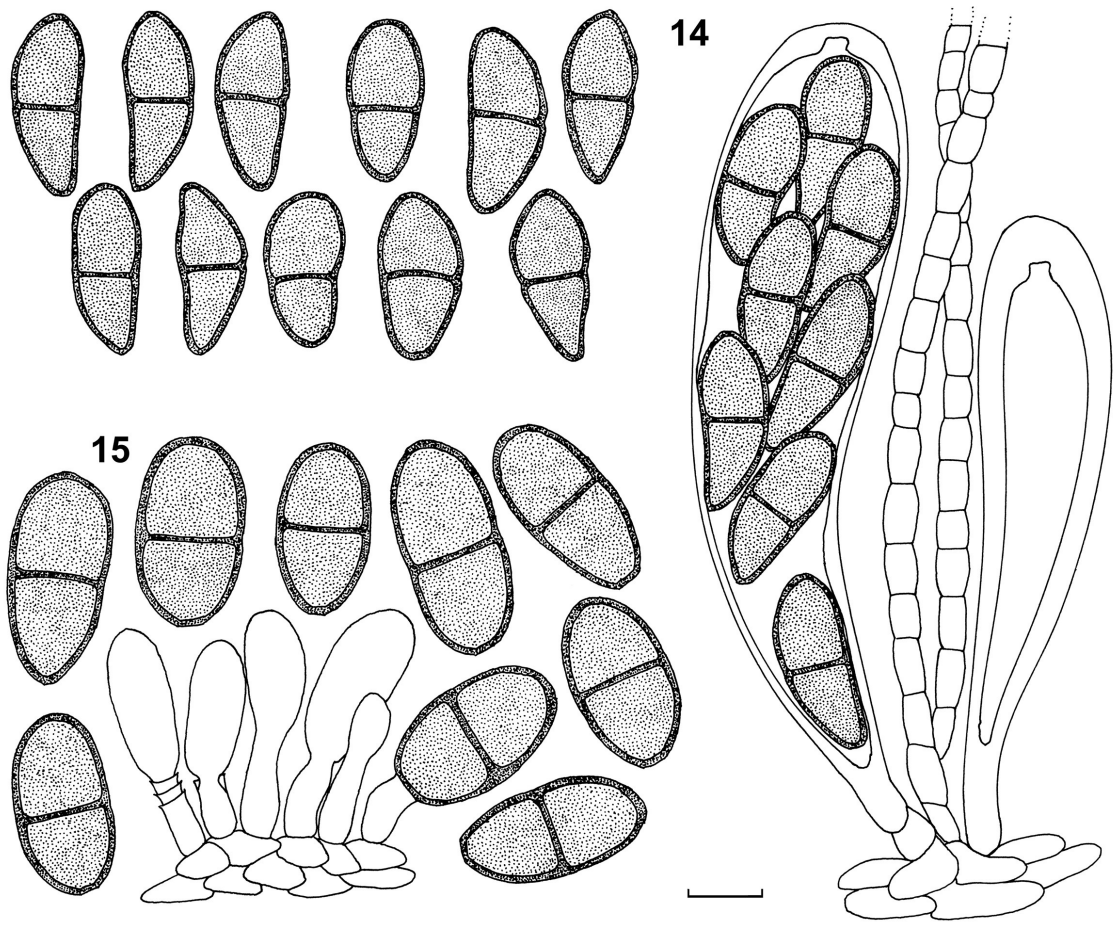
Known hosts: *Malus*, *Quercus*.

Known geographical range: Italy, Spain.

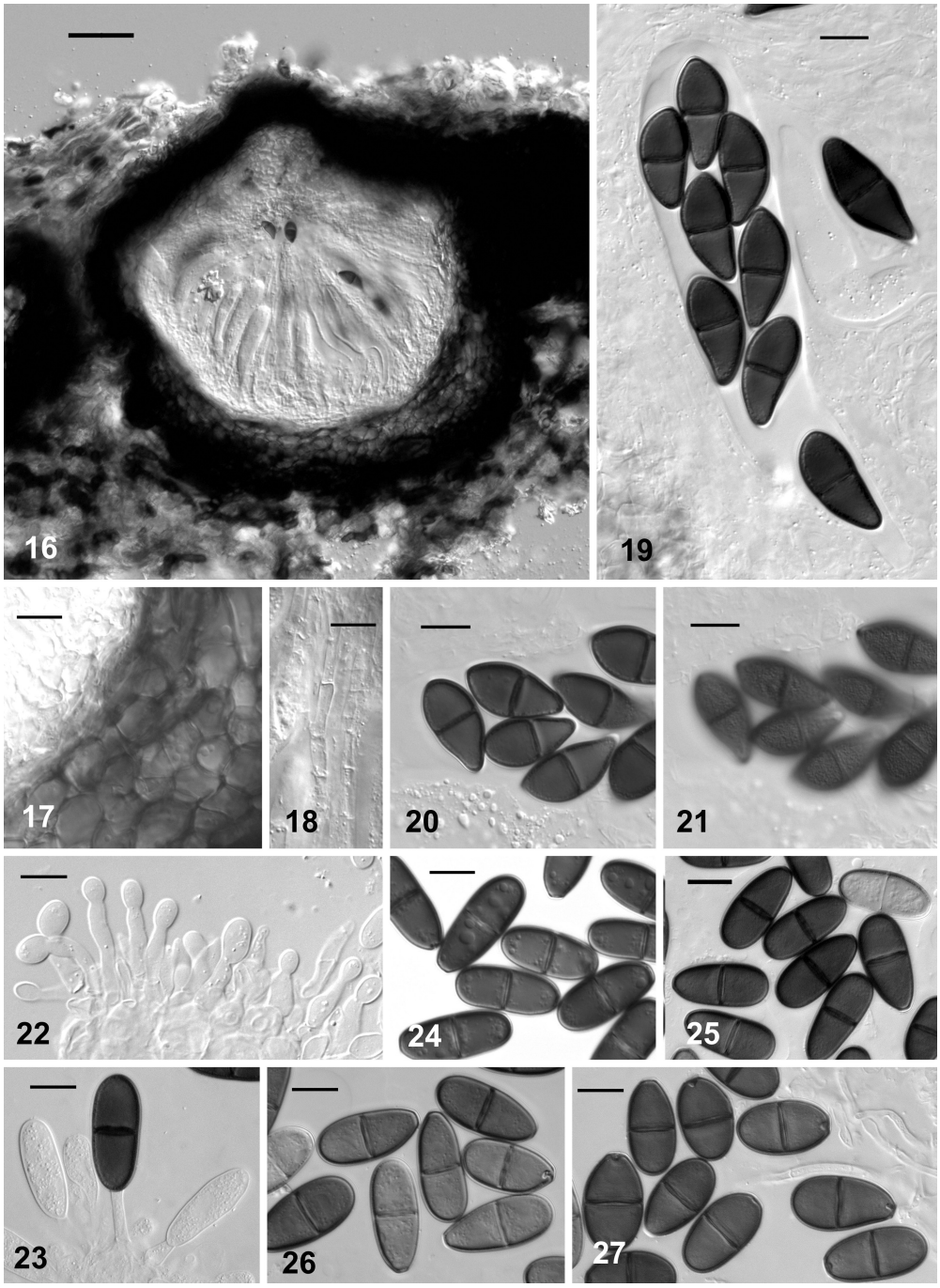
Specimens examined: SPAIN. ARAGON: Tarazona. On dead twigs of *Quercus ilex*, 18 Dec 2002, *J. Luque*, (HOLOTYPE of *B. iberica*, LISE 94944, culture ex type CBS 115041). SPAIN. ARAGON: Monzón. On dead twigs of *Quercus ilex*, 24 Sep 1999, *N. Ibarra*, (HOLOTYPE of *Do. iberica*, LISE 94942, culture ex type CBS 115035).

Cultures examined: See TABLE I.

Notes. This species is similar to *B. sarmentorum* but can be distinguished on characteristics of the asci, ascospores and conidia. Thus, in *B. iberica* the asci are shorter and more clavate, the ascospores characteristically taper towards the base, and on average the conidia are slightly longer.



Figures 14–15. *Botryosphaeria iberica* and its anamorph *Dothiorella iberica*. 14. Mature and immature asci, pseudoparaphyses and ascospores of LISE 94944. 15. Conidia and conidiogenous cells of LISE 94942. Scale bar = 10 µm. (drawings by Dr. Alan J.L. Phillips).



FIGURES 16–27. *Botryosphaeria iberica* and its anamorph *Dothiorella iberica*. 16–21. LISE 94944. 16. Vertical section through an ascoma. 17. Details of the ascoma wall. 18. Broad, frequently septate pseudoparaphyses. 19. Clavate ascus bearing eight ascospores. 20, 21. Ascospores in two different levels of focus to show the roughened inner surface of the ascus wall. 22. Conidiogenous cells of CBS 115041. 23. Conidiogenous cells of CBS 115035. 24–27. Conidia. 24. CBS 115041. 25. CBS 115035. 26. CBS 115040. 27. CBS 113188. Scale bars: 16 = 50 µm, 17–27 = 10 µm.

Botryosphaeria quercicola A.J.L. Phillips, nom. nov. FIGS. 28–36

≡ *Otthia quercus* Fuckel, Jahrb. Nassauischen Vereins Naturk., 23–24: 170. 1870 [1869].

?*Anamorph*. *Diplodia quercus* Fuckel, Jahrb. Nassauischen Vereins Naturk., 23–24: 170. 1870 [1869].

Ascomata dark brown to black, globose pseudothecia, multiloculate, individual locules 250–300 µm diam, immersed in the host and erumpent at maturity, ostiolate, ostioles central, circular, papillate; wall up to 100 µm thick, composed of up to 12 layers of thick-walled *textura angularis*, cells 17–34 × 10–18 µm, inner layers hyaline, thin-walled and flattened. *Pseudoparaphyses* 3.6–4.8 µm wide, thin-walled, hyaline, frequently septate, constricted at septum. *Asci* 110–165 × 27.5–38 µm, arising from base of ascoma, cylindrical to clavate, thick-walled, bitunicate with a well developed apical chamber, stipitate, 8-spored, irregularly biseriate. *Ascospores* brown, (28.3–)30.7–33.9(–35.1) × (10.4–)11.8–13.9(–15.5) µm, mean ± S.D. of 30 ascospores = 32.3 ± 2.5 × 12.9 ± 1.6 µm, 1–2 septate at maturity, constricted at septum, thin-walled, wall internally roughened, oval to broadly fusiform, both ends rounded, widest in the middle or upper third. *Conidiomata* eustromatic, uni- or multi-loculate, ostiolate, ostiole central, papillate. *Conidiogenous cells* cylindrical, discrete, indeterminate and proliferating percurrently to produce 2–3 annellations or proliferating at the same level to form periclinal thickenings. *Conidia* (24–)28.8–30.8(–38) × (11–)15.9–17.1(–21.2) µm, mean ± S.D. of 50 conidia = 29.8 ± 3.6 × 16.5 ± 2.1 µm, L/W ratio = 2.1, hyaline, aseptate, moderately thick walled, wall smooth, eguttulate, oval, both ends rounded. *Spermatiogenous* cells discrete or integrated, hyaline, smooth, cylindrical, holoblastic or proliferating via phialides with periclinal thickenings, 10–18 × 1.5–4 µm. *Spermatia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 4.5–9 × 1.5–2 µm.

Known hosts: *Quercus* species.

Known geographical range: Germany.

Specimen examined: GERMANY: Hessen. On *Quercus*, date unknown, *K.W.G.L. Fuckel*, *Fungi Rhenani 534* (HOLOTYPE in G).

Notes. A new name is proposed rather than a new combination since *B. quercus* could be confused with *B. quercuum*. The description of *D. quercus* was taken from Fungi Rhenani 534. Although Fuckel regarded this as the anamorph of *O. quercus*, the connection between the two has not been demonstrated definitively through culture of

ascospores. *Diplodia quercus* is similar to *Diplodia corticola* except that conidia of the latter are wider (Alves *et al.*, 2004).

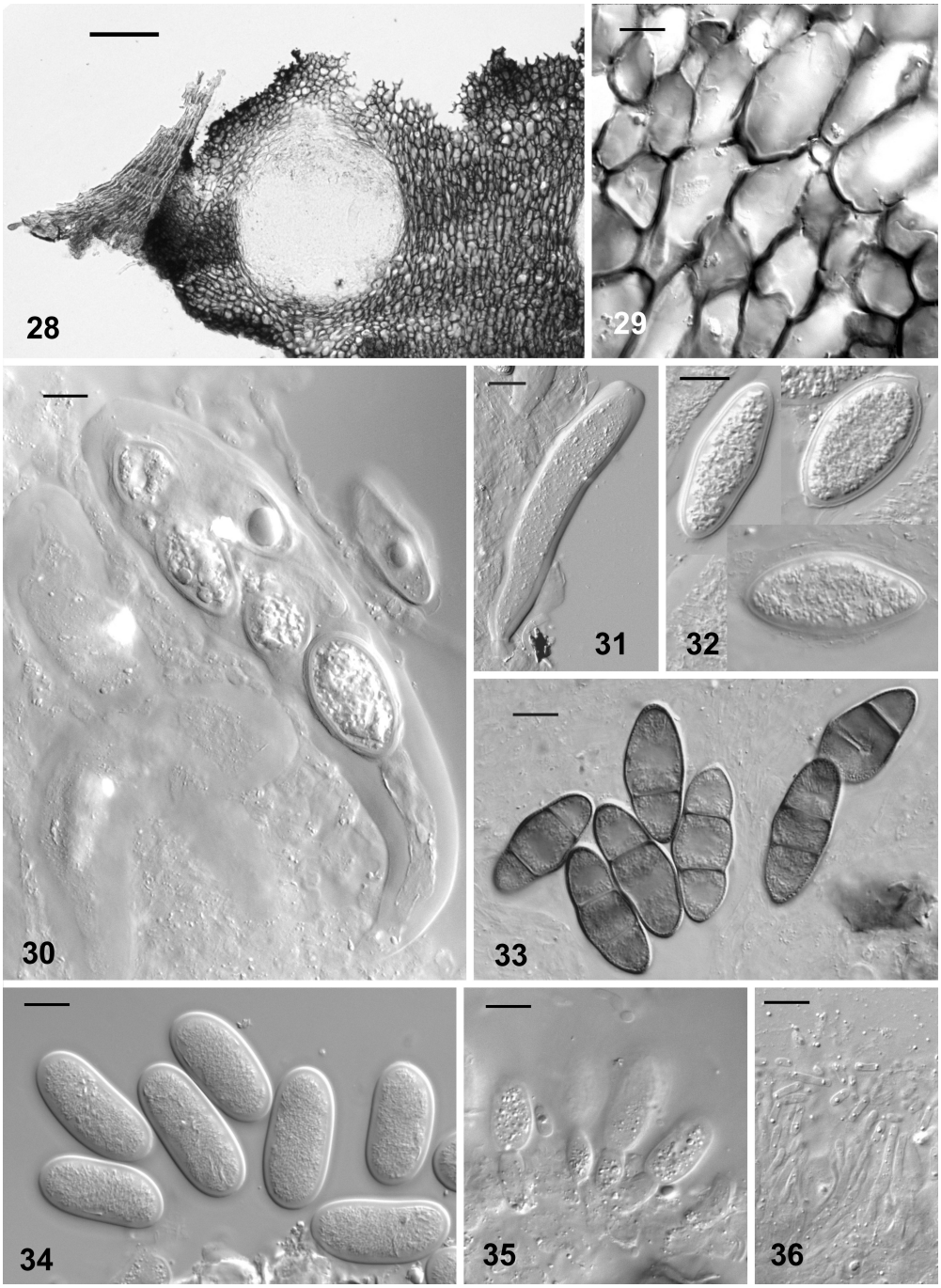
Otthia spiraeae (Fuckel) Fuckel, Jahrb. Nassauischen Vereins Naturk., 23–24: 170. 1870 [1869]. FIGS. 37–41

≡ *Cucurbitaria spiraeae* Fuckel, Fungi Rhenani Exs. No. 975 (1863).

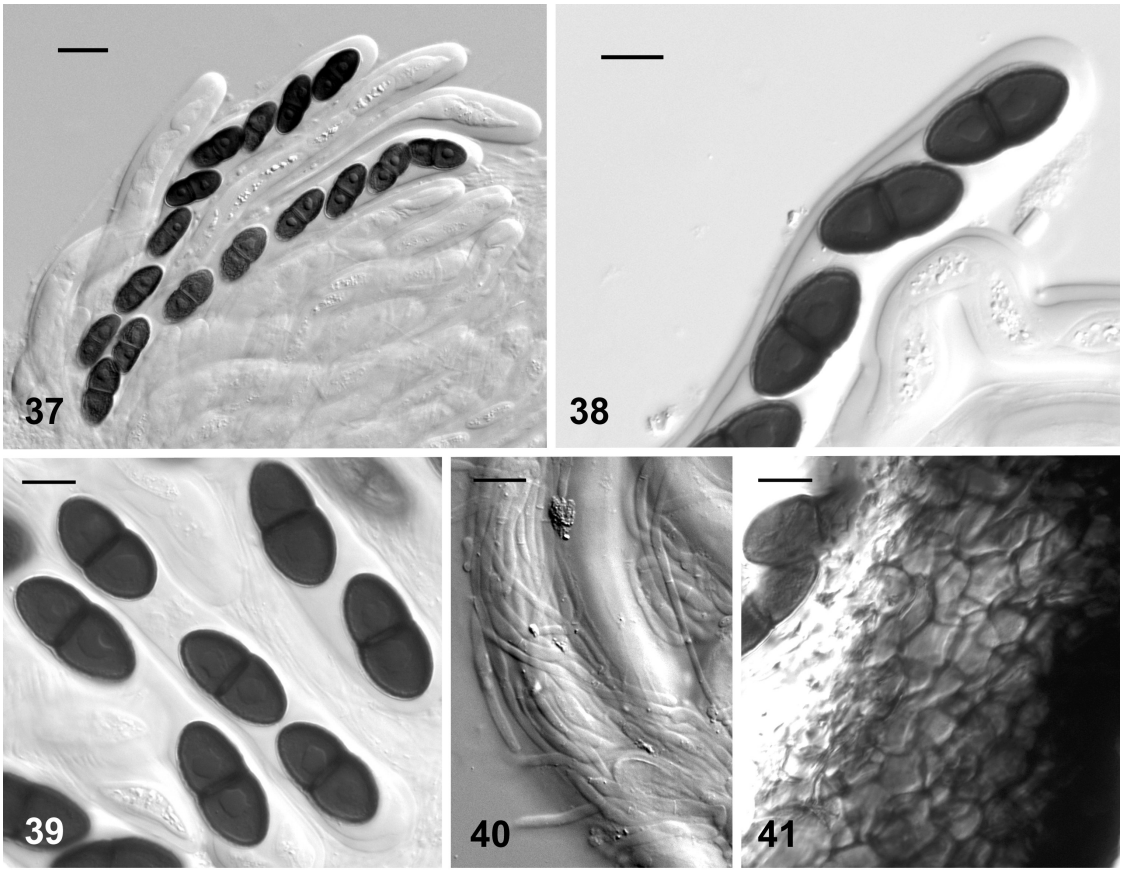
Anamorph. Not known.

Ascomata stromatic, dark brown to black, subcortical, necks erumpent at maturity; wall 60–70 µm wide, composed of 10–12 layers of thick-walled *textura angularis*, cells 7–12 × 5–10 µm. *Pseudoparaphyses* 1.5–2.5 µm wide, thin walled, hyaline, sinuous, unbranched, infrequently septate, not constricted at septum. *Asci* 210–230 × 19–21 µm, cylindrical, bitunicate, containing eight obliquely uniseriate ascospores. *Ascospores* (22.2–)24.6–25.3(–29.5) × (10.5–)11.5–11.9(–13.9) µm, mean ± S.D. of 30 ascospores = 25.0 ± 1.2 × 11.7 ± 0.7 µm, dark brown, one-septate, slightly constricted at the septum, oval, both ends rounded, moderately thick-walled.

Specimens examined: *Cucurbitaria spiraeae*; GERMANY. On dry twigs of *Spiraea opulifolia*, date unknown, *K.W.G.L. Fuckel, Fung. Rhen. No. 975* (HOLOTYPE K 104853).



Figures 28–36. *Botryosphaeria quercicola* and its anamorph *Diplodia quercus*, (Fungi Rhenani 534). 28. Vertical section through an ascoma. 29. Details of the ascoma wall. 30. Clavate ascus. 31. Immature ascus. 32. Hyaline, aseptate ascospores. 33. Brown, one- and two-septate ascospores. 34. Hyaline, aseptate conidia. 35. Conidiogenous cells. 36. Spermatiophores and spermatia. Scale bars: 28 = 100 µm, 29–36 = 10 µm.



FIGURES 37–41. *Otthia spiraeae* K(M)104853 (Fungi Rhenani 975). 37. Cylindrical asci bearing eight brown-walled, obliquely uniseriate ascospores. 38. Details of the ascus tip. 39. Ascospores. 40. Pseudoparaphyses. 41. Details of the ascoma wall. Scale bars: 37 = 20 µm, 38–41 = 10 µm.

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CHAPTER

4

Mycopathologia
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Morphology and phylogeny of *Botryosphaeria dothidea* causing fruit rot of olives

ABSTRACT

The taxonomic position of the causal agent of fruit rot of olives was determined from fresh collections of the fungus from central Greece. In culture it formed two types of conidia, namely fusiform, hyaline, aseptate conidia typical of the genus *Fusicoccum*, and dark-walled, ovoid, elliptical or fusiform, 1–2-septate conidia that are not typical for *Fusicoccum*. A phylogenetic analysis based on ITS and EF1-α sequences placed the fungus within the same group as *Fusicoccum aesculi*, which is the anamorph of *Botryosphaeria dothidea*.

***Key words*:** *Botryosphaeria dothidea*, *Fusicoccum aesculi*, *Olea europaea*, systematics, taxonomy.

INTRODUCTION

The European olive (*Olea europaea* L.) is a long-lived, drought resistant, evergreen tree native to the Mediterranean basin. It is hardy and suffers from few major disease problems. The most serious are bird's eye spot, caused by *Spilocaea oleaginea* (Castagne) S. Hughes (Graniti, 1993), and verticillium wilt caused by *Verticillium dahliae* Kleb. (Thanassoulopoulos *et al*., 1979; Jiménez-Diaz *et al*., 1998). However, a fruit rot of olives has been known for many years (Maublanc, 1904; Petri, 1915; Sarejanni and Papa, 1952) and appears to be widespread in the Mediterranean region (Zachos and Tzavella-Klonari, 1979).

Since it was first described in 1883, the causal agent of olive fruit rot has undergone several taxonomic changes. It was first described by Von Thümen (1883) as *Phyllosticta dalmatica* Thüm. on olive fruits from Dalmatia, Croatia. Saccardo (1884) considered this to be a species of *Phoma* Sacc. and made the new combination *Phoma dalmatica* (Thüm.) Sacc. When Berlese and Voglino (1886) raised *Phoma* subgenus *Macrophoma* to generic status, they included *P. dalmatica* as *Macrophoma dalmatica* (Thüm.) Berl. & Vogl. However, Gigante (1934) regarded it to be a species of *Sphaeropsis* Sacc. and made the new combination of *Sphaeropsis dalmatica* (Thüm.) Gigante. Zachos and Tzavella-Klonari (1979) studied the pathogen in detail and reported two types of conidia. In one type the conidia were fusiform, hyaline or yellow-brown, aseptate or 1–2(–8) septate, and measured 20–34 × 6.5–9 μm. In the second type they were hyaline, yellow-brown or brown, shapes were ovoid, ellipsoid,

limoniform, or pyriform, with 1–5 transverse and 1–3 longitudinal septa, and they measured 10.6–23.5 × 6.5–9.5 µm. Since cultures derived from single conidia of either type produced both types of conidia, Zachos and Tzavella-Klonari (1979) considered it to be a single species. On account of the longitudinal and transverse septation and the dark wall of some conidia, they reclassified *M. dalmatica* as *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav. -Klon. Based on von Thümen's (1883) description, van der Aa and Vanev (2002) assigned *Phyllosticta dalmatica* to *Fusicoccum* Corda as *Fusicoccum dalmaticum* (Thüm.) Vanev.

We recently isolated a fungus from olive fruits that appeared to be a *Fusicoccum* sp., but in culture it formed brown, septate conidia in addition to the hyaline, fusiform ones typical of the genus. Therefore, the purpose of the work presented here was to determine the correct taxonomic position and name for the fungus that causes fruit rot of olives.

MATERIALS AND METHODS

Collection and isolation

Olive fruits showing typical rot symptoms were collected from Thessalia in central Greece. Conidiomata were dissected out and crushed in a drop of sterile water. The resulting suspension of conidia was spread over the surface of a plate of Difco potato dextrose agar (PDA) and incubated overnight at 22 °C. Single germinating conidia were transferred to fresh plates of PDA.

Morphological characterisation

Cultures were grown on PDA or oatmeal agar (OA) (Anonymous, 1968) at 22 °C with 12 h of light per day from mixed UV and daylight fluorescent tubes. Conidia oozing from mature conidiomata were mixed with a small drop of water on a glass slide, the water was allowed to evaporate and the conidia were mounted in lactic acid. The conidiogenous layer was dissected from conidiomata and mounted in lactic acid. Digital images were recorded with a Leica DFC320 camera. Conidial measurements were made with the Leica IM500 measurement module. The mean, standard deviation and 95 % confidence intervals were calculated from measurements of at least 50 conidia of each isolate. Dimensions of conidia are presented as the lower and upper 95 % confidence limits with minimum and maximum dimensions in parentheses.

TABLE 1. Isolates included in this study

Accession no.[1]	Species	Host	Collector	Locality	Genbank	
					ITS	**EF1-α**
CBS 112877	*Botryosphaeria australis*	*Vitis vinifera*	F. Halleen	South Africa	*AY 343385*[2]	*AY 343346*
CBS 112872	*B. australis*	*V. vinifera*	F. Halleen	South Africa	*AY 343388*	*AY 343347*
CBS 112549[3]	*B. corticola*	*Quercus suber*	A. Alves	Portugal, Aveiro	*AY 259100*	*AY 573227*
CMW 8000	*B. dothidea*	*Prunus* sp.	B. Slippers	Switzerland, Crocifisso	*AY 236949*	*AY 236898*
CBS 110302	*B. dothidea*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259092*	*AY 573218*
CBS 110300	*B. dothidea*	*Populus nigra*	A.J.L. Phillips	Portugal, Braga	*AY 640253*	*AY 640256*
CAP 111	*B. dothidea*	*Olea europea*	I. Rumbos	Greece, Thessalia	AY 786320	AY 786317
CAP 125	*B. dothidea*	*Olea europea*	I. Rumbos	Greece, Thessalia	*AY 640254*	*AY 640257*
CAP 126	*B. dothidea*	*Olea europea*	I. Rumbos	Greece, Thessalia	AY 786321	AY 786318
CAP 127	*B. dothidea*	*Olea europea*	I. Rumbos	Greece, Thessalia	AY 786322	AY 786319
CMW 10125	*B. eucalyptorum*	*Eucalyptus grandis*	H. Smith	South Africa, Mpumalanga	*AF 283686*	*AY 236891*
CMW 10126	*B. eucalyptorum*	*E. grandis*	H. Smith	South Africa, Mpumalanga	*AF 283687*	*AY 236892*
CBS 110299	*B. lutea*	*V. vinifera*	A.J.L. Phillips	Portugal, Oeiras	*AY 259091*	*AY 573217*
CMW 9076	*B. lutea*	*Malus* x *domestica*	S.R. Pennycook	New Zealand	*AY 236946*	*AY 236893*
CBS 112555	*B. obtusa*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259094*	*AY 573220*
CMW 9080	*B. parva*	*P. nigra*	G.J. Samuels	New Zealand	*AY 236942*	*AY 236887*
CBS 110301	*B. parva*	*V. vinifera*	A.J.L. Phillips	Portugal, Palmela	*AY 259098*	*AY 573221*
CMW 9074	*B. rhodina*	*Pinus* sp.	T. Burgess	Mexico	*AY 236952*	*AY 236901*
CMW 7772	*B. ribis*	*Ribes* sp.	B. Slippers, G. Hudler	USA, New York	*AY 236935*	*AY 236877*
CMW 7773	*B. ribis*	*Ribes* sp.	B. Slippers, G. Hudler	USA, New York	*AY 236936*	*AY 236878*
CBS 112553	*B. stevensii*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259093*	*AY 573219*
CBS 112878	*Fusicoccum viticlavatum*	*V. vinifera*	F. Halleen	South Africa	*AY 343380*	*AY 343341*
CBS 112977	*F. viticlavatum*	*V. vinifera*	F. Halleen	South Africa	*AY 343381*	*AY 343342*
CBS 110887	*F. vitifusiforme*	*V. vinifera*	J.M. van Niekerk	South Africa	*AY 343383*	*AY 343343*
CBS 110880	*F. vitifusiforme*	*V. vinifera*	J.M. van Niekerk	South Africa	*AY 343382*	*AY 343344*

[1]Acronyms of culture collections: CAP – A.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 the GenBank public database. All others were obtained in this study.
[3]Isolate accession numbers in bold signify cultures ex-type, or from samples that have been linked morphologically to the type material.

DNA extraction and amplification

The procedures described by Alves *et al.* (2004) were used to extract genomic DNA from fungal mycelium and to amplify part of the nuclear rRNA operon using the primers ITS1 and ITS4 (White *et al.*, 1990). 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. 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.* (2002). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 55 ºC, and 1½ min at 72 ºC, and a final extension period of 10 min at 72 ºC. In some cases where amplification of the EF1-α region was not accomplished, a second PCR was performed using as template 1 μl of the first PCR amplification.

DNA sequencing and analysis

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described elsewhere (Alves *et al.*, 2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The sequences of the ITS (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) and partial EF1-α regions were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for both DNA regions of additional *Botryosphaeria* species were taken from GenBank (TABLE 1).

The ITS and EF1-α sequences were aligned with MegAlign (DNASTAR Inc, Madison, Wisconsin) and manual adjustments were made where necessary. Phylogenetic analyses of sequence data were done using PAUP* version 4.0b10 (Swofford, 2003). All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. The ITS and EF1-α sequences were combined and aligned and a partition homogeneity test was conducted in PAUP (Swofford, 2003) to examine the possibility of a joint analysis of the two data sets. 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. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Robustness of the branches was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993).

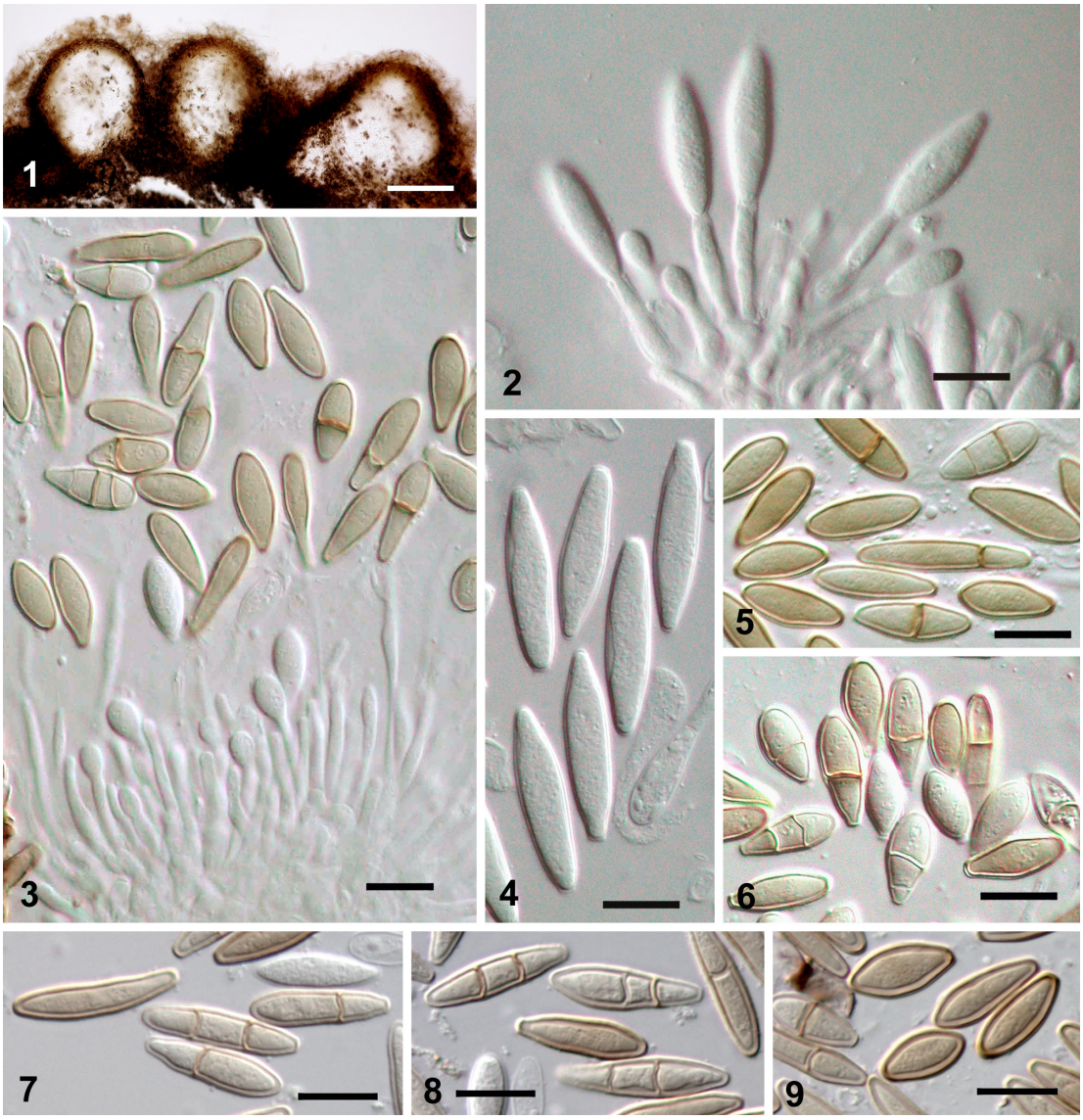
RESULTS

Morphological characterisation

Pycnidia on olive fruits were brown to black and immersed in the host, breaking through the epidermis to become partially erumpent. Conidia were hyaline, aseptate, thin-walled, fusiform to subclavate, apex subacute, base truncate with a minute marginal frill, (19.5–)22.3–23.6(–27.7) × (4.6–)5.2–5.5(–6.9) µm, mean ± S.D. of 33 conidia = 22.9 ± 2.0 × 5.3 ± 0.5 µm, L/W ratio of 4.35 ± 0.5.

Colonies developing on OA and PDA were initially colourless, becoming dark olive from the centre with the dark colouration ultimately spreading to the entire colony. Conidiomata started to form after 10 days on OA at 22 ºC. Conidiomata (FIG. 1) were unilocular and pycnidial, or multilocular and eustromatic and were initially covered with olivaceous, appendage-like hyphae. The wall was dark brown to black, composed of thick-walled, dark brown *textura angularis* in the outer parts with hyaline, thin-walled cells towards the inner layers. Ostioles were circular, central, and well-defined. Conidiophores, when present, were hyaline, cylindrical, smooth and sparingly branched. Conidiogenous cells (FIGS. 2, 3) were hyaline, smooth, thin-walled, cylindrical, initially holoblastic, becoming enteroblastic, producing one or more conidia apically, proliferating internally to form periclinal thickenings and typical phialides (Sutton, 1980) or proliferating percurrently resulting in 2–3 close- or wide-spaced annellations. Conidia were either hyaline, thin-walled and aseptate (FIG. 4), or brown, thick-walled and one- or two-septate (FIGS. 5–9). The hyaline conidia were smooth, aseptate, fusiform with a subacute apex and a rounded or truncate base that often bore a minute basal frill. Conidia measured (23.8–)26.4–27.4(–31.1) × (4.5–)5.3–5.5(–6.2) µm with mean and standard deviation of 50 conidia = 26.9 ± 1.7 × 5.4 ± 0.4 µm, length width ratios were in the range of (4.1–)4.9–5.2(–6.2) and the mean and standard deviation = 5.0 ± 0.5. The brown-walled conidia had a smooth, moderately thick, pale brown wall and were 0–2(–3) -septate, subglobose, clavate, fusiform-elliptical or clavate, with a truncate base. Conidia measured (9.7–)15.1–15.9(–22.9) × (3.1–)5.0–5.1(–6.7) µm with mean and standard deviation of 150

conidia = 15.5 ± 2.7 × 5.1 ± 0.6 µm, length width ratios were in the range of (1.6–)3.0–3.3(–5.2) and the mean and standard deviation = 3.1 ± 0.8.



FIGURES 1–9. *Fusicoccum* anamorph of *Botryosphaeria dothidea* causing fruit rot of olives. 1. Sectioned pycnidia from oatmeal agar cultures. 2. Condiogenous cells and developing fusiform, hyaline conidia. 3. Conidiogenous cells and dark-walled, septate conidia. 4. Hyaline, aseptate conidia. 5–9. Dark-walled, 0–2-septate conidia. Scale bars: 1. = 100 µm, 2–9 = 10 µm.

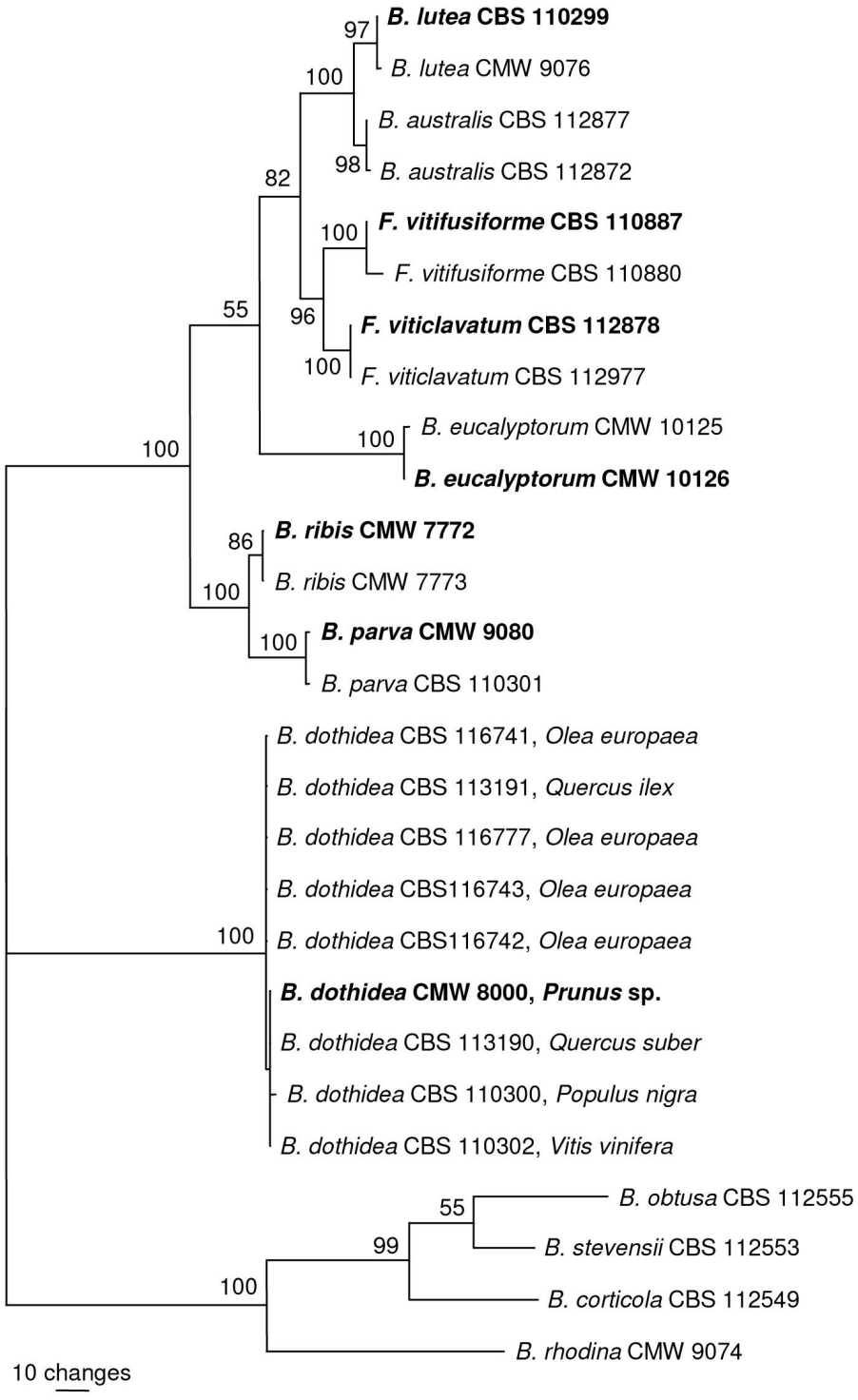


Figure 10. One of four most parsimonious trees obtained from combined ITS and EF1-α sequence data. Cultures linked to the type material are indicated in bold. Host names are provided for the *B. dothidea* isolates. Bootstrap values from 1000 replications are shown at the nodes. The tree was rooted to *B. obtusa*, *B. stevensii*, *B. corticola* and *B. rhodina*. The bar represents 10 changes.

Phylogenetic analysis

Four isolates from olives were chosen for sequencing the ITS and EF1-α. ITS and EF1-α sequences of a further 19 isolates (TABLE 1), representing five isolates of *B. dothidea* and seven other *Botryosphaeria* species with *Fusicoccum* anamorphs, were retrieved from GenBank and included in the analysis. Since the focus of this study was to determine the position of the isolates from olives in relation to other species with *Fusicoccum* anamorphs, the tree was rooted to four species with *Diplodia* anamorphs. New ITS and EF1-α sequences were deposited in GenBank. A partition homogeneity test (P = 0.34) indicated that the ITS and EF1-α trees reflect the same underlying phylogeny and could be combined in a single analysis. After alignment, the combined dataset consisted of 850 characters including gaps. Of these, 519 characters were constant while 52 variable characters were parsimony-uninformative. Using the remaining 279 characters, 4 equally parsimonious trees were retained after heuristic searches in PAUP, one of which is shown in FIG. 10. The four trees differed only in the positions of some isolates within the terminal clades with no differences in overall phylogeny. The eight species with *Fusicoccum* anamorphs were clearly distinguished and the species clades were supported by bootstrap values in excess of 98 % except for the *B. ribis* clade, which received 86 % support. The four isolates from olive fruits clustered within the clade containing isolates identified as *B. dothidea*. One of those (CMW8000) was isolated from the ex-epitype of *B. dothidea* (Slippers *et al*., 2004), and in that respect it can be regarded as authentic for the name. The *B. dothidea* clade formed a lineage separate from all other species with *Fusicoccum* anamorphs.

DISCUSSION

The fungus studied here and the one illustrated by Zachos and Tzavella-Klonari (1979) are clearly not species of *Phyllosticta* since the conidia lack the mucilaginous, apical appendages or caps that are characteristic of that genus (van der Aa, 1973; van der Aa and Vanev, 2002). Furthermore, the transfer to *Phoma* by Saccardo (1884) is not suitable, according to the current concept of that genus, since the conidiogenous cells of the isolates from olives are longer and more conspicuous than those found in any species of *Phoma*. Although the fungus was known for many years as *Macrophoma dalmatica* the genus *Macrophoma* has been the source of considerable confusion. The name has been applied indiscriminately to Phoma-like fungi with relatively large conidia and to species with dark conidia. When Berlese and Voglini (1886) raised *Phoma* subgenus *Macrophoma* to the status of genus, they included species with hyaline, thin-walled conidia as well as species with brown, thick-walled

conidia. However, they did not designate a species as type of the genus. Petrak and Sydow (1927) took the first listed species, *M. macrosperma* (Karst.) Berl. & Vogl., as lectotype, but showed that *Sphaeria pinea* Desm. provided an earlier epithet and consequently they proposed *M. pinea* (Desm.) Petrak & Sydow as type. Subsequently, Petrak (1961) showed that *M. pinea* is a later homonym of *M. pinea* Pass. (≡ *Dothiorella pinea* (Pass.) Petr.& Syd.) and so he replaced the name of the lectotype species with *M. sapinea* (Fr.) Petrak. Thus, the type species of *Macrophoma* (*M. sapinea*) has brown, thick-walled conidia. For this reason, Sutton (1980) regarded *Macrophoma* as a synonym of *Sphaeropis*. Clearly, *Macrophoma* is not a suitable genus for the fungus causing fruit rot of olives.

Gigante (1934) transferred *P. dalmatica* to *Sphaeropis* presumably because of the brown-walled conidia. However, the distinction between *Sphaeropsis* and *Diplodia* has never been clear. Percurrent proliferation of conidiogenous cells has been regarded as more typical of *Sphaeropsis* than of *Diplodia* as defined by Sutton (1980). Since percurrent proliferation of conidiogenous cells is common in *Diplodia mutila* (Alves *et al*., 2004), the type species of *Diplodia*, there is no valid reason to separate these two genera and *Sphaeropsis* is considered to be a later synonym of *Diplodia*.

Morphologically the fungus studied in this paper correlated well with the one reported by Zachos and Tzavella-Klonari (1979). While Zachos and Tzavella-Klonari (1979) regarded *Camarosporium* to be a suitable genus, the hyaline, thin-walled, aseptate conidia they also report are not typical of that genus and suggest *Fusicoccum*. Mode of conidiogenesis and morphology of the conidiogenous cells tend to confirm this. Dimensions and morphology of the hyaline conidia correlate well with *F. aesculi*, the anamorph of *B. dothidea* as defined by Slippers *et al*. (2004). Phylogenetically, the olive isolates were indistinct from other isolates of *B. dothidea*, including the ex-epitype strain. Therefore, we conclude that the fungus causing fruit rot of olives is *B. dothidea* and its anamorph is *Fusicoccum aesculi*.

The dark-walled conidia of the isolates from olive are unusual. Nevertheless, when Crous and Palm (1999) emended the concept of *Fusicoccum* they included conidia that can become olivaceous with age. Furthermore, conidia of some *Fusicoccum* become brown and develop septa when aged or just before germination. For example, conidia of *B. parva* and *B. ribis* develop two septa before germination and in *B. parva* the central cell often becomes darker than the two terminal cells (Slippers *et al*., 2004).

The inclusion of dark conidia in *Fusicoccum* makes the distinction between *Fusicoccum* and *Diplodia* less clear. As discussed by Alves *et al*. (2004) the separation of these two anamorph genera on the basis of conidium coloration is tenuous. Indeed, Denman *et al*. (2000) suggested that the two genera should be united under the older

name of *Fusicoccum*. Although this would be desirable in order to link one teleomorph genus with a single anamorph genus, no formal synonymies are made in this paper.

TAXONOMY

Anamorph: ***Fusicoccum aesculi*** Corda in Sturm, Deutschland flora 2: 111. 1829.

= *Fusicoccum dalmaticum* (Thüm.) Vanev, in van der Aa and Vanev, A revision of the species described in *Phyllosticta* p. X. 2002.

≡ *Phyllosticta dalmatica* Thüm., Boll. Soc. Adriat. Sci. Nat., Trieste 8: 240. 1883.

= *Phoma dalmatica* (Thüm.) Sacc., Syll. Fung. 3: 157. 1884.

= *Macrophoma dalmatica* (Thüm.) Berl. & Voglino, Atti Soc. Venet.-Trent. Sc. Nat., Padova : 196. 1886.

= *Sphaeropsis dalmatica* (Thüm.) Gigante, Boll. R. Staz. Patol. Veg. n.s. 14: X. 1934.

= *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav-Klon. [as "*dalmatica*"], Annls Inst. Phytopath. Benaki n.s. 12: 69. 1979.

Teleomorph: ***Botryosphaeria dothidea*** (Moug. : Fr.) Ces. & De Not., Comm. Soc. Crittog. Ital. 1: 215. 1863.

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CHAPTER

5

Fungal Diversity

2006

21:141–155

Characterisation and epitypification of *Botryosphaeria corticis*, the cause of blueberry cane canker

ABSTRACT

Botryosphaeria corticis was collected from a commercial field of *Vaccinium corymbosum* cv. Bluecrop in New Jersey, USA. The connection between the anamorph and the teleomorph was confirmed through isolations made from single ascospores. The fungus was characterised in terms of morphology of the teleomorph on the host, the anamorph in pure culture, and sequences of the ITS1/ITS2 regions of the ribosomal DNA operon. Morphologically the fungus compared well with the protologue of *B. corticis*. Phylogenetic analyses showed that the isolates from New Jersey reside in a clade together with isolates of the same species from North Carolina and this clade is sister to *B. dothidea*. The specimen from New Jersey is designated as epitype.

***Key words*:** *Botryosphaeriaceae*, *Fusicoccum*, ITS, phylogeny, taxonomy

INTRODUCTION

Botryosphaeria Ces. & De Not. is a species rich genus of plant parasites, saprobes and endophytes with a worldwide distribution on a wide range of mainly woody hosts (Denman *et al*., 2000). The genus is well circumscribed morphologically and its features have been documented in detail (Denman *et al*., 2000; Slippers *et al*., 2004a). Morphological features of the teleomorphs vary little from one species to another, but the anamorphs display a wide range of characters that discriminate the species.

Up to 18 anamorph genera have been associated with *Botryosphaeria* (Denman *et al*., 2000). Phylogenetic studies based on ITS sequences led Jacobs and Rehner (1998) and Denman *et al*. (2000) to recognise two main clades, one that corresponded to species with anamorphs in *Diplodia* Fr. and the other to species with anamorphs in *Fusicoccum* Corda. Subsequent studies by Zhou and Stanosz (2001), Slippers *et al*. (2004a) and Alves *et al*. (2004) with more species and additional markers supported this view. Nevertheless, some authors (e.g., Pavlic *et al*., 2004) have continued to separate *Lasiodiplodia* Ellis & Everh. from *Diplodia* because of their phylogenetic (ITS) and morphological (striate conidia and paraphyses) distinctions. *Dothiorella* Sacc. was recently reinstated for species with thick-walled conidia that become brown and septate at an early stage of development (Phillips *et al*., 2005).

In ITS phylogenies the *Fusicoccum* clade is composed of two sub-clades. *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., the type species of *Botryosphaeria*, lies in one of these together with *B. mamane* D.E. Gardner, *B. corticis* (Demaree & M.S. Wilcox) Arx & E. Müll. (Slippers *et al*., 2004a; Zhou and Stanosz, 2001; Alves *et al*., 2004; Phillips *et al*., 2005) and *Fusicoccum dimidiatum* (Penz.) D.F. Farr (Farr *et al*., 2005). All other species with *Fusicoccum* anamorphs fall within a second sub-clade. Several new species have recently been described in *Botryosphaeria* and many of these appear in the larger *Fusicoccum* sub-clade that does not contain *B. dothidea* (Smith *et al*., 2001; Denman *et al*., 2003; van Niekerk *et al*., 2004; Slippers *et al*., 2004b, c; Slippers *et al*., 2005; Farr *et al*., 2005). All species in the non-*B. dothidea* sub-clade are well-characterised by detailed morphological and molecular data, and ex-type or ex-epitype cultures are available for all of them.

In contrast, the sub-clade bearing *B. dothidea* is less well characterised. Slippers *et al*. (2004a) clarified the status of *B. dothidea*, proposed a neotype and an epitype and provided a corresponding ex-epitype culture. However, the other two species that fall within this sub-clade are less well studied and none of the cultures available for them can be linked to their corresponding type specimens. To stabilise these names and provide authentic cultures that can be used as standards, epitype specimens should be selected and cultures derived from them made available in long-term culture collections. The two known ex-type cultures of *B. mamane* are no longer extant (G. Stanosz pers. comm.). Recent attempts to re-collect and isolate *B. mamane* from the type location were unsuccessful (P.W. Crous, pers. comm.). Few cultures of *B. corticis* are available in publicly accessible culture collections. Thus, no isolates are available in IMI or CBS. Although six cultures are available in ATCC, none of these can be connected to the type specimen (BPI 598729).

The objectives of the present study were to collect a specimen of *B. corticis* that is suitable as epitype, to prepare ex-epitype cultures that can be made available for future studies, to fully characterise the species in terms of morphology and assess its phylogenetic relationships with known species.

MATERIALS AND METHODS

Isolates

Canes of *Vaccinium corymbosum* with typical symptoms of infection by *B. corticis* were collected from Hammonton, New Jersey, USA (-74.756, 39.639). Asci and ascospores were dissected from ascomata and spread over the surface of plates of Difco potato dextrose agar (PDA). After incubating overnight at 25 ºC, single

germinating ascospores were transferred to fresh plates of ½ strength PDA and checked microscopically to ensure that a single spore had been transferred. Cultures were stored on ½ strength PDA slopes at 5 ºC.

Morphology

To induce sporulation, cultures were grown on water agar supplemented with pieces of autoclaved poplar twigs and incubated on the laboratory bench (20–25ºC) where they received indirect daylight. Culture characteristics were recorded on Difco corn meal agar (CMA) plates incubated as for sporulation.

For microscopy, the contents of ascomata were dissected out and mounted in 100 % lactic acid. For observations of conidiogenesis, the conidiogenous layer was dissected out and mounted in 100 % lactic acid. Measurements of ascospores and conidia were made with the Leica IM 500 measurement module from images recorded on a Leica DFC 320 digital camera. From measurements of 50 conidia and ascospores the mean, standard deviation and 95 % confidence intervals were calculated. Spore dimensions are given as the range of dimensions with extremes in parentheses. Dimensions of other structures are given as the range of at least 20 measurements.

DNA isolation, amplification and phylogeny

The procedures described by Alves *et al*. (2004) were used to extract genomic DNA and to amplify part of the nuclear rRNA cluster with the primers ITS1 and ITS4 (White *et al*., 1990). 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). The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. The cycle sequencing procedure has already been described elsewhere (Alves *et al*., 2004). The sequences of the ITS (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in GenBank. Nucleotide sequences of additional *Botryosphaeria* species were retrieved from GenBank (TABLE 1).

The ITS sequences 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 phylogenetic analysis using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses were carried out using PAUP* version 4.0b10 (Swofford, 2003) for maximum-parsimony (MP) analysis and Mr Bayes v3.0b4 (Ronquist and Huelsenbeck, 2003) for the Bayesian analysis. The aim of this study was to determine the phylogenetic position of *B. corticis* within the species with *Fusicoccum* anamorphs. For this reason the trees were rooted to two species in the sister group of *Botryosphaeria* spp. with *Diplodia* anamorphs (Denman *et al*., 2000; Alves *et al*., 2004), namely *B. stevensii* Shoemaker and *B. obtusa* (Schwein.) Shoemaker. Trees were visualised with TreeView (Page, 1996).

Maximum-parsimony analysis was performed using the heuristic search option with 1000 random taxa additions 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 by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo (MCMC) method were performed. 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+Γ) was used. Four MCMC chains were run simultaneously, starting from random trees, for 10^6 generations. Trees were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated from the remaining 9000 trees. The analysis was repeated three times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis.

RESULTS

Phylogenetic analysis

The ITS dataset consisted of 34 ingroup and 2 outgroup taxa. New sequences were deposited in GenBank (TABLE 1) and the alignments in TreeBase (S1443). The alignment contained 554 characters including coded alignment gaps. Of these 554 characters 413 were constant and 16 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 125 parsimony informative characters resulted in 28 equally parsimonious trees with TL = 202, CI = 0.728, RI = 0.930 and HI = 0.213. The Bayesian analysis resulted in the tree shown in FIG. 1, which is essentially the same as the trees resulting from MP analysis. Two major clades (A and B) supported by high statistical support (MP bootstrap values of 98 % and 94 % respectively, and posterior probabilities of 1.00) were distinguished. Clade A consists of 7 species of *Botryosphaeria* and 4 species of *Fusicoccum* anamorphs. Not all species in this clade could be fully differentiated by the ITS sequence data. Thus, *B. parva* Pennycook & Samuels could not be distinguished unambiguously from *B. ribis* Grossenb. & Duggar and the *F. viticlavatum* Niekerk & Crous clade did not receive any significant bootstrap or posterior probability support. Nevertheless, all these species can be differentiated when ITS sequence data are combined with protein coding genes such as the translation elongation factor 1-α or β-tubulin genes (van Niekerk *et al*., 2004).

Clade B consisted of four clearly differentiated and well supported sub-clades corresponding to four known species including the type species of the genus, *B. dothidea*. Anamorphs of these four species are known to be *Fusicoccum*-like, but species names have been applied to only two of them, namely *F. aesculi* Corda (*B. dothidea*) and *F. dimidiatum* (Penz.) D.F. Farr (teleomorph unknown). MP and Bayesian analyses placed *B. corticis* in a well-supported clade sister to *B. dothidea*.

TABLE 1. Isolates studied.

Isolate number[1]	Identity	Host	Locality	Collector	ITS GenBank[2]
CBS 119046	*B. australis*	*Rubus* sp.	Alentejo, Portugal	E. Diogo	DQ299244
CMW 9072	*B. australis*	*Acacia* sp.	Melbourne, Australia	J. Roux	*AY339260*
CBS 119047	*B. corticis*	*Vaccinium corymbosum*	New Jersey, USA	P.V. Oudemans	DQ299245
CBS 119048	*B. corticis*	*Vaccinium corymbosum*	New Jersey, USA	P.V. Oudemans	DQ299246
ATCC 22927	*B. corticis*	*Vaccinium* sp.	North Carolina, USA	R.D. Millholland	DQ299247
ATCC 22928	*B. corticis*	*Vaccinium* sp.	North Carolina, USA	R.D. Millholland	DQ299248
CBS 115476	*B. dothidea*	Prunus sp.	Crocifisso, Switzerland	B. Slippers	*AY236949*
CBS 110300	*B. dothidea*	*Populus nigra*	Braga, Portugal	A.J.L. Phillips	*AY640253*
CBS 110302	*B. dothidea*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259092*
CBS 116741	*B. dothidea*	*Olea europaea*	Thessalia, Greece	I. Rumbos	*AY640254*
CBS 115766	*B. eucalypticola*	*Eucalyptus rossii*	Tidinbilla, NSW, Australia	M.J. Wingfield	*AY615143*
CMW 6539	*B. eucalypticola*	*Eucalyptus rossii*	Orbost, Victoria, Australia	M.J. Wingfield	*AY615141*
CBS 115791	*B. eucalyptorum*	*Eucalyptus grandis*	South Africa	H. Smith	*AF283686*
CMW 10126	*B. eucalyptorum*	*Eucalyptus grandis*	South Africa	H. Smith	*AF283687*
CBS 110299	*B. lutea*	*Vitis vinifera*	Oeiras, Portugal	A.J.L. Phillips	*AY259091*
CMW 9076	*B. lutea*	*Malus* × *domestica*	New Zealand	S.R. Pennycook	*AY236946*
GS 97-59	*B. mamane*	*Sophora chrysophylla*	Hawaii	D. Gardner	*AF246930*
GS 97-58	*B. mamane*	*Sophora chrysophylla*	Hawaii	D. Gardner	*AF246929*
CBS 112555	*B. obtusa*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259094*
CBS 110301	*B. parva*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259098*
CMW 9081	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	*AY236943*
STE-U 1775	*B. protearum*	*Leucadendron* sp.	South Africa	S. Denman	*AF452539*
STE-U 4398	*B. protearum*	*Leucadendron* sp.	Portugal	S. Denman	*AF452531*
CMW 7054	*B. ribis*	*Ribes rubrum*	New York, USA	N.E. Stevens	*AF241177*
CBS 115475	*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers	*AY236935*
CBS 112553	*B. stevensii*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259093*
UAMH 6800	*F. arbuti*	*Arbutus menziesii*	BC, Canada	A. Funk	*AY819725*
CBS 116131	*F. arbuti*	*Arbutus menziesii*	Washington, USA	M. Elliott	*AY819720*

[1]Acronyms of culture collections: ATCC – American Type Culture Collection; CAP – A.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; GS – G. Stanosz, University of Wisconsin, USA; STE-U – University of Stellenbosch, South Africa.

[2]Sequence numbers in italics were retrieved from the GenBank public database. All others were obtained in this study.

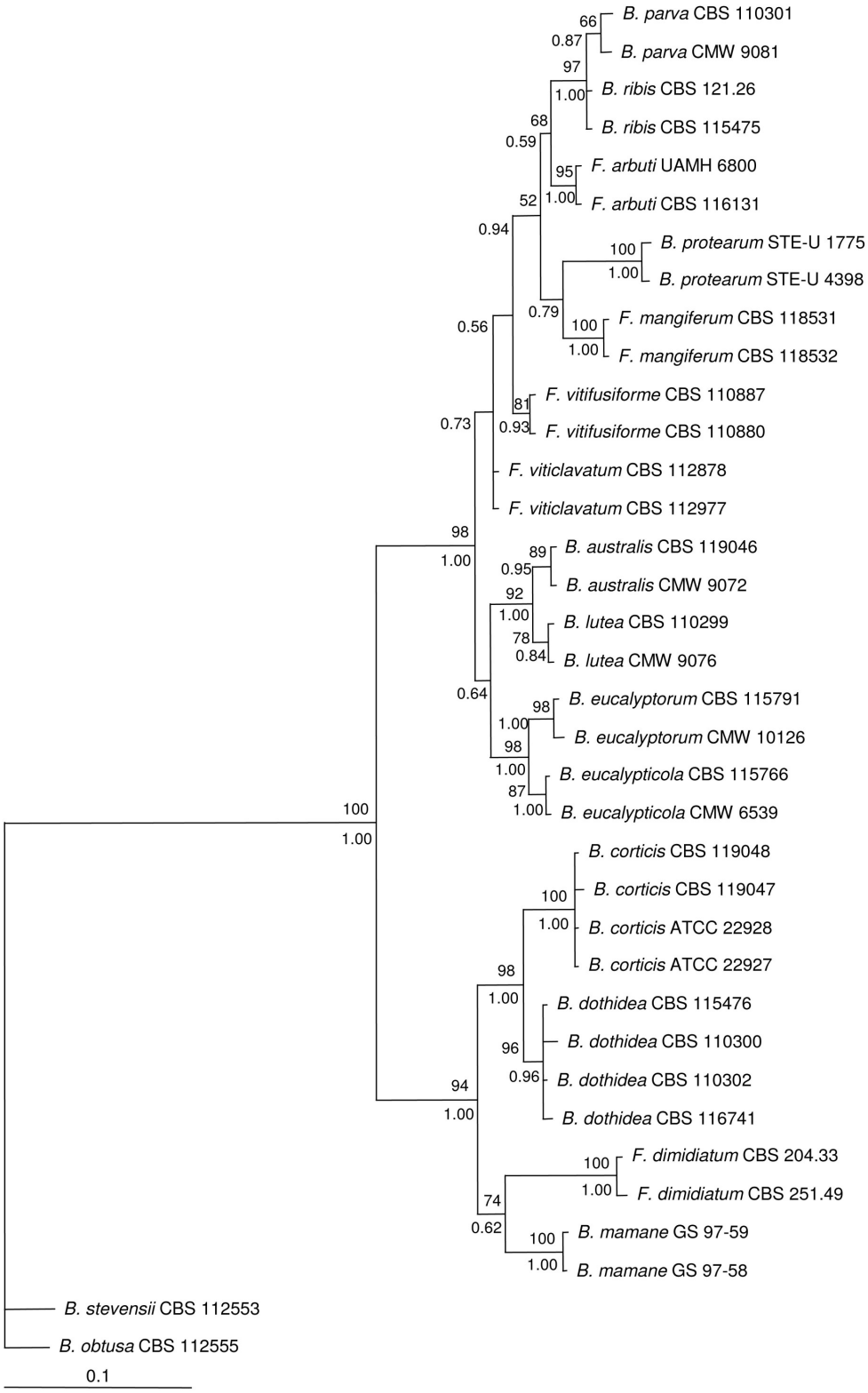


FIGURE 1. Bayesian tree resulting from analysis of ITS sequence data for *Botryosphaeria* species with *Fusicoccum* anamorphs. The numbers above the branches indicate pooled posterior percentages from three independent Bayesian analyses, each consisting of 10^6 Markov Chain Monte Carlo generations (GTR+I+Γ model), with a burn-in of 10^3 generations. Numbers below the branches indicate MP bootstrap support percentages from 10^3 pseudoreplicates. The tree was rooted to *B. stevensii* and *B. obtusa*.

TAXONOMY

The holotype of *B. corticis* (BPI 98729) was collected from *V. corymbosum* growing at Atkinson, North Carolina, USA in February 1940 by J.B. Demaree. The host was reported as *V. australe*, but this is now generally regarded as a synonym of *V. corymbosum* (VanderKloet, 1988). Since no cultures ex-type are available it was recollected and the fungus cultured. This collection (CBS-H 19706) is designated as epitype below.

Botryosphaeria corticis (Demaree & M. S. Wilcox) Arx & Müller, *Beitr. Kryptfl. Schweiz* **11**: 42 (1954). FIGS 2–8

≡ *Physalospora corticis* Demaree & Wilcox, *Phytopathology* **32**: 1074 (1942).

Anamorph: A *Fusicoccum* species. FIGS 9–17

Ascomata were abundant on the host, but conidiomata were not seen on any of the samples examined. *Ascomata* (FIGS. 2, 3) embedded in the host becoming partially erumpent at maturity, up to 250 µm diam., conical with a dark brown to black wall composed of up to six cell layers of thick-walled *textura angularis* giving way to hyaline, thinner-walled cells lining the ascomata (FIG. 4). *Asci* (FIGS. 5, 6) 145–165 × 25–28 µm, hyaline, clavate and stipitate, bitunicate with a thick endotunica and well-developed apical chamber, eight-spored, irregularly biseriate, formed amongst hyaline, thin-walled, septate *pseudoparaphyses* (FIG. 7). *Ascospores* (FIG. 8) ellipsoid to fusoid, (24–)25.5–33(–34.5) × (9.5–)10–12.5(–13.5) µm, 95 % confidence limits = 28.5–30.1 × 11.2–11.9 µm ($\bar{x}$ ± S.D. of 32 = 29.3 ± 2.4 × 11.6 ± 1 µm, L/W = 2.5 ± 0.23), aseptate, hyaline, thin-walled, widest in the middle to upper third.

Ascospores germinated within 24 h at 25 ºC and formed unbranched germ tubes. Colonies on CMA 28–40 mm diam. after 7 days at 25 ºC, initially white becoming olive green with clumps of loosely aggregated hyphae.

Conidiomata (FIG. 9) developing in culture on pieces of poplar twigs after 14 days and producing conidia after 28 days, solitary to aggregated, dark brown to black, globose, up to 450 µm diam. *Conidiophores* (FIG. 11) cylindrical, 7.5–14 × 3.5–4.5 µm, hyaline, smooth, thin-walled, septate, branched in the upper parts, lining the entire inner surface of the conidiomata. *Conidiogenous cells* (FIGS. 10–12) lageniform, 12.5–17.5 × 2.5–4.5 µm, hyaline, thin-walled, smooth, holoblastic producing a single conidium at the tip, rarely proliferating at the same level giving rise to periclinal thickenings. *Conidia* (FIG. 13) fusiform, (20.5–)23.5–32.5(–34.5) × (5–)5.5–7(–7.5) µm, 95 % confidence limits = 27.7–30.2 × 6.2–6.7 µm ($\bar{x}$ ± S.D. of 26 = 28.9 ± 3.4 × 6.4 ± 0.7 µm, L/W = 4.5 ± 0.46), widest in the middle to upper third, hyaline, thin-

walled, smooth, apex acute, base truncate with a minute marginal frill. *Microconidiomata* globose, dark brown to black. *Microconidiophores* (FIG. 14) cylindrical, 11–14 × 2–3 µm, hyaline, branched. *Microconidiogenous cells* (FIGS. 15, 16) 14.5–20.5 × 1.5–2.3 µm, hyaline, thin-walled, smooth, producing conidia at their tips, proliferating internally to form periclinal thickenings. *Microconidia* (FIG. 17) rod-shaped with obtuse ends, 4.1–6 × 1.5–2 µm, hyaline, thin-walled, smooth.

Habitat: On stems of *Vaccinium* species.

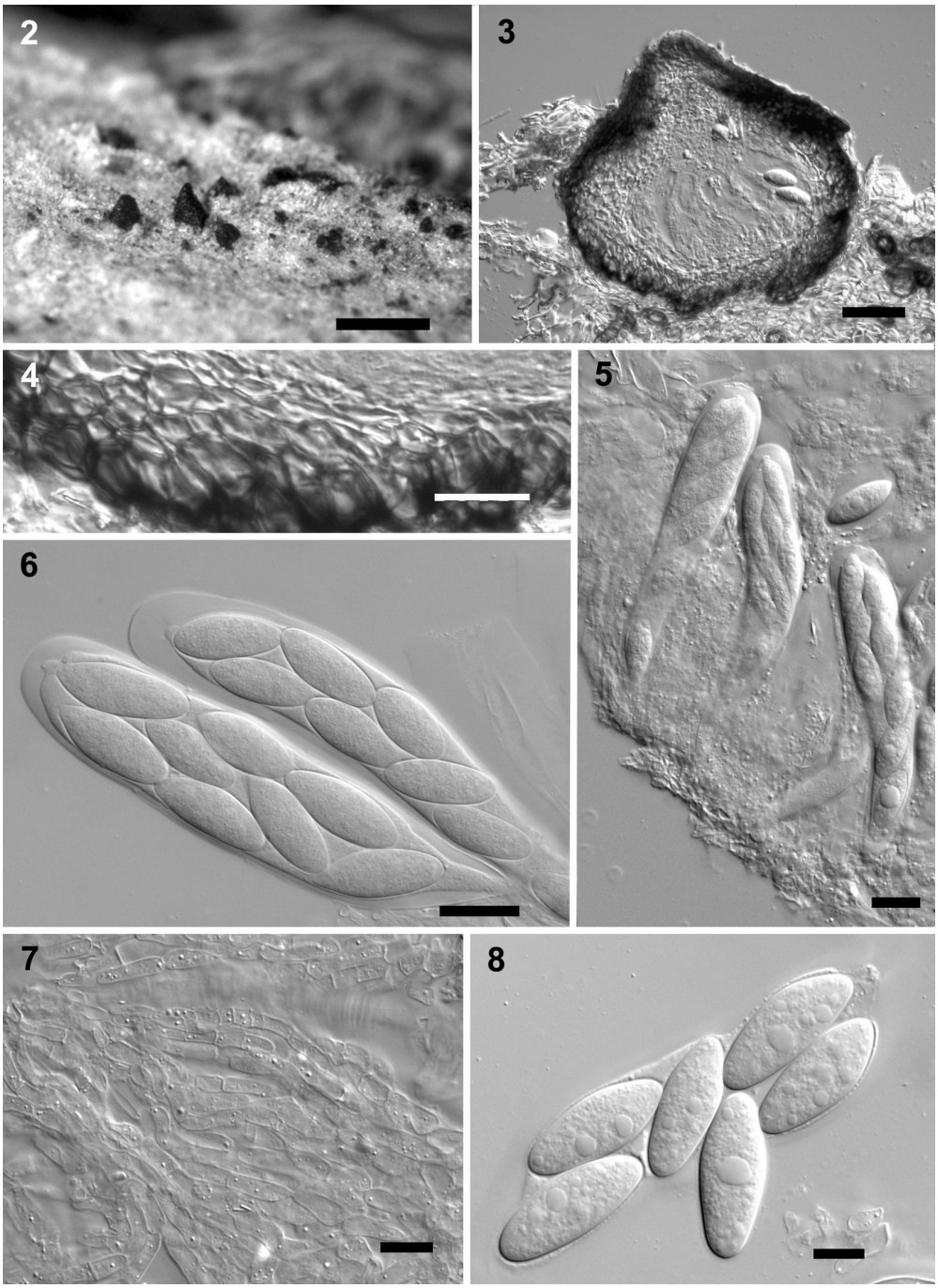
Known distribution: USA.

Material examined: USA, New Jersey, Hammonton, on stems of *Vaccinium corymbosum*, cv. Bluecrop, May 2005, P.V. Oudemans (CBS-H 19706; **epitype designated here**, cultures ex-epitype CBS 119047, CBS 119048; holotype in BPI 598729). USA, North Carolina, on stems of *V. corymbosum*, R.D. Millholland (ATCC 22927, ATCC 22928)

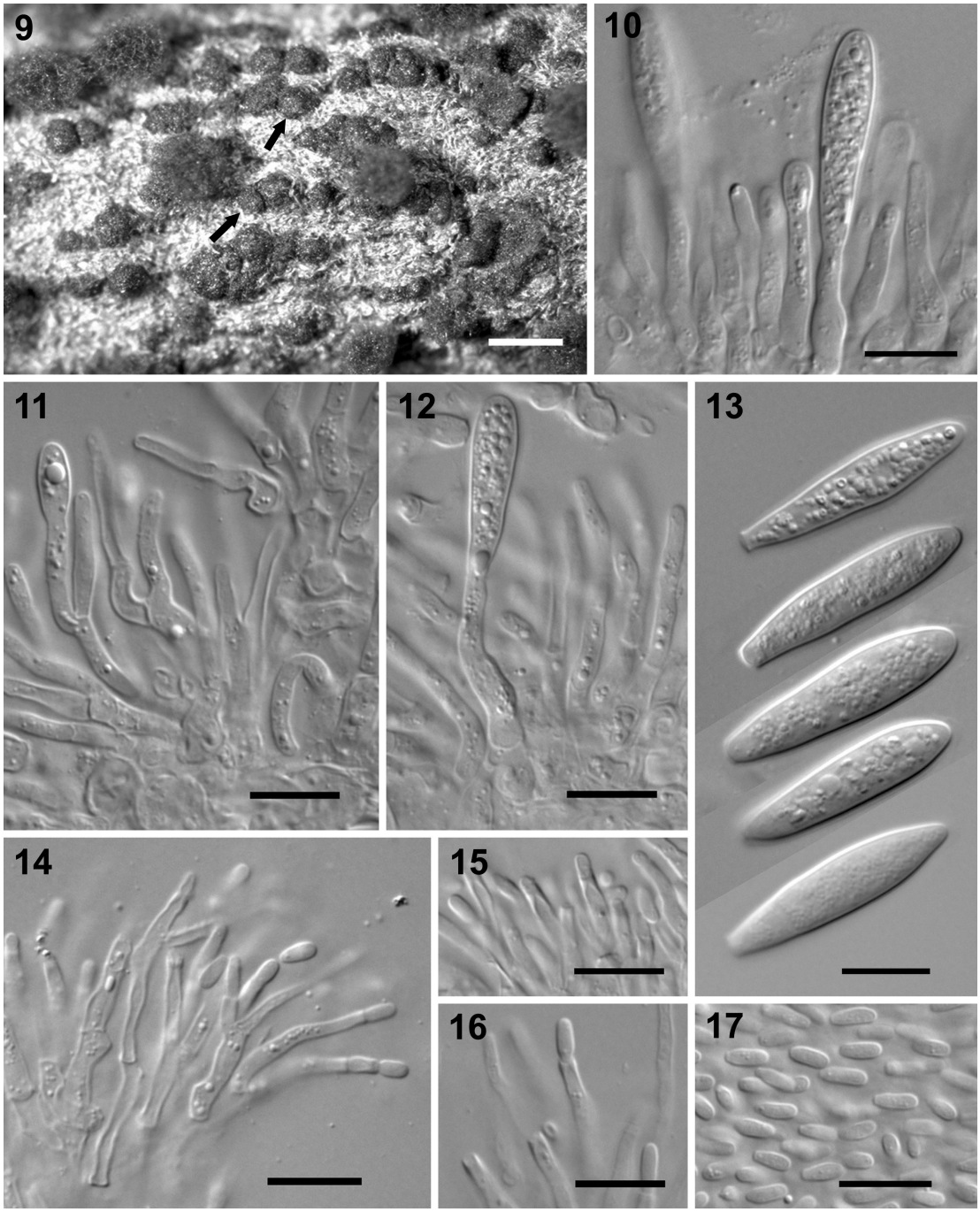
DISCUSSION

Botryosphaeria corticis was first described by Demaree and Wilcox (1942) as *Physalospora corticis* Demaree & M.S. Wilcox. Von Arx and Müller (1954) considered this to be a species of *Botryosphaeria* and made the new combination *Botryosphaeria corticis* (Demaree & M.S. Wilcox) Arx & E. Müll. The morphology of ascomata, asci and ascospores of the specimens examined in this study correlated well with the description of *Physalospora corticis* provided by Demaree and Wilcox (1942). These features are commonly associated with *Botryosphaeria* and the transfer by von Arx and Müller (1954) to *Botryosphaeria* is supported. Phylogenetic analysis of the isolates from *V. corymbosum* placed them within the genus *Botryosphaeria* and within the clade containing *B. dothidea*. The slightly larger ascospores and conidia of *Botryosphaeria corticis* distinguish it from its closest relative, *B. dothidea*.

The specimen of *B. corticis* examined here correlated well with the protologue and is proposed herein as epitype. The type of *B. corticis* was collected from a North Carolina field of *V. corymbosum* while the epitype proposed here was collected from the same host in New Jersey. Two isolates of *B. corticis* from North Carolina were included in the phylogenetic study (ATCC 22927 and ATCC 22928) and the ITS sequences of these did not differ significantly from cultures prepared from the proposed epitype. Thus, the species in New Jersey and the one in North Carolina can be considered to be the same. For this reason we consider that the proposed epitype is representative of the species.



FIGURES 2–8. *Botryosphaeria corticis* Herb CBS-H 19706 (epitype). 2. Ascomata on a cane of *Vaccinium corymbosum*. 3. Longitudinal section through an ascoma. 4. Section of an ascoma wall. 5. Asci. 6. Asci with eight ascospores and well-developed apical chamber. 7. Pseudoparaphyses. 8. Ascospores. Bars: 2 = 500 µm; 3 = 50 µm; 4, 6 = 20 µm; 5, 7, 8 = 10 µm.



Figures 9–17. *Fusicoccum* anamorph of *Botryosphaeria corticis* CBS 119047 (culture ex-epitype of *B. corticis*). 9. Conidiomata (arrowed) formed in culture on a piece of Poplar twig. 10–12. Conidiogenous cells. 13. Conidia. 14. Microconidiophores. 15. Microconidiogenous cells showing periclinal thickenings. 17. Microconidia. Bars: 9 = 500 µm; 10–17 = 10 µm.

Demaree and Wilcox (1942) did not apply a name to the anamorph of *B. corticis*, and they made no comments on what form genus it could be allied to. The hyaline, fusoid, aseptate conidia produced holoblastically on conidiogenous cells that proliferate internally to form periclinal thickenings, and the eustromatic, pycnidial conidiomata indicate that this is a species of *Fusicoccum*. We have chosen not to provide a name for this element of the holomorph since the teleomorph is well known, well characterised and appears to be common in nature. Nevertheless, the distinguishing features of this and other *Botryosphaeria* species are seen in the anamorphs.

The *Botryosphaeria* species with *Fusicoccum* anamorphs included in this study clustered in two clades in both the Bayesian and the MP analyses. Both clades were supported by high posterior probabilities and bootstrap values. It thus appears as if *Fusicoccum* consists of two phylogenetic groups. Morphological differences between the two groups are, however, less distinct. Nonetheless, the group consisting of *B. dothidea*, *B. corticis*, *B. mamane* and *F. dimidiatum* have conidia that are more fusoid than the other group in which the conidia tend to be more ellipsoid.

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CHAPTER

6

Fungal Diversity
2006
23:1–15

Multi-gene genealogies and morphological data support *Diplodia cupressi* sp. nov., previously recognised as *D. pinea* f. sp. *cupressi*, as a distinct species

ABSTRACT

The causal agent of Diplodia canker of cypress (*Cupressus* spp.) in the Mediterranean region was originally thought to represent a sub-population of the pine pathogen *Diplodia pinea* and was referred to as *D. pinea* f. sp. *cupressi*. In the USA a similar fungus causing canker and dieback of *Juniperus* spp. was referred to as *Diplodia mutila* (teleomorph: *Botryosphaeria stevensii*). The aim of this study was to characterise the cypress pathogen in terms of morphology and sequences of the ITS region, the β-tubulin and translation elongation factor 1-α genes. Phylogenetic analyses showed that the cypress canker pathogen resides in a clade together with other *Diplodia* species. It is, however, distinct from both *D. pinea* and *D. mutila* and more closely related to *Botryosphaeria tsugae*. The distinct phylogenetic position is supported by differences in conidial morphology and it is, therefore, described as *Diplodia cupressi* sp. nov.

***Key words*:** *Botryosphaeriaceae*, *Cupressus*, *Diplodia*, ITS, phylogeny, taxonomy

INTRODUCTION

A Diplodia canker of Italian cypress (*Cupressus sempervirens* L.) was described by Solel *et al*. (1987) in Israel. Since then the disease has been reported from other countries including Morocco (Frisullo and Graniti, 1990), Italy (Evidente *et al*., 1996), South Africa (Linde *et al*., 1997), Greece (Xenopoulos and Tsopelas, 2000), and Tunisia (Intini *et al*., 2005). Isolations made from bark and outer xylem rings of cankers yielded a fungus that according to Solel *et al*. (1987) was very closely related to *Diplodia pinea* (Desm.) J. Kickx f. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & B. Sutton). They considered the cypress pathogen to represent a distinct subpopulation of the plurivorous species *D. pinea*, and named it *D. pinea* f. sp. *cupressi*. It differed from *D. pinea* by the smaller conidia, and the lack of ornamentation on the inner surface of the conidium wall. Also, unlike *D. pinea*, which is a common shoot blight and canker pathogen of *Pinus* species, the cypress isolates were unable to cause any disease symptoms in artificial inoculations of pine trees (Solel *et al*., 1987; Linde *et al*., 1997; Xenopoulos and Tsopelas, 2000).

Swart *et al*. (1993) challenged the close relationship of *D. pinea* and *D. pinea* f. sp. *cupressi* and showed that both fungi are considerably different in terms of

morphological characters like conidium size and shape, growth rates on various culture media, and isozyme profiles. This led them to suggest that, in order to avoid further confusion, the cypress pathogen should be referred to as *Sphaeropsis* sp. until its taxonomy could be further elucidated. The observations of Swart *et al*. (1993) are further supported by molecular data, namely ITS and mt rDNA nucleotide sequence data (Zhou and Stanosz, 2001a, b) and ISSR fingerprinting (Zhou *et al*., 2001).

A fungus identified as *Botryosphaeria stevensii* Shoemaker (anamorph *Diplodia mutila* Fr.) was reported as the cause of a canker disease and dieback of *Juniperus* species in the United States (Tisserat *et al*., 1988; Flynn and Gleason, 1993; Stanosz and Moorman, 1997). Tisserat *et al*. (1988) observed the teleomorph and anamorph states of the fungus on *J. scopulorum* Sarg. in Kansas, and confirmed its pathogenicity by artificial inoculations of *J. scopulorum*, *J. virginiana* L., and *J. chinensis* L. Flynn and Gleason (1993) as well as Stanosz and Moorman (1997) reported only the anamorph in Pennsylvania and Iowa, respectively. On the basis of morphological and pathological data, as well as RAPD marker analysis, Stanosz *et al*. (1998) concluded that the cypress pathogen referred to as *D. pinea* f. sp. *cupressi* in Israel was the same species as the juniper pathogen identified as *B. stevensii* in the United States.

A close affinity between *D. mutila* and *D. pinea* f. sp. *cupressi* was noted by Swart *et al*. (1993), who showed that conidial dimensions of the two taxa were similar. However, Tisserat *et al*. (1988) reported differences between the juniper isolates and an isolate of *B. stevensii* obtained from *Malus pumila* Mill., as well as published descriptions of *B. stevensii*. Differences were noted in colony morphology, pigment production, radial growth rate and ability to rot apple fruit. Phylogenetic analyis of ITS and mt rDNA regions (Zhou and Stanosz, 2001a, b) and ISSR fingerprinting analysis (Zhou *et al*., 2001) showed that *B. stevensii* (*D. mutila*) isolates from juniper are closely related to the cypress pathogen *D. pinea* f. sp. *cupressi* but considerably different from *B. stevensii* isolates from *Malus pumila*.

The purpose of this study was to clarify the taxonomy of the fungus causing disease on cypress and juniper. For this we studied the type specimen and authentic cultures of *D. pinea* f. sp. *cupressi* in terms of morphological characteristics and nucleotide sequence data of ITS, EF1-α, and β-tubulin regions.

MATERIALS AND METHODS

Isolates

Single-conidial or ascospore isolates were prepared according to the methods described by Alves *et al.* (2004). Additional isolates were obtained from the

Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Isolates were maintained on ½ strength Difco PDA.

Morphology

Morphological characters were determined from isolates sporulating on 2 % water agar bearing autoclaved poplar twigs and incubated at 25 ºC under fluorescent light to induce sporulation. Structures were mounted in 100 % lactic acid and digital images were recorded with a Leica DFC 320 camera on a Leica DMR HC microscope. Measurements were made with the Leica IM500 measurement module. From 50 measurements of each type of structure the mean, standard deviation and 95 % confidence intervals were calculated. Dimensions are given as the range of dimensions with minimum and maximum dimensions in parentheses followed by mean and 95 % confidence limits.

DNA isolation, PCR amplification and sequencing

The procedures described by Alves *et al*. (2004) were used to extract genomic DNA from fungal mycelium and to amplify part of the nuclear rRNA cluster using the primers ITS1 and ITS4 (White *et al*., 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene respectively. 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). The amplification conditions for EF1-α and β-tubulin regions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 55 ºC, and 90 s at 72 ºC, and a final extension period of 10 min at 72 ºC. In some cases where amplification of the EF1-α region was not accomplished, a second PCR was performed using 1 μL of the first PCR amplification as template.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described by Alves *et al*. (2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA) and were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were

checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for all three DNA regions of additional *Botryosphaeria* species were taken from GenBank (TABLE 1).

Phylogenetic analyses

The ITS, EF1-α and β-tubulin sequences were aligned with ClustalX version 1.83 (Thompson *et al*., 1997), using pairwise alignment parameters of gap opening = 10, gap extension = 0.1, and multiple alignment parameters of 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 (insertions/deletions) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses of sequence data were done using PAUP* v.4.0b10 (Swofford, 2003) for Maximum-parsimony (MP) and Neighbour-joining (NJ) analyses and Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian analyses. The outgroup taxa selected for rooting the trees were *B. lutea* and *B. ribis*. Trees were visualized with TreeView (Page, 1996).

The kimura-2-parameter nucleotide substitution model (Kimura 1980) was used for distance analysis. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and alignment 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 by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were tree length (TL) consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo method were performed with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck, 2003). 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. 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 1000 trees were discarded as the burn-in phase of each

analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was done three times starting from different random trees to ensure that trees from the same tree space were being sampled during each analysis.

The validity of the incongruence length difference (ILD) test (= partition homogeneity test in PAUP*) for determining whether multiple data sets should be combined has been questioned (Cunningham, 1997; Barker and Lutzoni, 2002). In this study we adopted the method of assessing combinability of data sets by comparing highly supported clades among trees generated from different data sets to detect conflict. High support typically refers to bootstrap support values of ≥ 70 % and Bayesian posterior probabilities ≥ 95 % (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different data sets, this suggests the genes share similar phylogenetic histories, and phylogenetic resolution and support could ultimately be increased by combining the data sets.

RESULTS

Phylogenetic analyses

Approximately 550, 400 and 300 bases were determined for the ITS, β-tubulin and EF1-α genes, respectively, of the isolates (TABLE 1). New sequences were deposited in GenBank (TABLE 1) and the alignments in TreeBase (S1542). Sequences of the three genes were aligned and analysed separately by maximum parsimony and Bayesian analysis, and the resulting trees were compared. No major conflicts were detected between single gene phylogenies indicating that the genes could be combined, thus resulting in increased phylogenetic resolution.

The sequence alignment of 23 isolates (TABLE 1), including the two outgroup species, consisted of 579 characters for the ITS region, 331 for the EF1-α gene and 413 for the β-tubulin gene, including alignment gaps. Indels were coded separately and added to the end of the alignment as characters 1326–1397. In the analyses, alignment gaps were treated as missing data.

The combined dataset consisted of 1397 characters, of which 1043 were constant and 83 variable characters were parsimony-uninformative. Maximum parsimony analysis of the remaining 271 parsimony-informative characters resulted in a single most parsimonious trees (TL = 518 steps, CI = 0.8205, HI = 0.1795, RI = 0.8818, RC = 0.7235). Bayesian and NJ analyses produced trees with the same topology as the MP tree. The Bayesian analysis was done three times and the resulting trees in each

run were identical. The MP tree is shown in FIG. 1 with MP bootstrap supports above and posterior probabilities below the branches.

Phylogenetic analyses clearly separated the ingroup taxa into several clades which correspond to known species as well as two *D. pinea* morphotypes. The two species with *Lasiodiplodia* anamorphs (*B. rhodina* (Berk. & M.A. Curtis) Arx and *L. gonubiensis* Pavlic *et al*.) appear as a highly supported and distinct clade at the base of the ingroup. In phylogenetic terms *D. cupressi* is most closely related to *B. tsugae*, with both species forming a larger clade together with several isolates identified as *B. stevensii*.

Morphology

The two isolates received as *D. pinea* f. sp. *cupressi* (CBS 261.85 and CBS 168.87) sporulated well in culture. Conidia were of the type associated with *Diplodia mutila*. Thus, they were hyaline, thick-walled and aseptate with both ends rounded, or with a truncate base. Morphology in culture corresponded with the characteristics of a specimen lodged by Z. Solel in IMI 303475. These specimens and cultures differed from *D. pinea* in which the conidia are brown and larger. Conidia of the isolates from *Cupressus* are somewhat wider than typical for *D. mutila*. Since the isolates from *Cupressus* are phylogenetically and morphologically distinct, it is described here as a new species in *Diplodia*.

TAXONOMY

Diplodia cupressi A.J.L. Phillips & A. Alves, sp. nov. FIGS 2–10

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa, usque 300 µm diametro. Cellulae conidiogenae 12.5–20 × 4–4.5 µm, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1–4 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia 23.5–28.5 × 13.5–15.0 µm, hyalinae, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, cum aetas colorescentia uno cum septo. Microconidiophorae cylindricae, hyalinae usque 10 µm longae, 2.5–3 µm latae. Cellulae microconidiogenae 10–14 × 2–2.5 µm, cylindricae, hyalinae, holoblasticae, phialidibus typicus periclinater spissescentibus. Microconidia hyalinae, unicellulares, 4–5 × 1.5 µm.

TABLE 1. Isolates studied.

Isolate number[1]	Identity	Host	Locality	Collector	ITS[2]	EF1-α	β-tubulin
CBS 110299	*B. lutea*	*Vitis vinifera*	Oeiras, Portugal	A.J.L. Phillips	*AY259091*	*AY573217*	DQ458848
CBS 115475	*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers	*AY236935*	*AY236877*	*AY236906*
CBS 230.30	*B. stevensii*	*Phoenix dactylifera*	California, U.S.A.	L.L. Huillier	DQ458886	DQ458869	DQ458849
CBS 112553	*B. stevensii*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259093*	*AY573219*	DQ458850
CBS 112554	*B. stevensii*	*Pyrus communis*	Monte da Caparica, Portugal	A.J.L. Phillips	*AY259095*	DQ458870	DQ458851
JL 375	*B. stevensii*	*Fraxinus excelsior*	Llanars, Catalonia, Spain	J. Luque	DQ458887	DQ458871	DQ458852
CMW 7060	*B. stevensii*	*Fraxinus excelsior*	Maarseveen, Netherlands	H.A. van der Aa	*AY236955*	*AY236904*	*AY236933*
CBS 112549	*B. corticola*	*Quercus suber*	Aveiro, Portugal	A. Alves	*AY259100*	*AY573227*	DQ458853
CBS 112547	*B. corticola*	*Quercus ilex*	Córdoba, Spain	M.E. Sanchez	*AY259110*	DQ458872	DQ458854
CBS 418.64	*B. tsugae*	*Tsuga heterophylla*	British Columbia, Canada	A. Funk	DQ458888	DQ458873	DQ458855
CBS 112555	*B. obtusa*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259094*	*AY573220*	DQ458856
CBS 119049	*B. obtusa*	*Vitis* sp.	Italy	L. Mugnai	DQ458889	DQ458874	DQ458857
CBS 124.13	*B. rhodina*	Unknown	U.S.A.	J.J. Taubenhaus	DQ458890	DQ458875	DQ458858
CAA 006	*B. rhodina*	*Vitis vinifera*	California, USA	T.J. Michailides	DQ458891	DQ458876	DQ458859
CBS 115812	*L. gonubiensis*	*Syzygium cordatum*	Eastern Cape, South Africa	D. Pavlic	DQ458892	DQ458877	DQ458860
CBS 168.87	*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458893	DQ458878	DQ458861
CBS 261.85	*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458894	DQ458879	DQ458862
CBS 393.84	*D. pinea*	*Pinus nigra*	Putten, Netherlands	H.A. van der Aa	DQ458895	DQ458880	DQ458863
CBS 109725	*D. pinea*	*Pinus patula*	Habinsaran, South Africa	M.J. Wingfield	DQ458896	DQ458881	DQ458864
CBS 109727	*D. pinea*	*Pinus radiata*	Stellenbosch, South Africa	W.J. Swart	DQ458897	DQ458882	DQ458865
CBS 109943	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	DQ458898	DQ458883	DQ458866
CBS 109944	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458899	DQ458884	DQ458867
CBS 113424	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458900	DQ458885	DQ458868

[1]Acronyms of culture collections: CAA – A. Alves, Universidade de Aveiro, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; JL – J. Luque, IRTA, Spain. Isolates in bold are cultures ex-type.
[2]Sequence numbers in italics were retrieved from the GenBank public database. All others were obtained in this study.

Conidiomata up to 300 µm diameter, solitary, separate, uniloculate, dark brown to black, globose, ostiolate, wall composed of thick-walled textura angularis, becoming thin-walled and hyaline toward the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 12.5–20 × 4–4.5 µm, hyaline, smooth, holoblastic forming conidia at their tips, proliferating internally giving rise to periclinal thickenings or proliferating percurrently with 1–4 close or widely spaced annellations, formed from the inner wall of the pycnidium. *Conidia* (21.5–)23.5–28.5(–30.5) × (12.0–)13.5–15.0(–16.0) µm, 95 % confidence limits = 24.4–25.4 × 13.9–14.5 µm, ($\bar{x}$ ± S.D. of 50 = 24.9 ± 1.9 × 14.2 ± 0.9 µm, L/W = 1.76 ± 0.18) thick-walled, wall up to 2 µm wide, ovoid with both ends rounded, aseptate, hyaline and remaining so for a long time, becoming brown and one-septate after discharge from the pycnidia. *Microconidiophores* hyaline, smooth, cylindrical, up to 10 µm long, 2.5–3 µm wide. *Microconidiogenous cells* discrete or integrated, hyaline, smooth, cylindrical, holoblastic or proliferating via determinate phialides with periclinal thickening, 10–14 × 2–2.5 µm. *Microconidia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 4–5 × 1.5 µm.

Etymology: Named for the host genus it was first reported on, namely *Cupressus*.

Teleomorph: An unknown *Botryosphaeria* sp.

Habitat: On cankered stems of *Cupressus* and *Juniperus* species.

Known distribution: Greece, Israel, Italy, Morocco, South Africa, Tunisia, U.S.A.

Material examined: ISRAEL, Bet Dagan, dried culture from cankered stems of *Cupressus sempervirens*, 1986, Z. Solel (HERB IMI 303475; **holotype**, culture ex-type CBS 168.87).

DISCUSSION

The taxonomy of the fungal pathogen causing canker of cypress and juniper has been confused. Solel *et al*. (1987) considered it to be a forma specialis of the plurivorous pine pathogen *D. pinea* (= *S. sapinea*) and named it *D. pinea* f. sp. *cupressi*, while in the United States it was identified as *B. stevensii* or its anamorph *D. mutila*.

In this paper we studied the type specimen and authentic cultures of *D. pinea* f. sp. *cupressi* in terms of morphological characteristics and nucleotide sequences of ITS, EF1-α, and β-tubulin regions. On the basis of the morphological and multigene sequence data we recognize the cypress and juniper canker pathogen as a new species and describe it as *D. cupressi* sp. nov.

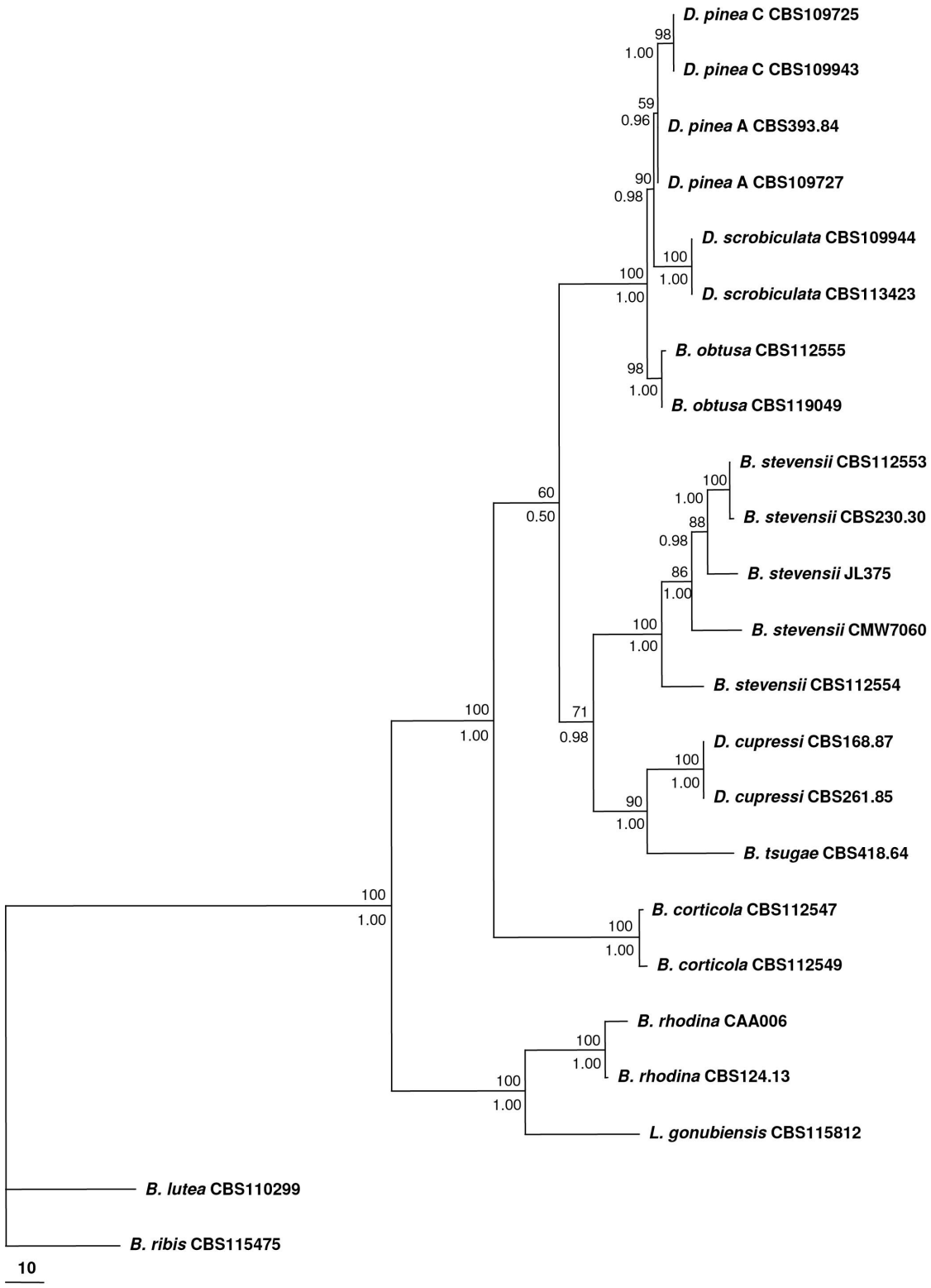
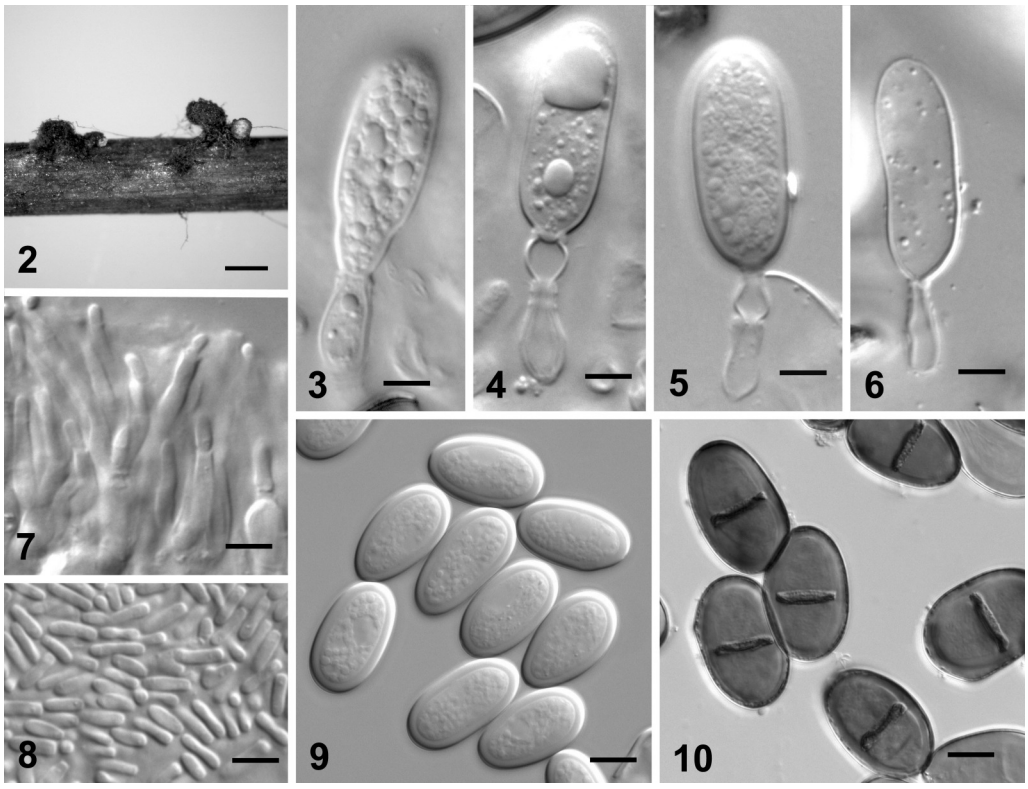


FIGURE 1. Single most parsimonious tree resulting from combined ITS, EF1-α and β-tubulin sequence data. Bootstrap support three independent Bayesian analyses below the nodes. The tree was rooted to *B. lutea* and *B. ribis*. The bar represents 10 changes.



FIGURES 2–10. *Diplodia cupressi* (from cultures ex-holotype). 2. Conidiomata formed in culture on an autoclaved pine needle. 3–6. Conidiogenous cells. 7. Microconidiogenous cells. 8. Microconidia. 9. Hyaline, aseptate, thick-walled conidia. 10. Brown, one-septate conidia. Bars: 2 = 500 µm; 3–10 = 10 µm.

Diplodia cupressi is clearly differentiated from *D. pinea* and *D. mutila* on the basis of morphological, cultural, and molecular characters. Conidia of *D. cupressi* are thick-walled, smooth, initially hyaline and aseptate and remain so for a long time, ultimately becoming one-septate and dark-walled after discharge from the pycnidia. In contrast, conidia of *D. pinea* become dark at an early stage of development and whilst within the pycnidial cavity. *Diplodia cupressi* differs from *D. pinea* not only in the stage at which the conidia become pigmented, there are some other characters that differentiate the two species. Conidia of *D. pinea* become septate just before germination, while in *D. cupressi* septation does not seem to be associated with germination. Furthermore, conidia of *D. pinea* are larger than those of *D. cupressi* with average lengths exceeding 40 µm. As discussed previously by Swart *et al*. (1993) and Stanosz *et al*. (1998) and confirmed by the data presented in this study *D. cupressi* differs markedly from *D. pinea* and cannot be considered merely as a host specialized sub-population within this species.

The thick-walled conidia that remain hyaline for a long time are characteristic of *Diplodia* as typified by *D. mutila* (Alves *et al*., 2004; Phillips *et al*., 2005). In this respect, *D. cupressi* closely resembles *D. mutila*. However, the conidia of *D. cupressi* are wider than typical of *D. mutila* (Alves *et al*., 2004) and correspond well with those described by Solel *et al*. (1987) in the original description of this fungus. They also correspond to the description of the fungus Tisserat *et al*. (1988) referred to as *B. stevensii*. Since they are larger than in the type of *D. mutila* and in the anamorph associated with the type of *B. stevensii* (Alves *et al*., 2004) we conclude that this fungus is not *B. stevensii* (*D. mutila*). This is supported by previous observations from Tisserat *et al*. (1988) who reported that several differences existed between *D. cupressi* and isolates obtained from apple and identified as *B. stevensii*.

Phylogenetic analyses of separate and combined nucleotide sequence data from three genes (ITS, EF1-α, and β-tubulin) place *D. cupressi* within Clade 1 of the *Botryosphaeriaceae* (Crous *et al*., 2006) but clearly separate it from all other *Diplodia* species. In phylogenetic terms *D. cupressi* is most closely related to *B. tsugae*, a species occurring on *Tsuga heterophylla* and known only from British Columbia (Funk, 1964), than to *B. stevensii*. Morphologically *D. cupressi* is easily separated from the anamorph of *B. tsugae*, which has much larger conidia (see TABLE 2).

Although no teleomorph has been described for this species it apparently does exist and is clearly a member of the genus *Botryosphaeria*. The teleomorph was reported by Tisserat *et al*. (1988) on dead branches of *J. scopulorum* and at the time identified as *B. stevensii*. However, no specimens of the teleomorph could be traced (Tisserat pers. comm.). Tisserat *et al*. (1988) established the connection between the teleomorphic and anamorphic states by culture of ascospores. According to these authors pseudothecia were immersed in the host, asci measured 120 $\times$ 16 µm; ascospores were hyaline, smooth, thick walled, elliptical to ovate, and 32–40(37) $\times$ 12–16(14) µm. Although dimensions of the asci fall within the range of the type of *B. stevensii,* the ascospores are much larger than were reported for the type of *B. stevensii*, which average 31.5 ± 2.3 $\times$ 11.4 ± 0.9 µm (Alves *et al*., 2004). Also, ascospores of *B. stevensii* are fusiform, widest in the middle, both ends obtuse, hyaline, thin-walled, smooth, aseptate, rarely becoming pale brown and 1- or 2-septate with age. It is thus apparent that the ascomycete reported by Tisserat *et al*. (1988) represents a previously undescribed species for which no name is provided due to the lack of a suitable specimen. It can be distinguished from its closest phylogenetic relative, *B. tsugae*, whose asci and ascospores are larger.

TABLE 2. *Botryosphaeria* and *Diplodia* species associated with Gymnosperms.

Teleomorph	Anamorph	Conidia		References
		colour/septation	size	
B. laricis (Wehm.) Arx & E. Müll.	A form of *Macrophoma sapinea*	dark, aseptate	17.5–44.1 × 11.2–21.0 µm	Smerlis (1970)
B. tsugae A. Funk	*Diplodia* sp.	hyaline, aseptate	36–41 × 18–22 µm	Funk (1964)
Botryosphaeria sp.	*D. cupressi* A.J.L. Phillips & A. Alves	hyaline, aseptate	23.5–28.5 × 13.5–15 µm	this work
"*B. stevensii*"	"*D. mutila*"	hyaline, aseptate	23–32 × 12–15 µm	Tisserat *et al*. (1988)
Unknown	*D. pinea* (Desm.) J. Kickx f.	dark, aseptate	30–45 × 10–16 µm	Punithalingam and Waterston (1970)
Unknown	*D. scrobiculata* J. de Wet *et al*.	dark, 1-3-septate	37.5–41.5 × 13–15.5 µm	de Wet *et al.* (2003)
Unknown	*D. cyparissa* Cooke & Harkn.	hyaline, aseptate	20–22 × 9 µm	Saccardo (1884)
Unknown	*D. juniperi* Westend.	dark, 1-septate	18–20 × 8–10 µm	Saccardo (1884)
Unknown	*D. kansensis* Ellis & Everh.	dark, 1-septate	20–27 × 12–15 µm	Saccardo (1895)
Unknown	*D. thujae* Sacc.	Unknown	20–25 × 10 µm	Saccardo (1884)
Unknown	*D. thujae* G.H. Otth	dark, 1-septate	20 × 9 µm	Saccardo (1895)
Unknown	*D. thujae* Westend.	dark, 1-septate	18–20 × 9–10 µm	Saccardo (1899)
Unknown	*D. thujana* Peck & Clinton	dark	18–23	Saccardo (1884)
Unknown	*D. thyoidea* Cooke & Ellis	dark	25–28 × 12–13 µm	Saccardo (1884)
Unknown	*D. virginiana* Cooke & Ravenel	dark, 1-septate	20–25 × 10 µm	Saccardo (1884)
Unknown	*D. megalospora* Berk. & M.A. Curtis	Unknown	37 × 12 µm	Saccardo (1884)
Unknown	*D. conigena* Desm.	dark, 1-septate	26–30 × 12–15 µm	Saccardo (1884)

Many *Botryosphaeria* and *Diplodia* species have been associated with Gymnosperms (TABLE 2). Cultures are lacking for most of these species thus making appropriate comparisons impossible. However, it can be seen from the data retrieved from the literature and summarized in TABLE 2 that none of the available names is suitable for *D. cupressi*.

Due to the close morphological resemblance *D. cupressi* has in the past been identified as *D. mutila* (Tisserat *et al*., 1988; Flynn and Gleason, 1993; Stanosz and Moorman, 1997). For this reason we included in this study several isolates obtained from different hosts and whose morphological features support their identification as *B. stevensii* or its anamorph *D. mutila*. These cultures differed phylogenetically from *D. cupressi* and grouped in a highly supported clade. Although closely related phylogenetically, some variation can be seen between these cultures in terms of nucleotide sequence from the three genes analysed (ITS, EF1-α, and β-tubulin). This variability falls within the range that can be found between different species in the genus *Botryosphaeria* and so these isolates probably represent a complex of cryptic species that needs to be resolved.

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CHAPTER

7

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Evaluation of amplified ribosomal DNA restriction analysis (ARDRA) as a method for the identification of *Botryosphaeria* species

ABSTRACT

The polymerase chain reaction (PCR) was used to amplify a rDNA fragment containing the internal transcribed spacers (ITS1-5.8S-ITS2) and the D1/D2 variable domains of the 28S rDNA from 10 species of the genus *Botryosphaeria* (Fungi, Ascomycota). Restriction analysis of the amplicons with frequent-cutting endonucleases (ARDRA) allowed the definition of 12 rDNA haplotypes. Each of the rDNA haplotypes could be unambiguously assigned to a single *Botryosphaeria* species, thus allowing clear identification of all the species tested. Intraspecific polymorphism was very low and detected only in *B. parva* and *B. dothidea*. Cluster analysis of banding patterns of the isolates corresponded well with known species delineations. The method described in this paper provides a simple and rapid procedure for the differentiation and identification of *Botryosphaeria* isolates at the species level.

***Key words*:** *Botryosphaeria*, rDNA, ITS, D1/D2 domains, species identification, ARDRA.

INTRODUCTION

Botryosphaeria species (Fungi, Ascomycota) have a worldwide distribution causing dieback, cankers, shoot blights, leaf spots, fruit and seed rots, and witches' brooms in a wide range of plant hosts of agricultural, forestry, ecological and economic importance. In addition, some species are saprophytic while others are endophytic (Barr, 1987; Denman *et al*., 2000, 2003; Sánchez *et al*., 2003).

Identification of these fungi at the species level is complicated by several aspects. Morphological diversity among the teleomorphs is often insufficient to allow clear species identification. For this reason, identification of species is based mainly on morphological characters of the anamorph, which is the most common form found in nature (Pennycook and Samuels, 1985; Jacobs and Rehner, 1998; Denman *et al*., 2000). These characters include size, shape, colour, septation, wall thickness and texture of the conidia, as well as details of conidiogenesis. Cultural aspects, such as colony morphology, chromogenicity, and temperature effects on mycelial growth rate have also been used for species recognition. However, some morphological characters exhibit extensive plasticity. Thus, size ranges of conidia of different *Botryosphaeria* species overlap, while age and state of maturity affect conidial pigmentation and

septation. Taxonomically meaningful morphological characters can also be influenced by the substrate on which the fungus is growing. Moreover, the diversity of hosts colonized by a single species and the occurrence of different species on the same host precludes the use of host association as a valid taxonomic character (Pennycook and Samuels, 1985; Jacobs and Rehner, 1998). Two closely related species may differ by minor morphological differences making identification a difficult task for a practitioner without experience of this group of pathogens. Therefore there is a need for tools that can provide accurate and reproducible identifications of *Botryosphaeria* species.

Ribosomal DNA (rDNA) sequences are universal and contain conserved and variable regions allowing discrimination of fungi at different taxonomic levels (Guarro *et al*., 1999). Non-coding regions, like the ITS (internal transcribed spacer), evolve more rapidly and are consequently more variable than coding regions. Within coding rDNA regions some domains like the D1 and D2 of the 28S rDNA gene are also known to be variable (Guarro *et al*., 1999). These regions are easy to access using universal fungal primers (White *et al*., 1990; O'Donnell, 1993) and the PCR (polymerase chain reaction) and have been used extensively in inter- as well as intraspecific comparisons of several fungi (Guarro *et al*., 1999). Sequence analyses of the ITS region alone (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001; Alves *et al*., 2004) or in combination with introns of protein-encoding genes such as β-tubulin and the translation elongation factor 1-alpha (EF1-α) (Slippers *et al*., 2004a, b; van Niekerk *et al*., 2004; Phillips *et al*., 2005) have made a significant contribution to resolving taxonomic problems in the genus *Botryosphaeria*. These genomic regions have been used to study relationships among species and to distinguish closely related *Botryosphaeria* spp. (Smith *et* al., 2001; Denman *et al*., 2003, Alves *et al*., 2004; Slippers *et al*., 2004a, b; van Niekerk *et al*., 2004; Phillips *et al*., 2005).

Amplified ribosomal DNA restriction analysis (ARDRA) has proved to be a suitable and rapid method for taxonomic studies of fungi (Guarro *et al*., 1999). This method is based on the PCR amplification of an rDNA fragment, followed by the digestion of the amplicon with frequent-cutting restriction endonucleases. The resulting fragments are then analysed by agarose gel electrophoresis (Guarro *et al*., 1999; Olive and Bean, 1999).

The purpose of our work was to develop a rapid procedure for the identification of *Botryosphaeria* species using the ARDRA technique. The method is based on the PCR amplification of the ITS + D1/D2 variable domains of the 28S rDNA and generation of restriction fingerprints, and was tested on 35 strains representing 10 species, including the most commonly reported species responsible for diseases in plants of economic importance.

MATERIALS AND METHODS

Fungal strains

The fungal strains used in this study are listed on TABLE 1. Cultures were maintained on half-strength Difco potato dextrose agar or oatmeal agar (Anonymous, 1968).

DNA extraction

Fungal strains were grown in potato dextrose broth for 5 days at approximately 25 ºC. Genomic DNA was extracted from fresh mycelium following an adaptation of the method of Pitcher *et al.* (1989) as published elsewhere (Alves *et al*., 2004).

PCR amplifications

PCR reactions were performed on a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA). The PCR primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (White *et al*., 1990) and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (O'Donnell, 1993) were supplied by MWG Biotech AG (Ebersberg, Germany). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania). The PCR reaction mixtures contained 1 × PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with $(NH_4)_2SO_4$; 1:1 v:v), 3 mM $MgCl_2$, 200 μM of each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50–100 ng of template DNA. Each reaction volume was made up to 50 μl with sterile water. Negative controls with sterile water instead of the template DNA were used in every PCR reaction. The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 1 min at 72 ºC, and a final extension period of 10 min at 72 ºC.

After amplification, 5 μL of each PCR product were separated by electrophoresis in 1% agarose gels in 1 × TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, and visualized on a UV transilluminator to assess PCR amplification.

Restriction analysis

The amplicons were digested with one of the following restriction endonucleases: *Alu*I, *Asu*I, *Hae*III, *Mbo*I (MBI Fermentas, Vilnius, Lithuania); *Nci*I, *Taq*I (Gibco BRL, Eggenstein, Germany). The reactions contained 10 μL of the PCR product that was digested with 2 U of enzyme for 14–16 h following the manufacturer's recommendations. DNA fragments were electrophoretically separated in 4 % Agarose 3:1 HRB™ (Amresco) gels using 1 × TAE buffer under a constant voltage of 80 V for

2.5 h. A molecular weight marker, GeneRuler™ 100 bp DNA Ladder plus (MBI Fermentas) was run on both sides of each gel. The procedure (DNA isolation, PCR amplification and restriction analysis) was performed twice to assess reproducibility of the method.

Computer-assisted analysis

Agarose gels were scanned on a Molecular Imager FX (BioRad) and recorded as TIFF images. The banding patterns were analysed with Quantity One (BioRad) software and converted to a two-dimensional binary matrix (1= presence of a band; 0= absence of a band). Similarity matrices between each pair of combined patterns were calculated with the Dice coefficient (Priest and Austin, 1993), since this coefficient is the best estimator for calculation of genetic divergence from DNA fragment patterns (Nei and Li, 1979). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA). The cophenetic correlation coefficient (*r*) was calculated to assess the goodness of the clustering method. Computer-assisted analysis was performed with the NTSYSpc2 program for Windows (Rohlf, 1993).

DNA sequencing

The D1/D2 regions from representatives of each species/haplotype were sequenced with the primers NL1 (5'-GCATATCAATAAGCGGAGGA-3') and NL4 (O'Donnell, 1993). Cycle sequencing reactions were as described previously (Alves *et al*., 2004), using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler.

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA) and were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database with the following accession numbers: AY928043 to AY928054.

Restriction map analysis

Sequence data of the ITS + D1/D2 regions of selected isolates representing each species or haplotype were analysed with the software Webcutter 2.0 available online (http://rna.lundberg.gu.se/cutter2/) to identify polymorphisms of restriction sites. Restriction maps (FIG. 3) were constructed for the six enzymes tested.

TABLE 1. Cultures used in this study.

Species (Teleomorph)	Anamorph	Accession no.[1]	Host	Locality
Botryosphaeria dothidea	***Fusicoccum aesculi***	**CMW 8000**[2]	*Prunus* sp.	Switzerland, Crocifisso
		CBS 110302	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CBS 113190	*Quercus suber*	Spain, Sevilla
		CBS 113191	*Quercus ilex*	Spain, Córdoba
		CMW 7780	*Fraxinus excelsior*	Switzerland, Molinizza
		CBS 110300	*Populus nigra*	Portugal, Braga
Botryosphaeria ribis	***Fusicoccum ribis***	**CMW 7772**	*Ribes* sp.	USA, New York
		CMW 7054	*Ribes rubrum*	USA, New York
		CMW 7773	*Ribes* sp.	USA, New York
Botryosphaeria parva	***Fusicoccum parvum***	**CMW 9081**	*Populus nigra*	New Zealand, TePuke
		CMW 9080	*Populus nigra*	New Zealand, TePuke
		CBS 110301	*Vitis vinifera*	Portugal, Palmela
Botryosphaeria lutea	***Fusicoccum luteum***	**CBS 110299**	*Vitis vinifera*	Portugal, Oeiras
		CAP 037	*Vitis vinifera*	Portugal, Sintra
Botryosphaeria corticola	***Diplodia corticola***	**CBS 112549**	*Quercus suber*	Portugal, Aveiro
		CBS 112545	*Quercus suber*	Spain, Cádiz
		CBS 112546	*Quercus ilex*	Spain, Huelva
		CBS 112552	*Quercus suber*	Portugal, Aveiro
		CBS 112071	*Quercus ilex*	Spain, Catalonia
Botryosphaeria stevensii	***Diplodia mutila***	CBS 112553	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CBS 230.30	Unknown	Unknown
Botryosphaeria obtusa	***Diplodia* sp.**	CBS 112555	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CAP 148	*Vitis* sp.	Italy
Botryosphaeria rhodina	***Lasiodiplodia theobromae***	CBS 124.13	Unknown	Unknown
		CBS 164.96	Fruit along coral reef coast	Papua New Guinea
		CAA 006	*Vitis vinifera*	USA, California
Botryosphaeria iberica	***Dothiorella iberica***	**CBS 115041**	*Quercus ilex*	Spain, Aragon
		CBS 115035	*Quercus ilex*	Spain, Aragon

		CBS 115039	*Quercus* sp.	Italy, Lazio
		CBS 113188	*Quercus suber*	Spain, Sevilla
		CBS 113189	*Quercus ilex*	Spain, Córdoba
Botryosphaeria sarmentorum	***Dothiorella sarmentorum***	**IMI 63581b**	*Ulmus* sp.	England, Warwickshire
		CBS 115038	*Malus* sp.	The Netherlands, Delft
		CBS 120.41	*Pyrus communis*	Norway
		CBS 165.33	*Prunus armeniaca*	Unknown

[1]Acronyms of culture collections: CAA – A. Alves, Universidade de Aveiro, Portugal; CAP – A.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; IMI – CABI Bioscience, Egham, U.K.

[2]Isolate accession numbers in bold signify cultures ex-type, or from samples that have been linked morphologically to the type material.

RESULTS

Restriction analysis of PCR amplicons

PCR reactions using primers ITS1 and NL4 yielded a single amplicon of about 1200 bp for each of the 35 strains tested. All amplicons were digested with each of the six restriction enzymes used, resulting in two to six fragments, depending on the enzymes and strains. In some cases, no restriction fragments were produced with the enzyme *Nci*I. The sum of the restriction fragments sometimes gave a size smaller than that of the undigested PCR products. This is because restriction fragments smaller than 50 bp were not taken into consideration since they were not clearly resolved by electrophoresis in 4% agarose gels. Also, some bands may represent two restriction fragments of the same size (doublets).

Previous tests revealed that restriction analysis of purified and unpurifed PCR products yielded the same result and that the components of the PCR reaction did not affect the digestion reaction (data not shown). Eliminating the PCR product purification step renders the method faster and cheaper and so all subsequent enzymatic digestions were performed with unpurified PCR products. Enzymatic digestion of amplicons obtained from independent analyses always yielded the same ARDRA patterns, thus showing the reproducibility of the procedure.

The enzymes *Mbo*I and *Alu*I generated five distinct restriction patterns, *Nci*I generated six, *Hae*III generated seven, *Taq*I and *Asu*I generated eight restriction patterns (FIG. 1). Each unique fingerprint produced by an endonuclease was designated by a letter. The ARDRA patterns obtained with the six restriction enzymes were combined into 12 rDNA haplotypes (TABLE 2), which are defined by the combination of the patterns obtained with the restriction enzymes. However, three enzymes (*Asu*I, *Taq*I and *Nci*I) were sufficient to resolve the same 12 rDNA haplotypes. Moreover, any of the following two enzyme combinations: *Asu*I/*Alu*I, *Asu*I/*Mbo*I, *Asu*I/*Nci*I and *Asu*I/*Taq*I allowed for *Botryosphaeria* species differentiation. Intraspecific polymorphism was detected only in *B. parva* and *B. dothidea*. The three *B. parva* strains studied produced two different *Taq*I restriction patterns while *B. dothidea* strain CBS 110300 produced an *Nci*I restriction pattern distinct from all other *B. dothidea* strains.

Cluster analysis

A cluster analysis was performed on the combination of ARDRA fingerprints. Using the combined data obtained with the six enzymes, a total of 56 restriction fragments was analysed. The dendrogram generated with the UPGMA method is shown in FIG. 2.

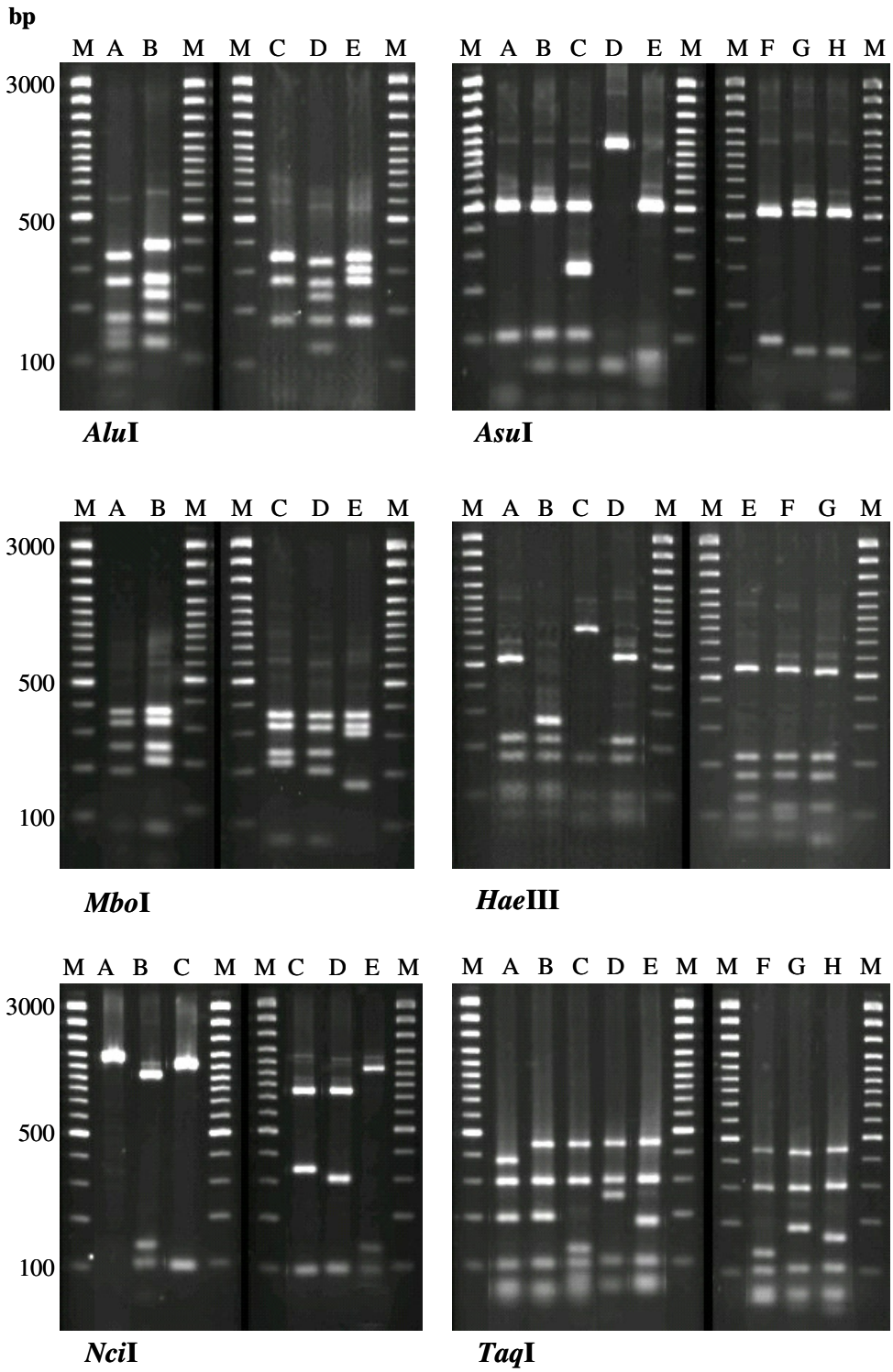


FIGURE 1. ARDRA patterns of the PCR-amplified rDNA fragment obtained by digestion with the enzymes *Alu*I, *Asu*I, *Hae*III, *Mbo*I, *Nci*I, and *Taq*I separated in 4 % Agarose 3:1 HRB™ gels. Each letter corresponds to a different restriction pattern. M. GeneRuler™ 100 bp DNA Ladder plus.

A cophenetic correlation coefficient of 0.94 was found between the cophenetic value matrix deduced from the dendrogram and the similarity matrix. An *r* value greater than 0.9 can be interpreted as a very good fit of the cluster analysis to the data (Rohlf, 1993).

The clustering was consistent with the division of the isolates into 12 different rDNA haplotypes and the corresponding 10 species tested. Species with *Fusicoccum* anamorphs were divided into two groups, namely, a cluster of *B. dothidea* isolates well separated from a cluster containing *B. lutea*, *B. ribis* and *B. parva* (approx. 90 % similarity). Within this last group *B. lutea* and *B. ribis* formed a sub-cluster at approx. 94 % similarity. The species with *Diplodia* anamorphs formed a cluster containing the *B. stevensii* and *B. obtusa* isolates (approx. 82 % similarity) that were well separated from another cluster containing only *B. corticola*. The two species with *Dothiorella* anamorphs (*B. sarmentorum* and *B. iberica*) grouped together at 96 % similarity level. *Botryosphaeria rhodina* (anamorph *Lasiodiplodia theobromae*) formed a cluster separated from all the other species.

TABLE 2. rDNA haplotypes generated by restriction analysis of the PCR-amplified rDNA among *Botryosphaeria* isolates

		ARDRA patterns					
Species	**rDNA haplotype**	***Alu*I**	***Asu*I**	***Hae*III**	***Mbo*I**	***Nci*I**	***Taq*I**
B. lutea	**1**	A	A	A	A	A	A
B. ribis	**2**	A	B	A	A	A	B
B. parva	**3**	A	C	B	A	A	B
B. parva	**4**	A	C	B	A	A	C
B. dothidea	**5**	B	D	C	B	B	D
B. dothidea	**6**	B	D	C	B	F	D
B. stevensii	**7**	A	E	D	B	C	E
B. obtusa	**8**	A	F	E	B	D	F
B. corticola	**9**	C	F	E	C	C	B
B. sarmentorum	**10**	D	G	F	D	D	G
B. iberica	**11**	D	H	F	D	D	G
B. rhodina	**12**	E	F	G	E	E	H

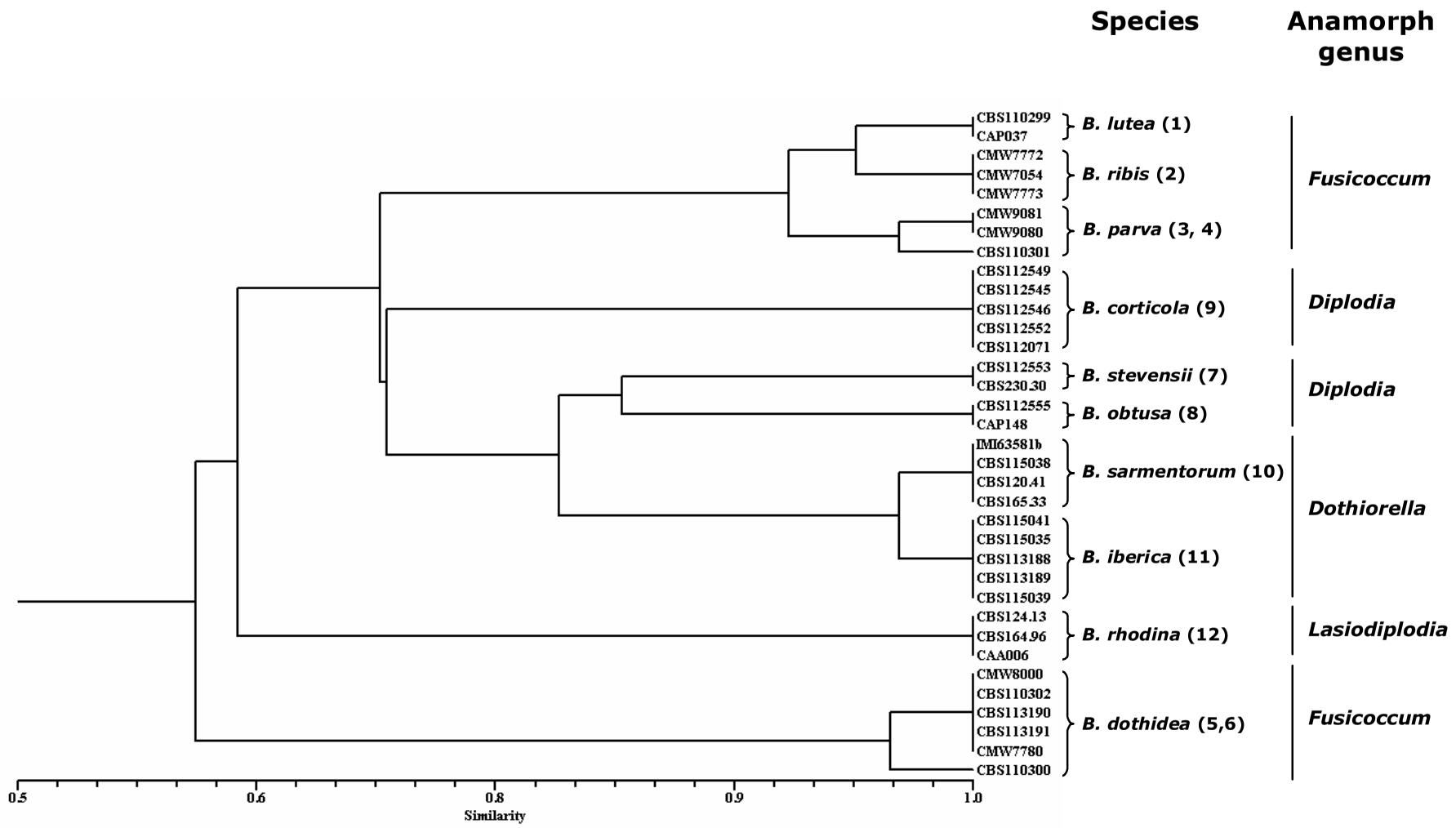


FIGURE 2. Dice/UPGMA cluster analysis of combined ARDRA fingerprints. Similarity is indicated as a percentage. Cophenetic correlation coefficient *r* = 0.94. Anamorphic genera are indicated to the right of the dendrogram. The numbers in parentheses indicate the haplotype.

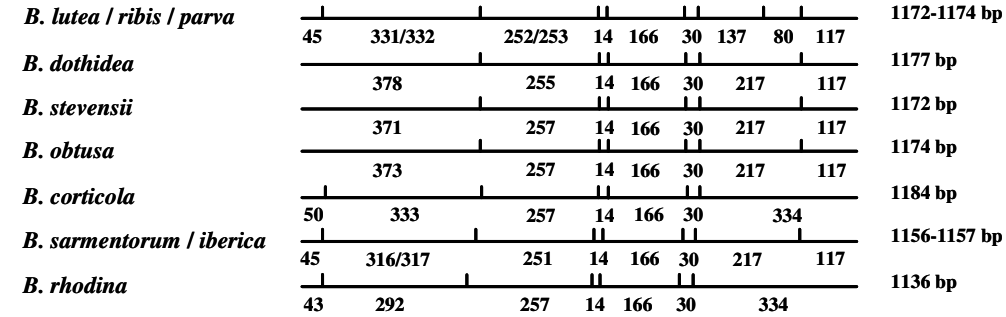
DNA sequence analysis

The D1/D2 region of the 28S rDNA of *Botryosphaeria* spp. as determined by DNA sequencing with primers NL1 and NL4 consisted of 614 bp. No size variation of the D1/D2 region was detected between or within species. The nucleotide sequences obtained were combined with the nucleotide sequences of the ITS regions retrieved from GenBank database allowing the accurate determination of the size of PCR amplicons: *B. lutea* CBS 110299 (1173 bp), *B. ribis* CMW 7772 (1174 bp), *B. parva* CMW 9081 and CBS 110301 (1172–1173 bp), *B. dothidea* CMW 8000 and CBS 110300 (1177 bp), *B. stevensii* CBS 112553 (1172 bp), *B. obtusa* CBS 112555 (1174 bp), *B. corticola* CBS 112549 (1184 bp), *B. sarmentorum* IMI 63581b (1156 bp), *B. iberica* CBS 115041 (1157 bp) and *B. rhodina* CBS 124.13 (1136 bp).

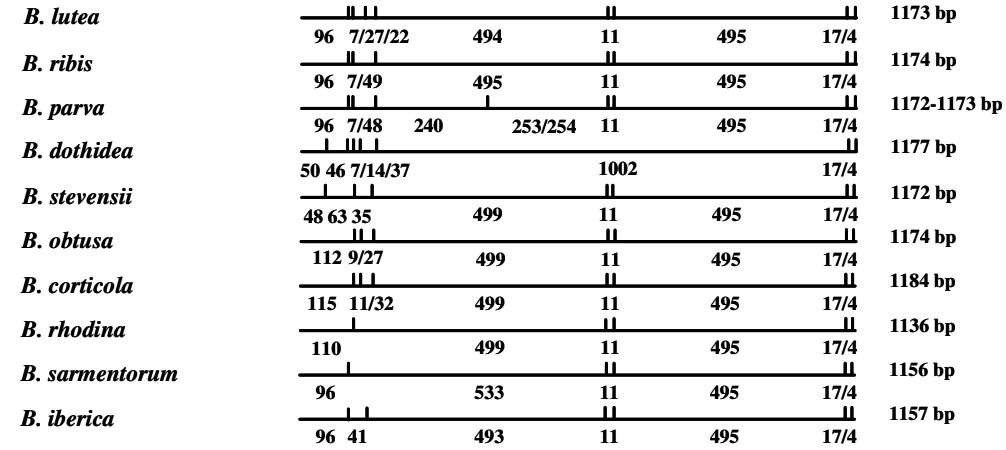
Restriction map analysis

Using the DNA sequence information from combined ITS + D1/D2 regions of representatives from each species/haplotype restriction maps for the six enzymes tested were constructed to determine precisely the restriction sites and the size of the fragments generated by each enzyme. This way it was possible to identify bands corresponding to doublets in *Asu*I restriction patterns (FIG. 1), and several small fragments undetected by agarose gel electrophoresis were also identified.

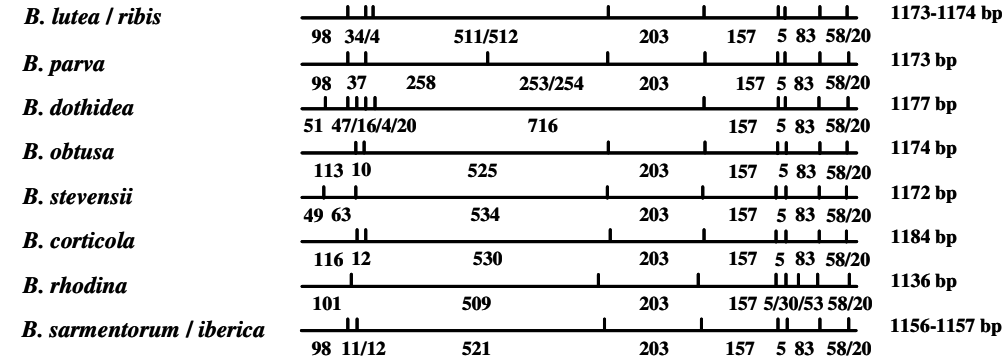
A. *Alu*I



B. *Asu*I



C. *Hae*III



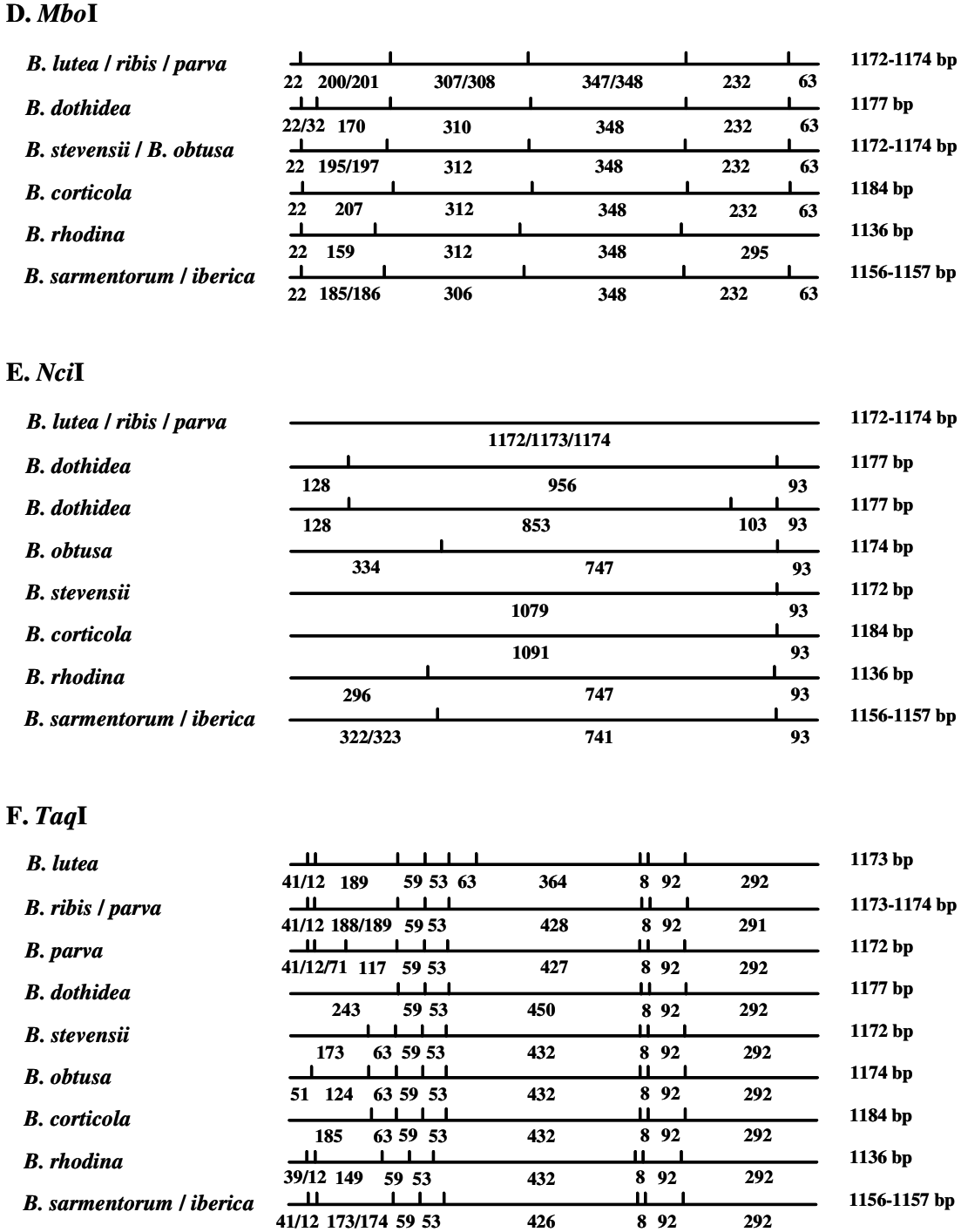


FIGURE 3. Restriction fragment length polymorphism maps of the PCR amplicons of *Botryosphaeria* spp. digested with the enzymes *Alu*I, *Asu*I, *Hae*III, *Mbo*I, *Nci*I, and *Taq*I. The total length of each fragment (in base pairs) is given in brackets and fragment lengths are given below each line.

DISCUSSION

The amplified rDNA fragment (approx. 1200 bp) included the ITS1-5.8S rDNA-ITS2 regions and the first 614 bp of the ribosomal large subunit gene (28S rDNA). The ITS region has proven useful in the phylogenetic analysis of *Botryosphaeria* species (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001) and other fungal taxa (e.g. Crous *et* al., 2001; Rodrigues *et* al., 2004). The D1/D2 domains of the 28S rDNA, although never studied in *Botryosphaeria* species, are frequently used for phylogenetic analysis of yeasts (Scorzetti *et al*., 2002) and less frequently for filamentous fungi (Abliz *et al*., 2004). The ARDRA procedure has been widely used for the identification of fungal phytopathogens (e.g. Edel *et al*., 1996; Ristaino *et al*., 1998; Chillali *et al*., 1998) and clinical fungi (e.g. Ahmed *et al*., 1999; Jackson *et al*., 1999). This method was recently applied to *Botryosphaeria* species (Slippers *et al*., 2004c). However, in that report it consisted of restriction analysis of the ITS region alone and was tested on a small set of species with *Fusicoccum* anamorphs.

The ARDRA procedure presented here allowed a clear differentiation of the isolates at the species level. Twelve rDNA haplotypes revealed by the restriction analysis could be clearly assigned to each of the 10 *Botryosphaeria* species. Cluster analysis is consistent with the division of the isolates into 12 haplotypes and 10 species which are readily identified in the dendrogram.

The ARDRA procedure tested appears to be an excellent tool for the differentiation of even cryptic species such as *B. ribis*/*B. parva* and *B. sarmentorum*/*B. iberica*. For example, *Botryosphaeria ribis* and *B. parva* have been the subject of much discussion (Jacobs and Rehner, 1998; Slippers *et al*., 2004a). These two species are difficult to separate on morphology and reliable identifications should be based on molecular methods. Sequence data from the ITS region alone cannot clearly discriminate between these species and it is necessary to combine these data with partial sequence of the EF1-α gene (Slippers *et al*., 2004a). Differentiation of these two species was impossible by ARDRA analysis of the ITS region alone (Slippers *et al*., 2004c). Interestingly, these two species are quite easily separated using the ARDRA procedure described here. Both species have distinctive *Hae*III and *Asu*I patterns. Our approach differs from the one previously reported (Slippers *et al*., 2004c) in using a larger portion of the ribosomal cluster. Digestion of this PCR amplicon produced restriction patterns with larger and more numerous fragments, making the patterns more discriminative and easier to score.

Botryosphaeria sarmentorum and *B. iberica*, two recently described *Botryosphaeria* species with *Dothiorella* anamorphs (Phillips *et al*., 2005) can be readily distinguished based on teleomorph features. However, this form is extremely rare, and the

anamorphic forms (*D. sarmentorum* and *D. iberica*) are the ones most frequently encountered and can be separated only by minor differences in the size and shape of the conidia (Phillips *et al*., 2005). In the same way as *B. ribis* and *B. parva* these two species can be differentiated by sequence analysis of the ITS and EF1-α regions (Phillips *et al*., 2005). As shown in the present work, these two species can be distinguished easily by the ARDRA patterns generated with the enzyme *Asu*I.

Intraspecific variability was detected only in *B. parva* and *B. dothidea* with the enzymes *Taq*I and *Nci*I respectively. For all the other species no polymorphisms were detected amongst the isolates tested. The strain CBS 110300 was described previously as *B. populi* (Phillips, 2000). However, phylogenetic analysis placed this isolate within the *B. dothidea* clade (van Niekerk *et al*., 2004; Phillips *et al*., 2005) and thus *B. populi* is considered a synonym of *B. dothidea*. The same can be seen on the dendrogram where isolate CBS 110300 clusters together with all the *B. dothidea* isolates. Although this isolate differs from the other *B. dothidea* isolates in the *Nci*I restriction patterns, this merely represents intraspecific variation as seen with the *B. parva* isolates.

The results of the present study demonstrate that ARDRA is a useful tool for the identification of the major species in the genus *Botryosphaeria*. Several new species have been described recently in the genus (Slippers *et al*., 2004b; van Niekerk *et al*., 2004; Slippers *et al*., 2004c), these as well as other species already described (Smith *et al*., 2001; Denman *et al*., 2003) would need to be studied to confirm that the ARDRA procedure is a valid method for species identification in *Botryosphaeria*. Although the technique distinguishes even some cryptic species it is possible that it does not differentiate between others that have not been tested. In this case, the procedure can be further improved by testing different restriction enzymes.

Sequencing of ITS as well as other genes (e.g. EF1-α, β-tubulin) has proven useful for the discrimination of *Botryosphaeria* species. ARDRA fingerprinting is an inexpensive and simple alternative to sequencing, and is especially appropriate when dealing with large sets of isolates (Guarro *et al*., 1999; Olive and Bean, 1999). In this respect, it is noteworthy that it was possible to discriminate between the 10 *Botryospheria* spp. by using combinations of only two restriction enzymes. This makes the identification procedure faster and easier, and greatly reduces the cost that would otherwise be close to those of DNA sequencing. The major advantages of the method are its simplicity, the universal availability of PCR primers, its reproducibility, and amenability to computer database analysis. It is possible to build a database of ARDRA patterns that can be used for routine identification of isolates by simple comparison of fingerprints.

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CHAPTER

8

Research in Microbiology

(submitted)

Rapid identification of *Botryosphaeria* species by PCR fingerprinting

ABSTRACT

The fingerprinting methods MSP-PCR and rep-PCR were used to discriminate between species of the genus *Botryosphaeria* (Fungi, Ascomycota) as well as associated anamorphic species. Several primers were tested with both methods and each primer allowed a clear identification of all the 24 species tested. Cluster analysis of banding patterns of the isolates corresponded well with known species delineations based on morphology and phylogenetic analysis. The methods described in this paper provide simple and rapid procedures that can be used for routine identification of *Botryosphaeria* isolates at the species level. Both methods are also useful to study intraspecific varibility

***Key words*:** *Botryosphaeria*, identification, fingerprinting, MSP-PCR, rep-PCR

INTRODUCTION

Species of *Botryosphaeria* are well known as pathogens, saprophytes and endophytes on a wide range of woody angiosperm and gymnosperm hosts (Barr, 1987; Denman *et al*., 2000). The classification and identification of *Botryosphaeria* species and related anamorphic fungi have traditionally been based on morphology. While the morphology of the teleomorphic states differs little between species, a wide range of morphologies is seen in the anamorphs and it is on this basis that species are distinguished (Jacobs and Rehner, 1998; Denman *et al*., 2000).

Species identification has relied heavily on morphological features of the conidia including size, shape, colour, septation, wall thickness and texture. However, some morphological characters exhibit extensive plasticity. Thus, size ranges of conidia of different *Botryosphaeria* species overlap, while 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 colonise a wide range of hosts and multiple species may occur on the same host (Jacobs and Rehner, 1998; Denman *et al*., 2000). Because species may differ by minor morphological features identification can be a difficult task for someone who is not familiar with these fungi. Therefore there is a need for tools that can provide accurate and reproducible identifications of *Botryosphaeria* species.

There has been an increasing interest in the application of polymerase chain reaction (PCR) technology for the identification of plant pathogenic fungi. Such

methods offer the advantage of reducing or eliminating the need for lengthy culturing and difficult morphological identification procedures (e.g. McDonald *et al*., 2000; Alves *et al*., 2005).

DNA based techniques provide practical markers for molecular typing a range of species of fungi. These methods are commonly used as tools in fungal taxonomy, allowing the discrimination of isolates from intrageneric to strain levels (Soll, 2000). Recently, Alves *et al*. (2005) developed an ARDRA (amplified ribosomal DNA restriction analysis) procedure for the identification of *Botryosphaeria* species. The method relies on the PCR amplification of a rDNA fragment followed by restriction analysis of the amplicons with frequent-cutting endonucleases. Using a combination of two restriction enzymes it was possible to identify ten *Botryosphaeria* species.

PCR-based genomic fingerprinting is a good alternative to methods that rely on specifically targeted primers. These techniques which analyse the whole genome, have been shown to be relatively robust and discriminatory (Olive and Bean, 1999; Soll, 2000). Microsatellites (tandem repeats of 1 to 5 base pairs) and minisatellites (tandem repeats of a basic motif 10 to 60 bp long) are ubiquitous components of eukaryotic genomes (Vogel and Scolnik, 1997). Microsatellite-primed polymerase chain reaction (MSP-PCR) uses single primers to generate DNA fingerprints that are useful to discriminate between fungal species and strains (Meyer *et al*., 1993, 1997; Thanos *et al*., 1996).

Repetitive-sequence-based polymerase chain reaction (rep-PCR) is a genomic fingerprinting technique that has proven useful for molecular typing and identification of bacterial species (Versalovic *et al.*, 1991, 1994). The technique is based on PCR amplification of DNA sequences, located between specific interspersed repeated sequences in prokaryotic genomes. Amplification generates an array of DNA fragments from the genomes of individual strains that can be separated in agarose gels, yielding highly specific DNA fingerprints. Several families of interspersed repetitive sequences are present in the genomes of bacterial species, e.g. the repetitive extragenic palindromic (REP) sequence, the enterobacterial repetitive intergenic consensus (ERIC), and the BOX element (Versalovic *et al*., 1991, 1994; Rademaker and de Bruijn, 1997; Olive and Bean, 1999).

Although rep-PCR primers were developed for repetitive elements in prokaryotic genomes, these primers have been applied with success in the fingerprinting of eukaryotic genomes including fungi (Gillings and Holley, 1997a). Thus, rep-PCR primers have been used to characterize variability at inter- and/or intra-specific levels of several fungal genera (van Belkum *et al*., 1993; Jedryczka *et al.*, 1999; Czembor and Arseniuk, 1999; Mehta *et al*., 2002a; de Arruda *et al*., 2003; Godoy *et al*., 2004).

Despite the widespread application of these PCR techniques, few studies have been performed regarding the applicability of MSP-PCR (Thanos *et al*., 1996; Meyer *et al*., 1993, 1997; Faggi *et al*., 2001) and rep-PCR (McDonald *et al*., 2000; Hierro *et al*., 2004; Pounder *et al*., 2005) for the identification of fungal species.

The PCR based method RAPD (random amplified polymorphic DNA) has been used to characterise populations of anamorphic species *Sphaeropsis sapinea* (Smith and Stanosz, 1995; Stanosz *et al*., 1996, 1999) while MSP-PCR has been used to characterize populations of *Botryosphaeria dothidea* (Ma *et al*., 2001, 2004; Ma and Michailides, 2002). This last technique proved useful for differentiating between *Botryosphaeria* species (Smith *et al*., 2001; Zhou *et al*., 2001). Zhou *et al*. (2001) using MSP-PCR (or ISSR fingerprinting) were capable of discriminating between ten *Botryosphaeria* species and associated anamorphic fungi.

The purpose of this work was to test the applicability of the MSP-PCR and rep-PCR genomic fingerprinting methodologies as tools for the identification of *Botryosphaeria* species and associated anamorphic fungi. The potential of both methods to discriminate between strains of the same species was also analysed.

MATERIALS AND METHODS

Fungal strains and cultural conditions

The fungal strains used in this study are listed on TABLE 1. A total of 53 strains representing 24 *Botryosphaeria* species or anamorphs were used. For comparative purposes three species of the closely related genus *Guignardia* were also included. Cultures were maintained on half-strength PDA (DIFCO) or oatmeal agar (Anonymous, 1968).

DNA isolation

Fungal strains were grown in potato dextrose broth for 5 days at approximately 25 ºC. Genomic DNA was isolated from fresh mycelium as described previously (Alves *et al*., 2004).

TABLE 1. Strains used this study.

Species				
Teleomorph	**Anamorph**	**Accession no.[1]**	**Host**	**Locality**
Botryosphaeria dothidea	*Fusicoccum aesculi*	CBS 115476	*Prunus* sp.	Switzerland, Crocifisso
		CBS 110302	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CBS 113190	*Quercus suber*	Spain, Sevilla
		CBS 113191	*Quercus ilex*	Spain, Córdoba
		CBS 110300	*Populus nigra*	Portugal, Braga
Botryosphaeria corticis	*Fusicoccum sp.*	CBS 119047	*Vaccinium corymbosum*	USA, New Jersey
		CBS 119048	*Vaccinium corymbosum*	USA, New Jersey
		ATCC 22927	*Vaccinium* sp.	USA, North Carolina
		ATCC 22928	*Vaccinium* sp.	USA, North Carolina
Botryosphaeria melanops	*Fusicoccum advenum*	CBS 116805	*Quercus* sp.	Germany, Munich
		CBS 116806	*Quercus* sp.	Germany, Munich
Botryosphaeria ribis	*Fusicoccum ribis*	CBS 115475	*Ribes* sp.	USA, New York
		CBS 121.26	*Ribes rubrum*	USA, New York
Botryosphaeria parva	*Fusicoccum parvum*	CMW 9081	*Populus nigra*	New Zealand, TePuke
		CBS 110301	*Vitis vinifera*	Portugal, Palmela
Botryosphaeria lutea	*Fusicoccum luteum*	CBS 110299	*Vitis vinifera*	Portugal, Oeiras
		CAP 037	*Vitis vinifera*	Portugal, Sintra
Botryosphaeria australis	*Fusicoccum australe*	CBS 119046	*Rubus* sp.	Portugal
		CAP 189	*Rubus* sp.	Portugal
Botryosphaeria corticola	*Diplodia corticola*	CBS 112549	*Quercus suber*	Portugal, Aveiro
		CBS 112545	*Quercus suber*	Spain, Cádiz
		CBS 112546	*Quercus ilex*	Spain, Huelva
Botryosphaeria stevensii	*Diplodia mutila*	CBS 112553	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CBS 230.30	*Phoenix dactylifera*	USA, California
Botryosphaeria tsugae	*Diplodia sp.*	CBS 418.64	*Tsuga heterophylla*	British Columbia, Canada
Unknown	*Diplodia cupressi*	CBS 168.87	*Cupressus sempervirens*	Israel, Bet Dagan
		CBS 261.85	*Cupressus sempervirens*	Israel, Bet Dagan
Botryosphaeria obtusa	*Diplodia sp.*	CBS 112555	*Vitis vinifera*	Portugal, Montemor-o-Novo
		CBS 119049	*Vitis* sp.	Italy
Unknown	*Diplodia pinea*	CBS 393.84	*Pinus nigra*	Netherlands, Putten
		CBS 109726	*Pinus patula*	South Africa, Habinsaran
		CBS 109727	*Pinus radiata*	South Africa, Stellenbosch
		CBS 109943	*Pinus patula*	Indonesia
Unknown	*Diplodia scrobiculata*	CBS 109944	*Pinus greggii*	Mexico
		CBS 113423	*Pinus greggii*	Mexico
Unknown	*Diplodia phoenicum*	JL 515	*Phoenix canariensis*	Spain, Barcelona

		JL 574	*Phoenix dactylifera*	Spain, Tarragona
Botryosphaeria rhodina	*Lasiodiplodia theobromae*	CBS 124.13	Unknown	USA
		CBS 164.96	Fruit along coral reef coast	Papua New Guinea
		CBS 356.59	*Theobroma cacao*	Sri Lanka, Agalawatta
		CBS 456.78	Cassava-field soil	Colombia
		CBS 447.62	*Citrus aurantium*	Suriname
		CBS 116459	*Gmelina arborea*	Costa Rica, San Carlos
Unknown	*Lasiodiplodia gonubiensis*	CBS 115812	*Syzygium cordatum*	South Africa, Eastern Cape
Unknown	*Lasiodiplodia crassispora*	CBS 110492	Unknown	Unknown
Botryosphaeria iberica	*Dothiorella iberica*	CBS 115041	*Quercus ilex*	Spain, Aragon
		CBS 115035	*Quercus ilex*	Spain, Aragon
Botryosphaeria sarmentorum	*Dothiorella sarmentorum*	IMI 63581b	*Ulmus* sp.	England, Warwickshire
		CBS 115038	*Malus pumila*	Netherlands, Delft
Botryosphaeria viticola	*Dothiorella viticola*	CBS 117009	*Vitis vinifera*	Spain, Vimbodí
		CBS 117006	*Vitis vinifera*	Spain, Vimbodí
Unknown	*Dothiorella coryli*	CBS 242.51	Unknown	Italy
		JL 599	*Corylus avellana*	Unknown
Guignardia philoprina	*Phyllosticta concentrica*	CBS 447.68	*Taxus baccata*	Netherlands, Baarn
Guignardia bidwellii	*Phyllosticta ampelicida*	CBS 111645	*Parthenocissus quinquefolia*	USA, Missouri
Guignardia citricarpa	*Phyllosticta citricarpa*	CBS 102374	*Citrus aurantium*	Brasil

[1]Acronyms of culture collections: ATCC – American Type Culture Collection, Virginia, USA; CAP – A.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; IMI – CABI Bioscience, Egham, U.K.; JL – Jordi Luque, IRTA – Institut de Recerca i Tecnologia Agroalimentàries, Barcelona, Spain.

PCR amplification and gel electrophoresis

The primers used for each fingerprinting method are listed in TABLE 2. All primers were synthesised by MWG-Biotech AG (Ebersberg, Germany). All PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers from MBI Fermentas (Vilnius, Lithuania), in a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA). PCRs were performed in 25 μl reaction mixtures containing 1 X PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 200 μM each nucleotide, 20 pmol of each primer (for MSP-PCR primers and primer BOX) and 50 pmol of each primer (for REP and ERIC primers), 1 U of *Taq* polymerase, and 50–100 ng of purified template DNA. For each PCR reaction an initial denaturation step at 95 ºC for 5 min was done; after that, 30 cycles of denaturation, annealing, and extension were performed, according to TABLE 2. A final extension step at 72 ºC for 10 min (for MSP-PCR) and 65 ºC for 18 min (for rep-PCR) was included. Negative control reactions without any template DNA were carried out simultaneously.

PCR products were separated by electrophoresis in 2 % agarose gels at 80 V for 3 h in Tris-acetate-EDTA buffer. A GeneRuler™ DNA Ladder Mix (MBI Fermentas) was run at both sides of each gel. The gels were stained with ethidium bromide, and visualized on a UV transilluminator to assess PCR amplification.

Computer-assisted analysis of genomic fingerprints

Agarose gels were scanned on a Molecular Imager FX (BioRad) and recorded as TIFF images. The banding patterns were analysed with GelCompar II software (Applied Maths). Gels were normalized using the same standard loaded at both sides. The "rolling disk" background subtraction option was applied. DNA bands detected by the software were carefully verified by visual examination to correct unsatisfactory detection. Variations in band intensity were not considered to be differences. Levels of similarity between the profiles were calculated with the band matching Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA) to produce a dendrogram.

TABLE 2. Primers and PCR conditions used in this study.

Method	Primer	Sequence (5'→3')	PCR conditions			Reference
			Denaturation	Annealing	Extension	
REP-PCR	Rep1R Rep2I	IIIICGICGICATCIGGC ICGICTTATCIGGCCTAC	94 ºC, 1 min	40 ºC, 1 min	65 ºC, 8 min	Versalovic *et al.* (1991)
ERIC-PCR	ERIC1 ERIC2	AAGTAAGTGACTGGGGTGAGC ATGTAAGCTCCTGGGGATTCAC	94 ºC, 1 min	52 ºC, 1 min	65 ºC, 8 min	Versalovic *et al.* (1994)
BOX-PCR	BOX A1R	CTACGGCAAGGCGACGCTGACG	94 ºC, 1 min	53 ºC, 1 min	65 ºC, 8 min	Versalovic *et al.* (1994)
MSP-PCR	$(GTG)_5$	GTGGTGGTGGTGGTG	94 ºC, 1 min	50 ºC, 1 min	72 ºC, 2 min	
	$(GTGC)_4$	GTGCGTGCGTGCGTGC	94 ºC, 1 min	50 ºC, 1 min	72 ºC, 2 min	
	$(CAG)_5$	CAGCAGCAGCAGCAG	94 ºC, 1 min	50 ºC, 1 min	72 ºC, 2 min	
	M13	GAGGGTGGCGGTTCT	94 ºC, 1 min	45 ºC, 1 min	72 ºC, 2 min	
	T3B	AGGTCGCGGGTTCGAATCC	94 ºC, 1 min	45 ºC, 1 min	72 ºC, 2 min	

RESULTS

The core sequence of the wild-type phage M13, specific to minisatellite sequences, the synthetic oligonucleotides $(CA)_8$, $(GTG)_5$, $(CAG)_5$, $(GTGC)_4$, $(GACA)_4$, which are specific to simple repetitive DNA (microsatellite) sequences, as well as primer T3B, which was derived from tRNA intergenic spacers were tested as single primers for MSP-PCR fingerprinting. In rep-PCR fingerprinting the primers tested were: REP1R/REP2I (REP-PCR), ERIC1/ERIC2 (ERIC-PCR), and BOXA1R (BOX-PCR).

An initial testing using a smaller set of strains was performed with all the above primers to determine their ability to generate DNA fingerprints and to evaluate the reproducibility of both methods. Primers $(CA)_8$ and $(GACA)_4$, were unable to generate DNA fingerprints from any of the strains tested, even after testing several annealing temperatures and were thus discarded. The remaining primers produced visible DNA fingerprints in agarose gels and were used to study a larger set of strains.

Reproducibility of the MSP-PCR and rep-PCR fingerprinting techniques was checked by comparing the banding profiles resulting from independent extractions and amplifications of the same strains (data not shown). In analyzing the reproducibility, comparison of the resulting fingerprint patterns yielded a similarity of ~90 to 95 % when the patterns were generated in the same PCR experiment and resolved on the same gel, while independent PCR and electrophoresis of repeated samples resulted in similarities of ~85 to 90 % (data not shown). These values are consistent with those from other studies (McDonald *et al*., 2000).

Both approaches yielded highly reproducible and complex genomic fingerprints, with several bands ranging in size from 200 to 3500 bp (MSP-PCR) and 100 to 5000 bp (rep-PCR) (FIG. 1). The number of bands generated in each fingerprint varied according to the strain and also with the primer used. Of all the primers tested rep-PCR primers gave rise to more complex DNA fingerprints than the MSP-PCR primers.

The ability of the selected primers to produce species-specific fingerprints was apparent. Strains that were deemed to represent different species according to conventional morphological as well as molecular phylogenetic criteria gave rise to distinct PCR fingerprints, whereas strains of the same species had similar banding patterns. The number and size of the multilocus PCR fingerprint bands were clearly species-specific. Based on the specific PCR fingerprints and the high inter-species variation of these banding patterns, a clear distinction between all species was possible. Although strains of the same species always generated very similar fingerprints, both methodologies (MSP-PCR and rep-PCR) were still capable of detecting intraspecific variability.

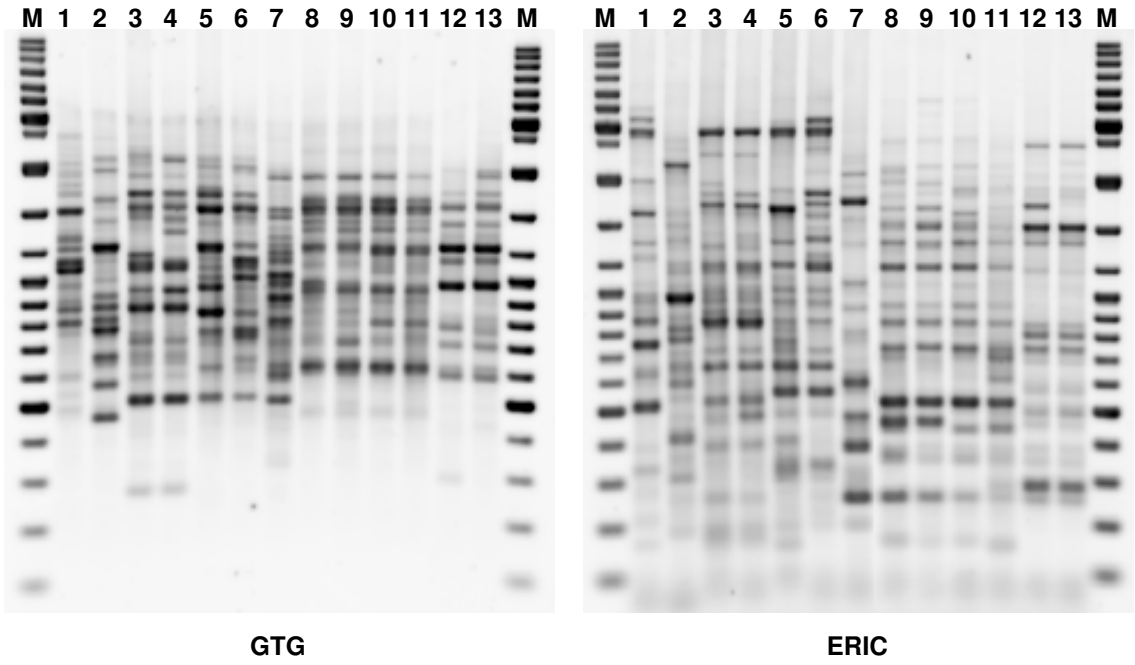

FIGURE 1. Examples of MSP-PCR and rep-PCR fingerprints obtained with primers GTG_5 and ERIC. M. GeneRuler™ DNA Ladder Mix. 1. *L. gonubiensis* CBS 115812. 2. *L. crassispora* CBS 110492. 3, 4, 5, 6. *L. theobromae* CBS 356.59, CBS 456.78, CBS 447.62 and CBS 116459. 7. *B. tsugae* CBS 418.64. 8, 9. *D. pinea* morphotype A CBS 393.84 and CBS 109727. 10, 11. *D. pinea* morphotype C CBS 109726 and CBS 109943. 12, 13. *D. scrobiculata* CBS 109944 and CBS 113423.

Cluster analyses were performed on the genomic fingerprints generated by each of the primers tested. Samples of the dendrograms generated with the UPGMA method are shown in figures 2 and 3. For all primers analysed the clustering of the strains was species-specific. Strains belonging to the same species typically clustered together at similarity values greater than 80 %. The only two exceptions found were the species *B. viticola* and *Dothiorella coryli*. The two strains of each species did not cluster together in some of the analyses, and when they grouped in the same cluster the similarity between the fingerprints was below 80 %. Also, the isolates identified as *Lasiodiplodia theobromae* (teleomorph *B. rhodina*) formed three separate and well defined clusters, each with similarity values well above 80 %.

MSP-PCR and rep-PCR fingerprints and cluster analysis did not reveal relationships at the genus level. For example, the three *Guignardia* species did not cluster separately from *Botryosphaeria* species. Also, *Botryosphaeria* species possessing the same anamorph genus were not grouped in clearly defined and separate clusters.

The fingerprints obtained with all the MSP-PCR primers were subjected to combined cluster analysis, and the same was performed for BOX, ERIC, and REP fingerprints. In both analyses the dendrograms produced were similar to those produced by cluster analysis of the non-combined fingerprints.

DISCUSSION

The identification of *Botryosphaeria* species and related anamorphs can be a difficult task if based solely on morphological and cultural characters. In this respect the application of molecular methods particularly PCR-based techniques may give a great contribute.

MSP-PCR and rep-PCR fingerprinting have proven useful in the molecular typing of fungal strains (van Belkum *et al*., 1993; Jedryczka *et al*., 1999; Czembor and Arseniuk, 1999; Mehta *et al*., 2002a; de Arruda *et al*., 2003; Godoy *et al*., 2004). Recently, some studies have addressed its application in the identification of species of yeasts (Thanos *et al*., 1996; Meyer *et al*., 1993, 1997; Hierro *et al*., 2004) and filamentous fungi (McDonald *et al*., 2000; Faggi *et al*., 2001; Pounder *et al*., 2005) showing that these methodologies have a great potential as diagnostic tools. In a previous work, Zhou *et al*. (2001) reported that MSP-PCR, which they referred to as ISSR fingerprinting, allowed the differentiation of ten *Botryosphaeria* species. In this study we tested the applicability of these fingerprinting methods to the identification of 24 *Botryosphaeria* species.

Of all the primers tested only the microsatellite repeats $(CA)_8$ and $(GACA)_4$ were unable to generate visible DNA fingerprints. It is possible that these repeats are not present in the genome of these fungi. Another possibility is that the primer annealing sites are at such a distance that amplification by *Taq* polymerase is impossible. Zhou *et al*. (2001) reported that no tetra-nucleotide repeat primers worked well for *Botryosphaeria* species. We tested two tetra-nucleotide repeat primers namely $(GACA)_4$ and $(GTGC)_4$. The results obtained with $(GACA)_4$ are in agreement with the ones reported by Zhou *et al*. (2001), but the primer $(GTGC)_4$ generated fingerprints with all the species studied.

Primers REP, ERIC, and BOX used for rep-PCR fingerprinting all proved useful to generate highly reproducible and discriminatory fingerprints with all the species tested. These primers are derived from repetitive sequences present in prokaryotic genomes (Versalovic *et al*., 1991, 1994). It is not known if these repetitive sequences are also present in eukaryotic genomes but it was shown that these primers are able to generate fingerprints in a wide diversity of eukaryotes (Gillings and Holley, 1997a).

Some studies have shown that these primers amplify regions that are not necessarily related with the prokaryotic REP, ERIC and BOX regions (Gillings and Holley, 1997b; Mehta *et al*., 2002b).

The MSP-PCR and rep-PCR fingerprinting methods presented here allowed a clear differentiation of the isolates at the species level. Fingerprinting profiles generated by all the primers tested readily discriminate between each of the 24 *Botryosphaeria* species as well as the 3 *Guignardia* species. Cluster analysis further supported this observation and clusters corresponding to each species are readily identified in the dendrograms. As can be seen in the dendrograms presented clusters formed at similarity values ≥ 80 % correspond to known species. Both procedures appear to be excellent tools for the identification of even cryptic species which can be easily distinguished by the fingerprints generated with any of the MSP-PCR or rep-PCR primers tested. A clear separation of the species according to the genus was not possible. Thus, *Botryosphaeria* and *Guignardia* are not separated in cluster analyses and the same is seen for the anamorph genera associated with *Botryosphaeria*. MSP-PCR and rep-PCR fingerprinting methods analyse regions of the genome that are extremely variable and are thus better suited for taxonomic studies at or below the species level (Rademaker and de Bruijn, 1997; Vogel and Scolnik, 1997).

In recent years sequence analyses of the ITS region alone (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001; Alves *et al*., 2004) or in combination with introns of protein-encoding genes such as β-tubulin and the translation elongation factor 1-alpha (EF1-α) (Slippers *et al*., 2004a, b; Phillips *et al*., 2005; Luque *et al*., 2005) have been used to study relationships among species and to distinguish closely related *Botryosphaeria* spp. Some cryptic species like *B. ribis* and *B. parva* or *B. lutea* and *B. australis*, as well as their *Fusicoccum* anamorphs, are very similar in morphological terms and reliable identifications are difficult to accomplish without the use of molecular methods (Slippers *et al*., 2004a, b). In fact, nucleotide sequences of the ITS region alone cannot clearly discriminate between the species and it is necessary to combine this region with partial sequence of the EF1-α gene (Slippers *et al*., 2004a, b). These four species are easily separated by their MSP-PCR or rep-PCR fingerprints and form separate clusters readily identified by cluster analyses.

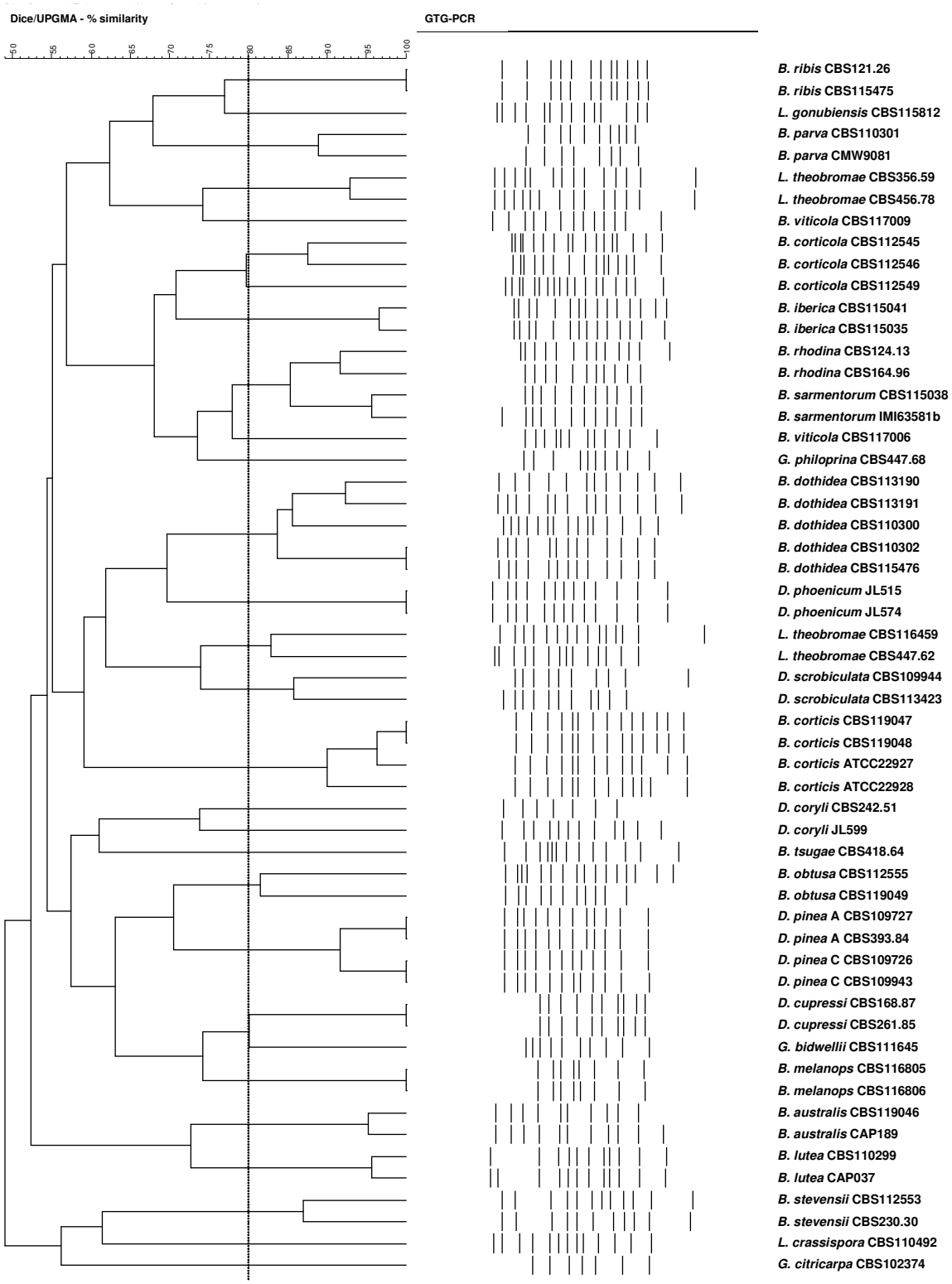

FIGURE 2. Dice/UPGMA cluster analysis of MSP-PCR fingerprints obtained with primer $(GTG)_5$. Similarity is indicated as a percentage.

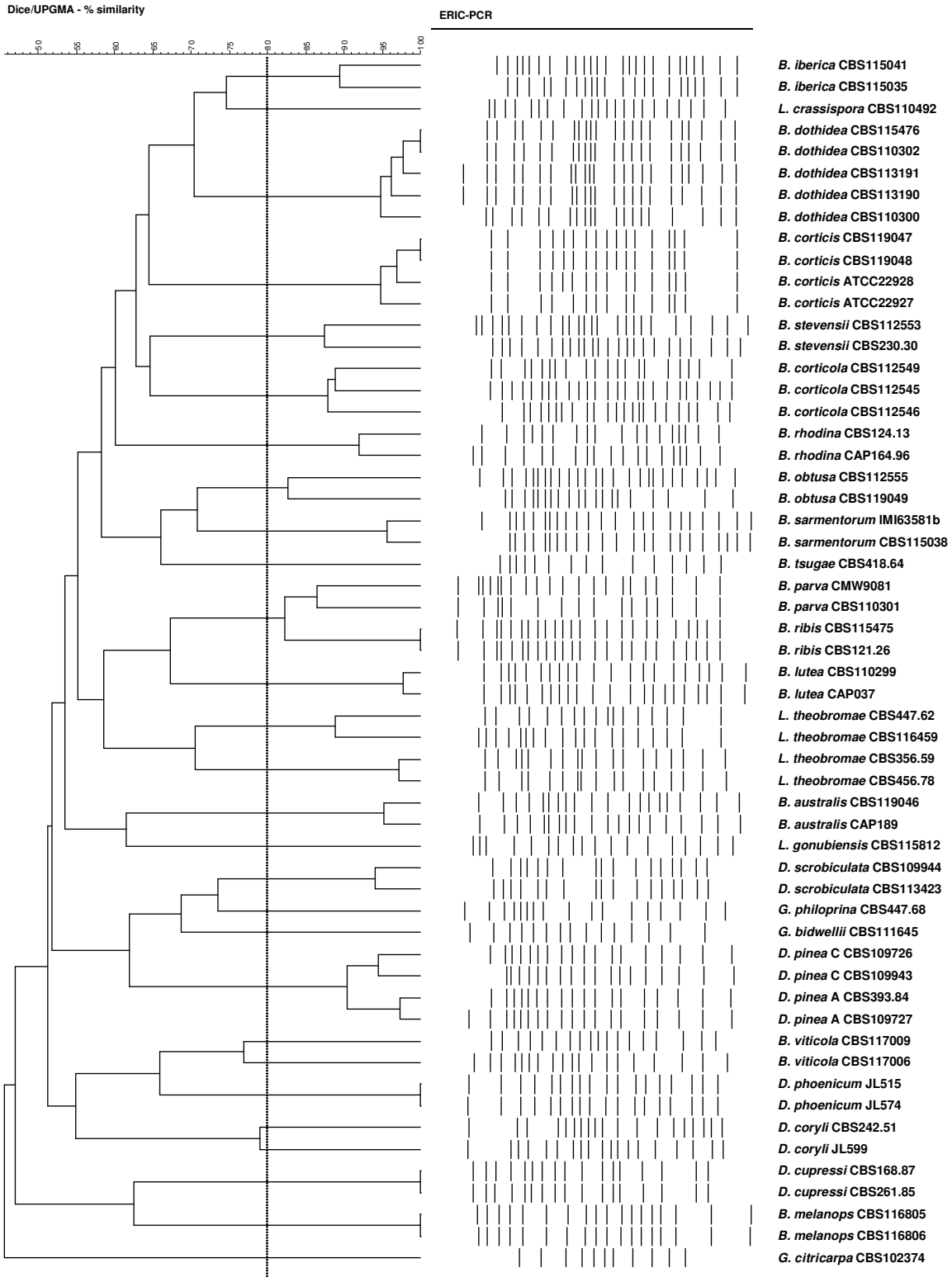

FIGURE 3. Dice/UPGMA cluster analysis of rep-PCR fingerprints obtained with primers ERIC1/ERIC2 (ERIC-PCR). Similarity is indicated as a percentage.

Botryosphaeria sarmentorum, *B. iberica*, and *B. viticola* are *Botryosphaeria* species with *Dothiorella* anamorphs (Phillips *et al*., 2005; Luque *et al*., 2005). These recently described species can be readily distinguished based on teleomorph features. However, this form is extremely rare, and the anamorphic forms (*D. sarmentorum*, *D. iberica*, and *D. viticola*) are more abundant in nature and the ones most frequently found. The anamorphs can be separated by minor differences in the size and shape of the conidia thus complicating the identification (Phillips *et al*., 2005; Luque *et al*., 2005). In the same way as other *Botryosphaeria species*, these can be differentiated by sequence analysis of the ITS and EF1-α regions (Phillips *et al*., 2005; Luque *et al*., 2005). The DNA fingerprints generated by MSP-PCR and rep-PCR allow a clear differentiation of these species.

Two isolates of *B. viticola* were analysed in this study. In the majority of the analysis these isolates did not appear in the same cluster. When describing *B. viticola*, Luque *et al*. (2005) reported that the isolate CBS 117006 exhibited some differences in culture morphology from the other isolates studied. Furthermore this isolate differed in ITS and EF1-α sequences (one substitution and one deletion in ITS and nine substitutions in EF1-α). These differences in nucleotide sequences and colony morphology were regarded as intraspecific variation. The results present here also show that strain CBS 117006 is markedly different from the other *B. viticola* strain tested (CBS 117009). On account of the differences in ITS and EF1-α sequences as well as MSP-PCR and rep-PCR fingerprints it is likely that the strain CBS 117006 identified as *B. viticola* may in fact represent a separate taxon. In order to confirm this hypothesis a larger set of similar isolates would need to be studied.

The same possibility may be true for the two strains identified as *D. coryli* (CBS 242.51 and JL 599) which have generated divergent MSP-PCR and rep-PCR fingerprints. These strains differ by one nucleotide substitution in the ITS region, as well as 5 nucleotide substitutions and one deletion in the EF1-α region (data not shown).

The isolates of *L. theobromae* (*B. rhodina*) analysed in this study revealed an extremely high genetic diversity forming three separate groups that can be easily identified in cluster analyses. These isolates although initially identified as members of the species *L. theobromae* are now deemed to represent three separate taxa based on morphological and phylogenetic evidences (Alves *et al*., unpublished).

Diplodia pinea is a latent, opportunistic pathogen of conifers that occurs worldwide. This species has long been recognised as being morphologically variable and three different morphotypes have been described. The 'A' and 'B' morphotypes were separated on cultural characteristics and minor differences in conidial morphology

(Palmer *et al*., 1987). The existence of two discrete morphotypes in North America was confirmed by RAPD markers (Smith and Stanosz, 1995). A 'C' morphotype, based on conidial dimensions, RAPDs and ITS sequence data, was reported from Indonesia (de Wet *et al*. 2000). De Wet *et al*. (2003) used sequences of six protein-coding genes and six microsatellite loci to separate the 'B' morphotype from the 'A' and 'C' morphotypes. They considered that the resulting phylogenies provided sufficiently robust evidence to regard the 'B' morphotype as a distinct species which, they described as *Diplodia scrobiculata*.

Diplodia scrobiculata is clearly separated from *D. pinea* on the basis of the DNA fingerprints produced by both methods. Also, strains belonging to different *D. pinea* morphotypes (A and C) although very closely related can be separated by their MSP-PCR and rep-PCR fingerprints. It is not clear if these morphotypes represent distinct taxa, but due to the large amount of bands shared in the DNA fingerprints it is likely that the differences between the morphotypes reflect only intraspecific variability. This, however, can only be verified through the comparison of a larger number of isolates.

The PCR fingerprinting methods tested although producing fingerprints that are very similar within strains of the same species are also capable of detecting intraspecific variation. As can be seen in the fingerprints it is possible to identify polymorphic bands between strains of the same species. Thus MSP-PCR and rep-PCR fingerprinting can be used to study genetic variability and population structure of *Botryosphaeria* species and both are a good alternative to RAPD analysis. The major advantage of MSP-PCR and rep-PCR when compared to RAPD is the capability to generate highly reproducible results. Higher reproducibility is related to the fact that longer primers are used and consequently PCR reactions are carried out at higher annealing temperatures (Vogel and Scolnik, 1997; Soll, 2000).

The results of this study demonstrate the usefulness of MSP-PCR and rep-PCR genomic fingerprinting as complementary or alternate strategies for routine identification of *Botryosphaeria* cultures. Recently a ARDRA procedure was described that could be used to identify *Botryosphaeria* species (Alves *et al*., 2005). Like ARDRA, MSP-PCR and rep-PCR fingerprinting are simple and fast alternatives to sequencing, which can be extremely useful when dealing with large sets of isolates. The advantages of the three methods are the simplicity, the universal availability of PCR primers, reproducibility, and amenability to computer database analysis. It is possible to build databases of fingerprints that can be used for routine identification of isolates by simple comparison of fingerprints.

The major advantage of MSP-PCR and rep-PCR over ARDRA is the fact that there is no need for a step involving the digestion of PCR amplicons with restriction endonucleases. In order to discriminate between ten *Botryospheria* spp. it was necessary to use combinations of two restriction enzymes in the ARDRA procedure (Alves *et al*., 2005). Using only a single primer or set of primers in MSP-PCR and rep-PCR it is possible to achieve higher levels of resolution. This makes the identification procedure much faster, easier, and greatly reduces the costs.

Pounder *et al*. (2005) tested the performance of rep-PCR using the DiversiLab system for identification of dermatophytes. These authors reported that the turnaround time for both identification and typing results is < 24 h once a pure culture is available versus several days to weeks for fungal identification and 2 days when using sequencing for identification. Similar timescales are applicable to the identification of *Botryosphaeria* species.

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CHAPTER

9

Unpublished

A re-evaluation of the anamorph genera *Diplodia* and *Lasiodiplodia* based on morphology and multi-gene sequence data

ABSTRACT

Phylogenetic analyses of combined sequence data from nuclear large- and small-subunit ribosomal DNA, ITS, EF1-α and β-tubulin support the genera *Diplodia* and *Lasiodiplodia* as distinct. It is shown that conidial striations are not a taxonomically meaningful morphological character to separate both genera since they also occur in *Diplodia phoenicum*. *Lasiodiplodia* is distinguished from *Diplodia* by the presence of paraphyses. A collection of isolates identified as *L. theobromae* was studied on the basis of sequence data from the ITS region and EF1-α gene. Phylogenetic analyses identified three well-supported clades within this group of isolates, one corresponding to *L. theobromae* and two others corresponding to potential cryptic species. The distinct phylogenetic position of the two clades is supported by differences in conidial morphology and these are, therefore, described as new species, *Lasiodiplodia pseudotheobromae* sp. nov. and *Lasiodiplodia parva* sp. nov.

***Key words*:** *Botryosphaeriaceae*, *Diplodia*, *Lasiodiplodia*, phylogenetic analysis, taxonomy

INTRODUCTION

Botryosphaeria Ces. & De Not. is a species-rich ascomycete genus with a cosmopolitan distribution (Barr, 1972, 1987; Sivanesan, 1984). It is known to occur on a great variety of gymnosperm and angiosperm hosts, where it is found on woody branches, twigs, herbaceous leaves or stems, and lichen talli (Barr, 1972, 1987). Some species are know as important pathogens causing dieback and cankers on a wide range of plant hosts, while others are saprophytic or endophytic (von Arx, 1981; Sivanesan, 1984; Barr, 1972, 1987; Smith *et al*., 1996; Mohali *et al*., 2005).

Due to the lack of variation among morphological features of the teleomorphs species in *Botryosphaeria* are characterised essentially on the morphology of the anamorphs (Pennycook and Samuels, 1985; Jacobs and Rehner, 1998; Denman *et al*., 2000) which reside in the Coelomycetes. More than 18 anamorph genera have been associated with *Botryosphaeria* (Denman *et al*., 2000). Phylogenetic analyses of the ITS region identified two clades (Jacobs and Rehner, 1998; Denman *et al*., 2000) which correspond to Diplodia-like and Fusicoccum-like anamorphs. For this reason,

Denman *et al*. (2000) considered that only two anamorph genera should be associated with *Botryosphaeria*, namely *Diplodia* Fr. and *Fusicoccum* Corda, and in so doing they suggested that *Lasiodiplodia* Ellis & Everh. and *Sphaeropsis* Sacc. should be regarded as synonyms of *Diplodia*. This view was supported by later studies with more isolates and species (Zhou & Stanosz, 2001; Alves *et al*., 2004).

The synonymy of *Sphaeropsis* under *Diplodia* has generally been accepted by most authors (e.g. de Wet *et al*., 2003; Burgess *et al*., 2004a, b; Pavlic *et al*., 2004). Nevertheless Xiao and Rogers (2004) recently described a new species in *Sphaeropsis* (*S. pyriputrescens* Xiao & J.D. Rogers) thus preferring to keep *Sphaeropsis* as a separate genus.

Because of the morphological (paraphyses and striate conidia) and phylogenetic (ITS and EF1-α) distinctions, some authors (Pavlic *et al*., 2004; Burgess *et al.*, 2006) have retained *Lasiodiplodia* as a separate genus from *Diplodia*.

The type species of *Lasiodiplodia*, *L. theobromae* (Pat.) Griff. & Maubl., is geographically widespread but is most common in the tropics and subtropics (Punithalingam, 1980). It is plurivorous and has been associated with approximately 500 host plants (Punithalingam, 1980). This apparently unspecialized plant pathogen has been reported to cause numerous diseases, namely dieback, root rot, fruit rots, leaf spot, witches' broom and many others (Punithalingam, 1980). Additionally it can occur as an endophyte (Rubini *et al*., 2005; Mohali *et al*., 2005). Although less frequently it has also been associated with keratomycosis and phaeohyphomycosis in humans (Punithalingam, 1976; Rebell and Forster, 1976; Summerbell *et al*., 2004). Due to its widespread occurrence, the large number of hosts and its known morphological variability it is possible that *L. theobromae* is composed of a number of cryptic species. Recently, Pavlic *et al*. (2004) described a new species *L. gonubiensis* Pavlic, Slippers & M.J. Wingf. on the basis of conidium morphology and dimensions, and ITS sequence data.

The purpose of this work was to determine genetic variability within a collection of strains originally identified as *L. theobromae* through a study of ITS and EF1-α nucleotide sequences. In addition, the genus-level phylogenetic relationship of *Lasiodiplodia* and *Diplodia* was determined through a study of multigene sequence data.

TABLE 1. Isolates included in this study.

Accession no.[1]	Teleomorph	Anamorph	Host	Country	Collector	Genbank[2]				
						SSU	LSU	ITS	EF1-α	β-tubulin
CBS 112553	*B. stevensii*	*D. mutila*	*Vitis vinifera*	Portugal	A.J.L. Phillips	ND	*AY928049*	*AY259093*	*AY573219*	*DQ458850*
CBS 230.30	*B. stevensii*	*D. mutila*	*Phoenix dactylifera*	U.S.A	L.L. Huillier	ND	ND	*DQ458886*	*DQ458869*	*DQ458849*
CBS 418.64	*B. tsugae*	*Diplodia* sp.	*Tsuga heterophylla*	Canada	A. Funk	ND	ND	*DQ458888*	*DQ458873*	*DQ458855*
CBS 112546	*B. corticola*	*D. corticola*	*Quercus ilex*	Spain	M.E. Sanchez	ND	ND	*AY259090*	ND	ND
CBS 112549	*B. corticola*	*D. corticola*	*Quercus suber*	Portugal	A. Alves	ND	*AY928051*	*AY259100*	*AY573227*	*DQ458853*
CBS 112555	*B. obtusa*	*Diplodia sp.*	*Vitis vinifera*	Portugal	A.J.L. Phillips	ND	*AY928050*	*AY259094*	*AY573220*	*DQ458856*
CBS 119049	*B. obtusa*	*Diplodia sp.*	*Vitis* sp.	Italy	L. Mugnai	ND	ND	*DQ458889*	*DQ458874*	*DQ458857*
CBS 393.84	Unknown	*D. pinea*	*Pinus nigra*	Netherlands	H.A. van der Aa	ND	ND	*DQ458895*	*DQ458880*	*DQ458863*
CBS 109727	Unknown	*D. pinea*	*Pinus radiata*	South Africa	W.J. Swart	ND	ND	*DQ458897*	*DQ458882*	*DQ458865*
CBS 109725	Unknown	*D. pinea*	*Pinus patula*	South Africa	M.J. Wingfield	ND	ND	*DQ458896*	*DQ458881*	*DQ458864*
CBS 109943	Unknown	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	ND	ND	*DQ458898*	*DQ458883*	*DQ458866*
CBS 109944	Unknown	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	ND	ND	*DQ458899*	*DQ458884*	*DQ458867*
CBS 113423	Unknown	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	ND	ND	*DQ458900*	*DQ458885*	*DQ458868*
CBS 168.87	Unknown	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	ND	ND	*DQ458893*	*DQ458878*	*DQ458861*
CBS 261.85	Unknown	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	ND	ND	*DQ458894*	*DQ458879*	*DQ458862*
JL 515	Unknown	*D. phoenicum*	*Phoenix canariensis*	Spain	J. Luque	ND	ND	ND	ND	ND
JL 574	Unknown	*D. phoenicum*	*Phoenix dactylifera*	Spain	J. Luque	ND	ND	ND	ND	ND
CBS 124.13	*B. rhodina*	*L. theobromae*	Unknown	U.S.A.	J.J. Taubenhaus	ND	*AY928054*	*DQ458890*	*DQ458875*	*DQ458858*
CBS 164.96	*B. rhodina*	*L. theobromae*	Fruit on coral reef coast	New Guinea	A. Aptroot	ND	ND	*AY640255*	*AY640258*	ND
CBS 175.26	*B. rhodina*	*L. theobromae*	Unknown	Unknown	K.B. Boedijn	-	-	ND	ND	-
CBS 190.73	*B. rhodina*	*L. theobromae*	*Persea americana*	Tanzania	W.S. Bos	-	-	ND	ND	-
CBS 287.47	*B. rhodina*	*L. theobromae*	*Musa sapientum*	Unknown	K. Sys	-	-	ND	ND	-
CBS 289.56	*B. rhodina*	*L. theobromae*	sail-cloth	New Guinea	M.B. Schol-Schwartz	-	-	ND	ND	-
CBS 306.58	*B. rhodina*	*L. theobromae*	*Cocos nucifera*	Unknown	G.H. Boerema	-	-	ND	ND	-
CBS 339.90	*B. rhodina*	*L. theobromae*	phaeohyphomycotic cyst	Canada	R.C. Summerbell	-	-	ND	ND	-
CBS 559.70	*B. rhodina*	*L. theobromae*	*Zea mays*	Unknown	H.A. van der Aa	-	-	ND	ND	-
CBS 111530	*B. rhodina*	*L. theobromae*	Unknown	Unknown	Unknown	-	-	ND	ND	-
CBS 112874	*B. rhodina*	*L. theobromae*	*Vitis vinifera*	South Africa	F. Halleen	-	-	ND	ND	-
CBS 113520	*B. rhodina*	*L. theobromae*	Unknown	Unknown	Unknown	-	-	ND	ND	-
CAA 006	*B. rhodina*	*L. theobromae*	*Vitis vinifera*	USA	T.J. Michailides	ND	ND	*DQ458891*	*DQ458876*	*DQ458859*

CMW 9074	*B. rhodina*	*L. theobromae*	*Pinus sp.*	Mexico	T. Burgess	-	-	*AY236952*	*AY236901*	-
CMW 10130	*B. rhodina*	*L. theobromae*	*Vitex donniana*	Uganda	J. Roux	-	-	*AY236951*	*AY236900*	-
STE-U 5051	*B. rhodina*	*L. theobromae*	*Vitis vinifera*	Argentina	M. Gatica	-	-	*AY343483*	*AY343369*	-
CBS 110492	Unknown	*L. crassispora*	Unknown	Unknown	Unknown	ND	ND	ND	ND	
CBS 115812	Unknown	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	ND	ND	*DQ458892*	*DQ458877*	*DQ458860*
CBS 116355	Unknown	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	-	-	*AY639594*	*DQ103567*	-
CBS 304.79	Unknown	*L. pseudotheobromae*	*Rosa* sp.	Netherlands	P.D. Wageningen	-	-	ND	ND	-
CBS 374.54	Unknown	*L. pseudotheobromae*	*Coffea* sp.	Zaire	Unknown	-	-	ND	ND	-
CBS 447.62	Unknown	*L. pseudotheobromae*	*Citrus aurantium*	Suriname	C. Smulders	ND	ND	ND	ND	ND
CBS 116459	Unknown	*L. pseudotheobromae*	*Gmelina arborea*	Costa Rica	K. Seifert	ND	ND	ND	ND	ND
CBS 116460	Unknown	*L. pseudotheobromae*	*Acacia mangium*	Costa Rica	K. Seifert	-	-	ND	ND	-
CBS 356.59	Unknown	*L. parva*	*Theobroma cacao*	Sri Lanka	A. Riggenbach	ND	ND	ND	ND	ND
CBS 456.78	Unknown	*L. parva*	Cassava-field soil	Colombia	O. Rangel	-	-	ND	ND	-
CBS 494.78	Unknown	*L. parva*	Cassava-field soil	Colombia	O. Rangel	ND	ND	ND	ND	ND
CBS 495.78	Unknown	*L. parva*	Cassava-field soil	Colombia	O. Rangel	-	-	ND	ND	-
CBS 115476	*B. dothidea*	*F. aesculi*	*Prunus* sp.	Switzerland	B. Slippers	ND	*AY928047*	*AY236949*	*AY236898*	*AY236927*
CBS 110299	*B. lutea*	*F. luteum*	*Vitis vinifera*	Portugal	A.J.L. Phillips	ND	*AY928043*	*AY259091*	*AY573217*	*DQ458848*

[1]Acronyms of culture collections: CAA – Artur Alves, Universidade de Aveiro, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; JL – J. Luque, IRTA, Barcelona, Spain; STE-U – Department of Plant Pathology, University of Stellenbosch, South Africa. Isolate accession numbers in bold signify cultures ex-type, or from samples that have been linked morphologically to the type material.

[2]Sequence numbers in italics were retrieved from the GenBank public database. All others were obtained in this study. ND – sequences not deposited in GenBank yet.

MATERIALS AND METHODS

Isolates and morphology

A collection of isolates previously identified as *Lasiodiplodia theobromae* was studied (TABLE 1). In addition, two isolates of *Diplodia phoenicum* (Sacc.) H.S. Fawc. & Klotz from *Phoenix canariensis* and *P. dactylifera* were included in the morphological and phylogenetic study. For studies on temperature effects on growth, isolates were grown on PDA. To induce sporulation, isolates were grown on autoclaved pine needles on 2% water agar and incubated at room temperature where they received diffused daylight.

DNA isolation, amplification and phylogeny

The procedures described by Alves *et al*. (2004) were used to extract genomic DNA from fungal mycelium. 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, 2005), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. All primers used were synthesised by MWG Biotech AG (Ebersberg, Germany).

Part of the nuclear rRNA cluster comprising the ITS region plus the D1/D2 variable domains of the ribosomal LSU gene was amplified using the primers ITS1 (White *et al*., 1990) and NL4 (O'Donnell, 1993) as described by Alves *et al*. (2005). Nucleotide sequences of the ITS and D1/D2 regions were determined as described previously (Alves *et al*., 2004, 2005) using the primers ITS4 (White *et al*., 1990) and NL1 (O'Donnell, 1993) as internal sequencing primers.

A portion of the ribosomal SSU gene was amplified with primers NS1 and NS4 (White *et al*., 1990). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 45 s at 94 ºC, 45 s at 48 ºC, and 1½ min at 72 ºC, and a final extension period of 10 min at 72 ºC. The nucleotide sequence of the SSU region was determined using the above primers along with the internal sequencing primers NS2 and NS3 (White *et al*., 1990).

The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene respectively. For some isolates the amplification of the EF1-α region was impossible using the primer set EF1-728F and EF1-986R. Thus, a new set of primers was designed based on the sequences of the elongation factor 1-alpha gene from *Neurospora crassa* (D45837) and *Aureobasidium pullulans* (U19723). The sequences were aligned and suitable regions for primer design were identified by visual inspection and the following set of primers was chosen: EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3') and EF1-1251R (5'-

CCTCGAACTCACCAGTACCG-3'). The amplification conditions for EF1-α and β-tubulin regions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 55 ºC, and 90 s at 72 ºC, and a final extension period of 10 min at 72 ºC.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described elsewhere (Alves *et al* 2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The nucleotide sequences were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for both DNA regions of additional *Botryosphaeria* species were taken from GenBank (TABLE 1).

Two datasets were analysed, a larger one consisting of ITS and EF1-α sequences to evaluate phylogenetic relationships within isolates identified as *L. theobromae*, and a smaller multigene dataset (SSU, LSU, ITS, β-tubulin and EF1-α) to determine genus level relationships.

The sequences were aligned with the alignment program 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 on indels (gaps) was incorporated into phylogenetic analysis using simple indel coding as implemented in the GapCoder software (Young and Healy, 2003).

Phylogenetic analyses of sequence data were done using PAUP* version 4.0b10 (Swofford 2003) for Maximum-parsimony (MP) analyses and Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian analyses. The outgroup taxa selected for rooting the trees were *B. lutea* and *B. dothidea*. Trees were visualized using the program 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 by 1000 bootstrap replications (Hillis and 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. 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. 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 1000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and 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 that trees from the same tree space were being sampled during each analysis.

The validity of the incongruence length difference (ILD) test (= partition homogeneity test in PAUP*) for determining whether multiple data sets should be combined has been questioned (Cunningham, 1997; Barker and Lutzoni, 2002). In this study we adopted the method of assessing combinability of data sets by comparing highly supported clades among trees generated from different data sets to detect conflict. High support typically refers to bootstrap support values ≥ 70 % and Bayesian posterior probabilities ≥ 95 % (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different data sets, this suggests the genes share similar phylogenetic histories and phylogenetic resolution and support could ultimately be increased by combining the data sets.

RESULTS

Phylogenetic analysis

Approximately 1100, 600, 550, 400 and 300 bases were determined for the SSU, LSU, ITS, β-tubulin and EF1-α genes, respectively, of the isolates sequenced in TABLE 1. The new set of primers was able to amplify the EF1-α region from those isolates that did not originate any amplicon with the set EF1-728F and EF1-986R. The sequencing of the amplified region showed that the lack of amplification was due to several mismatched bases in the primer EF1-728F. The new forward primer EF1-688F worked equally well with either of the reverse primers. However, the best results in

sequencing were obtained with the primer pair EF1-688F/ EF1-986R, and this was the one used for amplification and sequencing of the isolates.

Sequences of the five genes were aligned and analysed separately by maximum parsimony and Bayesian analysis, and the resulting trees were compared. No major conflicts were detected between the single gene phylogenetic analyses indicating that the datasets could be combined.

ITS and EF1-α phylogeny

For the ITS and EF1-α dataset the manually adjusted alignment of 47 isolates, including the two outgroup species, consisted of 545 characters for the ITS region, and 328 characters for EF1-α, including alignment gaps (data not shown). Indels were coded separately and added to the end of the alignment as characters 875–960. In the analyses, alignment gaps were treated as missing data.

The combined dataset contained 960 characters, of which 269 were parsimony informative, 73 were variable and parsimony uninformative and 618 were constant. Maximum-parsimony analysis of the combined dataset resulted in a single most parsimonious tree (tree length = 553 steps; CI = 0.7830; RI = 0.9230; RC = 0.7227; HI = 0.2170). The 50 % majority-rule consensus tree of 10 000 trees sampled during the Bayesian analysis showed an identical topology to the MP tree. The tree resulting from MP analysis is presented in FIG. 1 with MP bootstrap supports and Bayesian posterior probabilities above and below the branches, respectively.

Two main clades were identified. One corresponded to the anamorphic genus *Diplodia* (MP bootstrap 85 %; posterior probability 1.00) and the other corresponded to the anamorphic genus *Lasiodiplodia* (MP bootstrap 100 %; posterior probability 1.00). Within these two clades several sub-clades supported by high bootstrap values and Bayesian posterior probabilities could be identified that correspond to known species. Thus, within the *Diplodia* clade, eight sub-clades corresponding to known species could be distinguished. Bootstrap and posterior probabilities for the clades were generally high, but the complex of *D. pinea*, and *D. scrobiculata* was only partially resolved. The two isolates of *D. phoenicum* clustered at the base of the *Diplodia* clade (MP bootstrap 85 %; posterior probability 1.00).

The *Lasiodiplodia* clade was resolved into five sub-clades corresponding to *L. gonubiensis, L. crassispora* and a large clade containing isolates previously identified as *L. theobromae*. This large clade was further subdivided into three sub-clades referred to as clades I, II and III in FIG. 1.

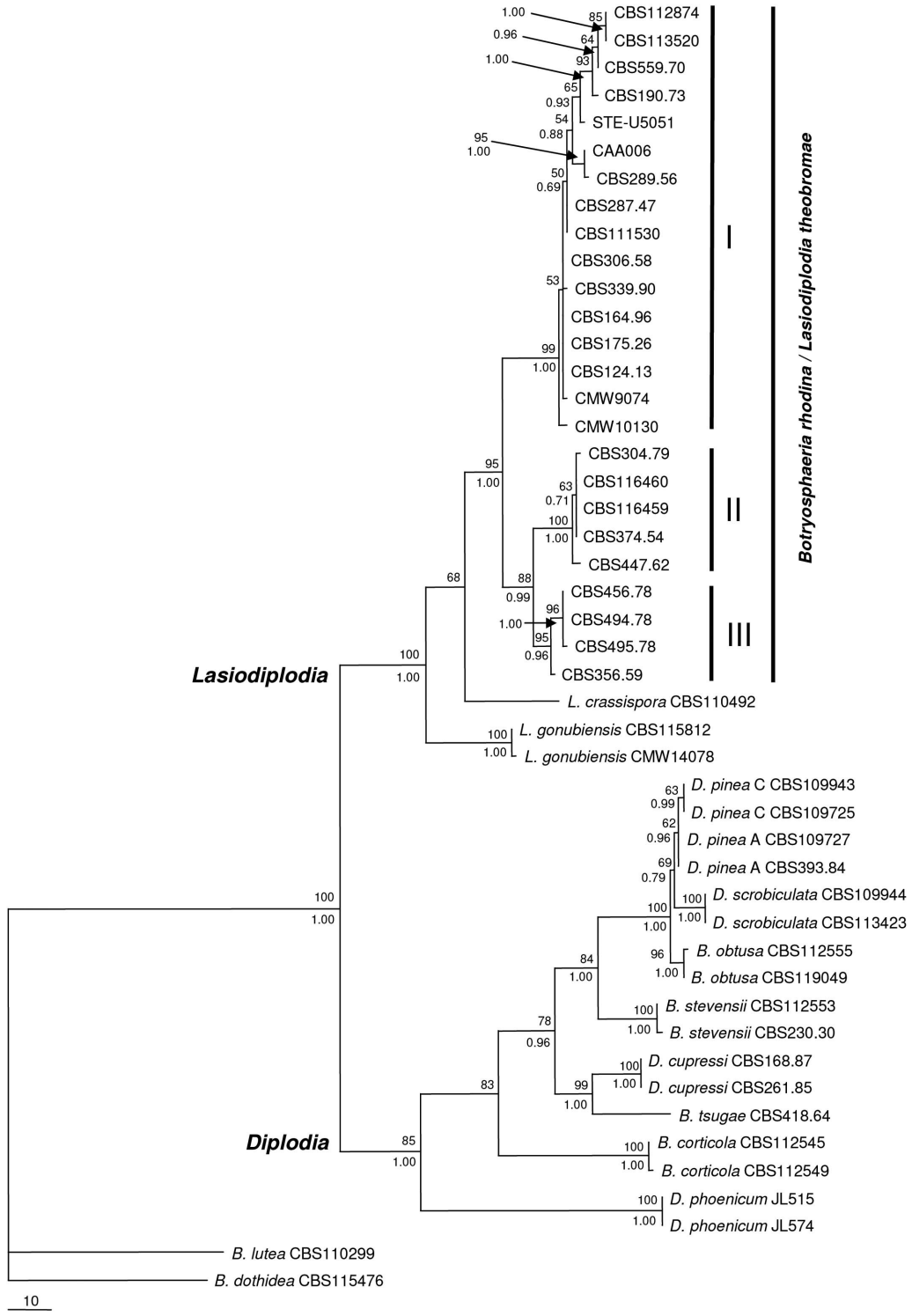


FIGURE 1. Maximum parsimony tree obtained from the combined analysis of ITS and EF1-α sequence data tree length = 553 steps; CI = 0.7830; RI = 0.9230; RC = 0.7227; HI = 0.2170). Bootstrap support values from 1000 replicates are shown above the branches and Bayesian posterior probabilities below the branches. The tree was rooted to *B. dothidea* and *B. lutea*.

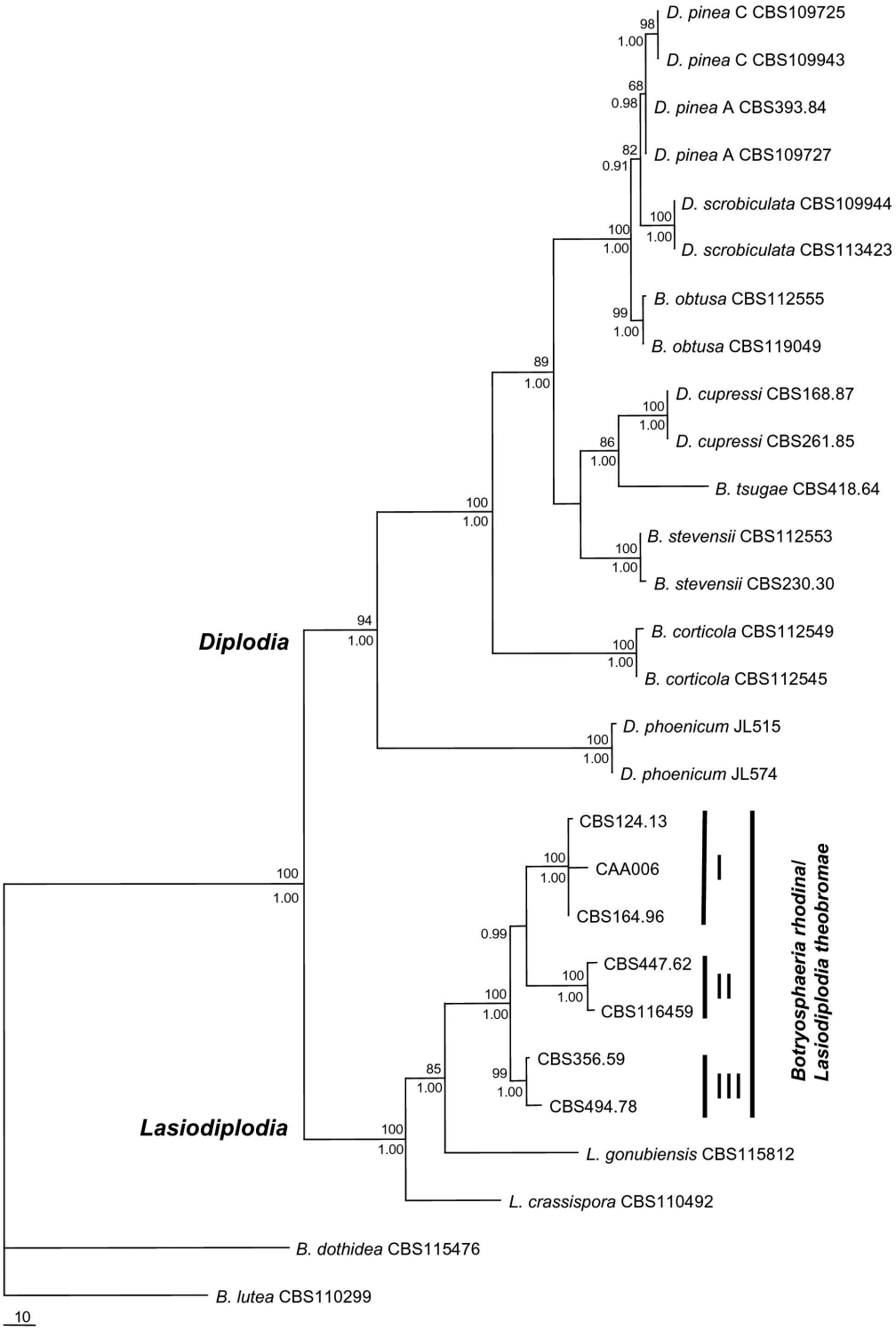


FIGURE 2. One of two most parsimonious trees obtained from combined SSU, LSU, ITS, EF1-α and β-tubulin sequences of *Lasiodiplodia* and *Diplodia* species. Bootstrap support values from 1000 replicates are shown above the branches and Bayesian posterior probabilities below the branches. The tree was rooted to *B. dothidea* and *B. lutea*.

Multi-gene phylogeny (SSU, LSU, ITS, β-tubulin and EF1-α)

For the multi-gene dataset (SSU, LSU, ITS, β-tubulin and EF1-α) the alignment of 28 isolates, including the two outgroup species, consisted of 1134 characters for the SSU region, 599 characters for the ITS region, 614 characters for LSU region, 356 characters for the EF1-α region, and 468 characters for the β-tubulin region, including alignment gaps (data not shown). Indels were coded separately and added to the end of the alignment as characters 3176–3278. In the analyses, alignment gaps were treated as missing data.

The combined dataset contained 3278 characters, of which 373 were parsimony informative, 160 were variable and parsimony uninformative and 2745 were constant. Maximum parsimony analysis of the combined dataset resulted in 2 equal, most parsimonious trees (tree length = 802 steps; CI = 0.7868; RI = 0.8857; RC = 0.6968; HI = 0.2132). The MP and Bayesian phylogenetic analyses generated trees with the same topology.

The MP phylogenetic tree is presented in FIG. 2 with MP bootstrap supports and Bayesian posterior probabilities above and below the branches, respectively. In this tree two main clades are readily identified. One clade corresponds to the anamorphic genus *Diplodia* (MP bootstrap 94 %; posterior probability 1.00) and the other corresponds to the anamorphic genus *Lasiodiplodia* (MP bootstrap 100 %; posterior probability 1.00). Within each clade several sub-clades corresponding to known species could be distinguished. Bootstrap and posterior probabilities for the clades were high. The two isolates of *D. phoenicum* clustered at the base of the *Diplodia* clade (MP bootstrap 100 %; posterior probability 1.00).

Representative isolates from the three clades identified in the *Lasiodiplodia theobromae* clade (clades I, II and III) in FIG. 1 grouped within a well-supported sub-clade in the combined multi-gene analysis.

TABLE 2. Conidial size and septation of some *Lasiodiplodia* species.

Species	Conidia		References
	septation	size	
Lasiodiplodia gonubiensis Pavlic *et al*.	1,3-septate	32–36 × 16–18.5 µm	Pavlic *et al*. (2004)
Lasiodiplodia nigra K.R. Appel & Laubert	1-septate	28–32 × 18–21 µm	Pavlic *et al*. (2004)
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	1-septate	26.2–27.0 × 14.0–14.4 µm	This work
Lasiodiplodia triflorae B.B. Higgins (= *L. theobromae*)	1-septate	22–25 × 13–16.5 µm	Pavlic *et al*. (2004)
Lasiodiplodia tubericola Ellis & Everh. (= *L. theobromae*)	1-septate	18–22 ×11–14 µm	Pavlic *et al*. (2004)
Lasiodiplodia crassispora Burgess & Barber	1-septate	27–30 × 14–17 µm	Burgess *et al*. (2006)
Lasiodiplodia rubropurpurea Burgess *et al*.	1-septate	24–33 × 13–17 µm	Burgess *et al*. (2006)
Lasiodiplodia venezuelensis Burgess *et al*.	1-septate	26–33 × 12–15 µm	Burgess *et al*. (2006)
Lasiodiplodia pseudotheobromae Phillips *et al*.	1-septate	23.5–32 × 14–18 µm	This work
Lasiodiplodia parva Phillips *et al*.	1-septate	16–23.5 × 10.5–13 µm	This work

Morphology

Morphology of all the isolates previously identified as *L. theobromae* that were induced to sporulate in culture corresponded with that commonly regarded as typical of the genus *Lasiodiplodia*. Thus, paraphyses were present within the conidiomata and the conidia were initially hyaline and aseptate, but in time a single median septum formed, the wall became dark brown and melanin granules deposited on the inner surface of the spore wall gave the conidia a striate appearance. Isolates in the three clades revealed by combined ITS and EF1-α analysis were morphologically distinct (TABLE 2). Thus, conidia of isolates in clade I (FIGS 3–12) were 21–31 × 13–15 µm (mean = 26.2 × 14.2 µm) and corresponded to those of *L. theobromae* (Punithalingam, 1976; Pavlic *et al*., 2004). Conidia of isolates in clade II (FIGS 13–20) were somewhat larger (23.5–32 × 14–18 µm, mean = 28 × 16 µm) while those in clade III (FIGS 21–25) were smaller (16–23.5 × 10.5–13 µm, mean = 20.2 × 11.5). The two isolates of *Diplodia phoenicum* did not produce paraphyses, but their conidia, initially hyaline and aseptate, became dark brown and developed striations more typical of *Lasiodiplodia* (FIGS 26–32).

DISCUSSION

The relationship between the anamorph genera *Diplodia*, *Sphaeropsis* and *Lasiodiplodia* has been the subject of some debate. Denman *et al.* (2000) regarded both *Sphaeropsis* and *Lasiodiplodia* as synonyms of *Diplodia*. The synonymy of *Sphaeropsis* under *Diplodia* has been followed by most authors and this is supported by morphological data (Phillips, 2002; Alves *et al*., 2004) as well as phylogenetic analyses of the ITS region alone (Denman *et al*., 2000, Alves *et al*., 2004) and in combination with the EF1-α gene and other genes (this work; Slippers *et al*., 2004a; Phillips *et al*., 2005).

Some authors, however, still accept *Lasiodiplodia* as a separate genus from *Diplodia* based on morphological characters and nucleotide sequence data (Pavlic *et al*., 2004; Burgess *et al*., 2006). The genus *Lasiodiplodia* as defined by the type species *L. theobromae* (Pat.) Griffon & Maubl. is characterised by the presence of paraphyses and longitudinal striations on the conidium wall. Both features are not found in any species of *Diplodia*, and this is the main reason why *Lasiodiplodia* and *Diplodia* are still retained as separate genera. Although paraphyses and striate conidia are seen in *D. gossypina* Cooke, Punithalingam (1976) regarded this species as a synonym of *L. theobromae*. In recent works (Slippers *et al*., 2004a; van Niekerk *et al*., 2004; Phillips *et al*., 2005; Burgess *et al*., 2006), as well as in the present study, phylogenetic

analysis of combined ITS and EF1-α nucleotide sequence data further supported the split between *Lasiodiplodia* and *Diplodia*.

In this work two isolates from *Phoenix* species were studied. These two isolates possess conidia that remain hyaline and aseptate for a long time, becoming dark and 1-septate with age, a feature that is common to both *Diplodia* and *Lasiodiplodia* species (Sutton, 1980; Denman *et al*., 2000). Conidia of these isolates developed longitudinal striations, a feature that would place them within *Lasiodiplodia*. However, these isolates lack paraphyses, which is a character that has been used to distinguish *Diplodia* from *Lasiodiplodia* (Sutton, 1980; Denman *et al*., 2000). Phylogenetic analyses placed the isolates from *Phoenix canariensis* and *P. dactylifera* as a distinct species within *Diplodia* and the name *D. phoenicum* (Sacc.) H.S. Fawc. & Klotz. was applied.

The inclusion of the isolates from *Phoenix* within *Diplodia* thus raises the question of the phylogenetic significance of striate conidia at genus level. Conidial striations are not a unique feature of *Lasiodiplodia* and can also be found in *Diplodia* as seen in *D. phoenicum*. Thus, the presence of longitudinal striations on the conidium wall is shown to be an unsuitable taxonomic character for the discrimination at the genus level. Nevertheless this is still one feature that is common to all species in *Lasiodiplodia* which can be separated from *Diplodia* species only by the presence of paraphyses.

In order to clarify the relationship between the two genera, a combined phylogenetic analysis of nucleotide sequence data from the five genes (SSU, LSU and ITS rDNA, β-tubulin and EF1-α) was performed (FIG. 2). As can be seen from the phylogenetic tree presented in FIG. 2 the species included in *Lasiodiplodia* are clearly separated from the species in *Diplodia* and both genera form two separate and highly supported monophyletic clades.

A synonymy of *Lasiodiplodia* under *Diplodia* has been suggested by Denman *et al*. (2000) and previously by other authors (Taubenhaus, 1915). In a recent study (Crous *et al*., 2006) phylogenetic analysis of nucleotide sequence data from the LSU gene showed that both genera could not be separated. However, in that study a *Diplodia* clade (including *Lasiodiplodia*) lacked statistical support and could not be fully resolved.

The phylogenetic analyses based on multi-gene sequence data presented here does not favour the synonymy hypothesis and clearly shows that *Lasiodiplodia* and *Diplodia* should be maintained as separate genera. On the basis of the morphological characters as well as molecular data it is apparent that these genera although distinct are intimately related to each other. It is possible that the genus *Lasiodiplodia* represents a lineage within *Diplodia* that has evolved in particular environmental

conditions namely tropical and sub-tropical regions where species of this genus are found.

The genetic variability within a collection of strains originally identified as *L. theobromae* was determined through a study of ITS and EF1-α nucleotide sequences. Morphological variability of conidial characters was also assessed within this group of isolates.

Lasiodiplodia pseudotheobromae and *L. parva* are recognized as two new species in the genus *Lasiodiplodia*, closely related to *L. theobromae*. Both species possess typical morphological features, namely slowly maturing conidia with thick walls and longitudinal striations caused by the deposition of melanin on the inner surface of the wall. Although *L. pseudotheobromae* and *L. parva* closely resemble *L. theobromae*, they can be separated on the size and shape of conidia. Thus, mature conidia of *L. pseudotheobromae* are larger, more ellipsoid and do not taper so strongly to the base as those of *L. theobromae*. Also, *L. parva* is easily distinguished from the other two species on its smaller conidia. *Lasiodiplodia pseudotheobromae* and *L. parva* produce a dark pink pigment in PDA cultures incubated at 35 ºC, a feature that is not observed in *L. theobromae*. Moreover, *L. pseudotheobromae* is capable of growing at 10 ºC while *L. parva* and *L. theobromae* cannot. These conidial (e.g. size, shape, and septation) and cultural characters (e.g. colony morphology, chromogenicity, and temperature effects on mycelial growth) have been used to distinguish closely related species within *Botryosphaeria* anamorphs (Slippers *et al*., 2004a, b; Phillips *et al*., 2005). Pavlic *et al*. (2004) distinguished *L. gonubiensis* from *L. theobromae* by its larger and multiseptate conidia. *Lasiodiplodia crassispora, L. venezuelensis* and *L. rubropurpurea* are also distinguished on the basis of morphological features of the conidia (Burgess *et al*., 2006).

Phylogenetic analyses of combined ITS and EF1-α (FIG. 1) as well as multi-gene sequence data (FIG. 2) clearly separated all the species in *Lasiodiplodia*. The results showed that the new *Lasiodiplodia* species described are closely related, but distinct from *L. theobromae*.

The teleomorph of *L. theobromae* is frequently referred to as *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx (von Arx, 1970, 1981). However, the subject surrounding the correct name to use for the sexual state of *L. theobromae* is in need of clarification. In 1867 Curtis collected the type material of *Physalospora rhodina* on branches of *Rosa rubiginosa* in South Carolina, USA, and listed the fungus as "*Sphaeria rhodina* B. & C.", on page 148 of a catalogue published in the *Geographical and Natural History Survey of North Carolina*, Raleigh 1867. Subsequently, Cooke (Grevillea 17: 92, 1889) published a formal description of this fungus under the name

Physalospora rhodina and attributed it to Berkley & Curtis. However, because Cooke was the first person to provide a formal description of this fungus under the name *Physalospora rhodina*, and that Curtis merely listed the fungus (without a description) under the name *Sphaeria rhodina* the species *P. rhodina* must be attributed to Cooke alone. Von Arx (1970) transferred this species to the genus *Botryosphaeria*. Thus, the correct name that should be applied to this fungus is *B. rhodina* (Cooke) von Arx.

Another important subject is related to the teleomorph-anamorph connection. As stated by Sivanesan (1984) the connection between the two states has yet to be absolutely proved, but there are some inconclusive reports in the literature. The connection between the anamorph and teleomorph was determined by Stevens (1925, 1926). Stevens (1925) made single ascospore isolations from a fungus he tentatively referred to as "*Physalospora gossypina*" found on cotton stems in Florida. He made further isolations from a similar ascomycete found on *Hicoria*, *Ilex*, *Liquidambar*, *Quercus* and *Vitis*. In all cases, conidia generated from single ascospore cultures were reportedly identical to those of *D. gossypina*. Later, Stevens (1926) determined that the fungus he called "*Physalospora gossypina*" was in fact the same as *Physalospora rhodina* Cooke. Since then there have been no reports that confirm this connection.

Stevens (1926) studied the perfect stage of the *Citrus* stem-end rot *Diplodia* and determined it to be a *Physalospora* species. The same fungus was also obtained from *Persea* and *Rosa*. The cultures produced from single ascospores of this material gave rise to conidia that agreed closely in appearance with *L. theobromae* (=*D. gossypina*). The cultures obtained from *Citrus*, *Persea* and *Rosa* exhibited good growth at 36–37 ºC and chromogenesis at this temperature on potato dextrose agar (Stevens, 1926). However, not all the cultures of "*Diplodia*" from *Citrus* and other hosts that were able to grow at 36–37ºC showed chromogenesis at this temperature, and a form unable to grow at this temperature also occurs on *Citrus* as well as other hosts (Stevens, 1926). Thus, according to Stevens (1926) the high temperature fungus should be considered merely as a variety within a single species (*Physalospora rhodina*), which is common on many hosts.

In this work we have shown that the fungus known as *L. theobromae* is a complex of different cryptic species. In fact, due to the large number of hosts associated with it and the number of synonyms listed for this species it is reasonable to assume that in the future more species may well be found within this complex. In view of these facts, it is impossible to determine exactly to which species the teleomorph name *B. rhodina* should be attributed. In order to clarify this issue it is imperative to collect fresh material that can be used as epitype, and to prepare ex-epitype cultures which in turn

can be used to fully characterise the species in terms of morphology and phylogenetic relationships with known species.

TAXONOMY

The two isolates of *D. phoenicum* that were studied had striate conidia, a character that would place them in *Lasiodiplodia*. On the other hand no paraphyses could be detected, which places them outside of *Lasiodiplodia*. However, in phylogenetic analyses, these two isolates clustered within the clade normally associated with *Diplodia* species. This suggests that conidial striations have little value at the genus level and this is not a valid character to separate *Lasiodiplodia* from *Diplodia*. These genera can be separated by the presence of paraphyses.

Lasiodiplodia theobromae (Pat.) Griff. & Maubl., *Bull. trimest. Soc. Mycol. Fr.* 25: 57 (1909)

= *Diplodia gossypina* Cooke, *Grevillea* **7**: 95 (1879). FIGS 2–11

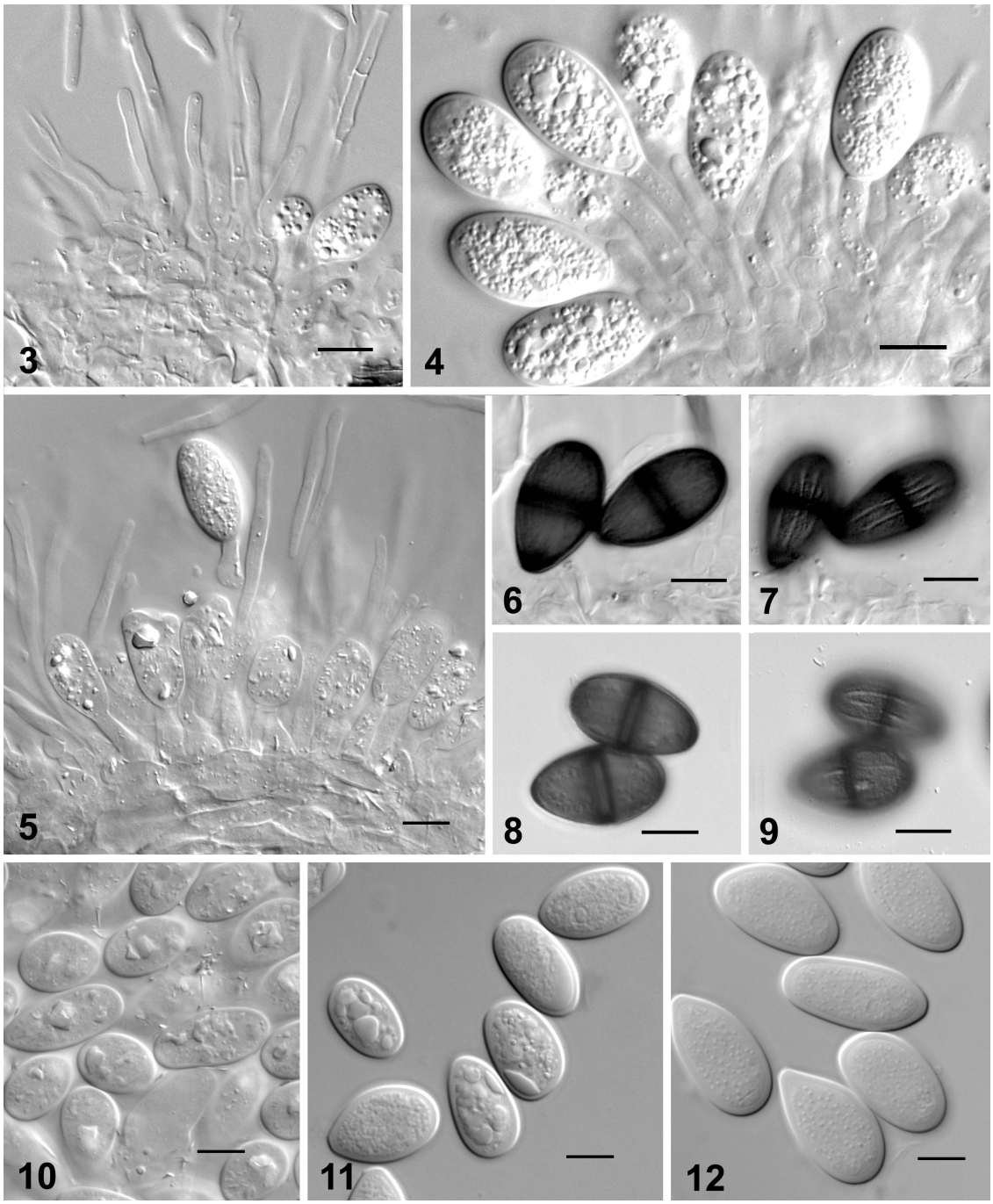
Teleomorph: ?*Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx, *Gen. Fungi Sporul. Cult.* (Lehr): 143 (1970).

≡ *Physalospora rhodina* Berk. & M.A. Curtis, *Grevillea* **17**: 92 (1889).

= *Botryosphaeria quercuum* (Berk. & M. A. Curtis) Arx & E. Müll., Beiträge zur Kryptogamenflora der Schweiz, 2 (1): 33 (1954).

Conidiomata formed on pine needles in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. Paraphyses hyaline, cylindrical, septate, occasionally branched, ends rounded, up to 55 µm long, 3–4 µm wide. Conidiogenous cells hyaline, thin-walled, smooth, cylindrical, holoblastic, proliferating percurrently to form one or two annellations, or proliferating at the same level giving rise to periclinal thickenings. Conidia subovoid to ellipsoid-ovoid, apex broadly rounded, tapering to truncate base, widest in middle to upper third, thick-walled, contents granular, initially hyaline and aseptate, remaining hyaline for a long time, becoming dark brown and one-septate only a long time after discharge from the pycnidia, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (19.0–)21.0–31.0(–32.5) × (12.0–)13.0–15.5(–18.5) µm, 95 % confidence interval = 26.2–27.0 × 14.0–14.4 µm ($\bar{x}$ ± S.D. = 26.2 ± 2.6 × 14.2 ± 1.2 µm, l/w ratio = 1.9 ± 0.2).

Cultures examined: CBS 164.96, CBS 111530.



FIGURES 3–12. *Lasiodiplodia theobromae* and *Diplodia gossypina*. FIGS 3, 4, 6, 7, 11, 12, *L. theobromae*. 3. Paraphyses. 4, 5. Conidiogenous cells and young conidia. 6, 7. Mature conidia in two different focal planes to show the longitudinal striations. 11, 12. Hyaline, immature conidia. FIGS 5, 8, 9, 10, *Diplodia gossypina* K118158. 5. Conidiogenous layer with paraphyses. 8, 9. Mature conidia in two different focal planes to show the longitudinal striations. 10. Conidia. Scale bars = 10 µm.

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous FIGS 13–19

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa. Cellulae conidiogenae, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1–2 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia 27.5–28.5 × 15.5–16.5 µm, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, primaria hyalinae, cum maturitate cinnamomescentia vel brunnescentia, longitudinaliter striata et unum septa formantia.

Conidiomata formed on pine needles in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. Paraphyses hyaline, cylindrical, mostly aseptate, sometimes branched; ends rounded, up 58 µm long, 3–4 µm wide arising amongst the conidiogenous cells. Conidiogenous cells hyaline, smooth, cylindrical, slightly swollen at the base, holoblastic, proliferating percurrently to form one or two closely spaced annelations. Conidia ellipsoidal, apex and base rounded, widest at the middle, thick-walled, initially hyaline and aseptate and remaining so for a long time, becoming one-septate and dark brown only some time after release from the conidiomata, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (22.5–)23.5–32.0(–33.0) × (13.5–)14.0–18.0(–20.0) µm, 95 % confidence limits = 27.5–28.5 × 15.5–16.5 µm ($\bar{x}$ ± S.D. = 28.0 ± 2.5 × 16.0 ± 1.2 µm, l/w ratio = 1.7 ± 0.2).

Etymology: Named for its resemblance to *L. theobromae*.

Holotype: Culture ex-holotype CBS 116459. Other cultures examined CBS 116460.

Note: This species differs from *L. theobromae* on the shape of its mature conidia, which are more ellipsoidal and do not taper so strongly to the base. The conidia are somewhat larger as revealed by the mean dimensions and the 95 % confidence limits.

Lasiodiplodia parva A.J.L. Phillips, A. Alves & Crous FIGS 21–25

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa. Cellulae conidiogenae, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1–2 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia 19.8–20.5 × 11.4–11.7 µm, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, primaria hyalinae, cum maturitate cinnamomescentia vel brunnescentia, longitudinaliter striata et unum septa formantia.

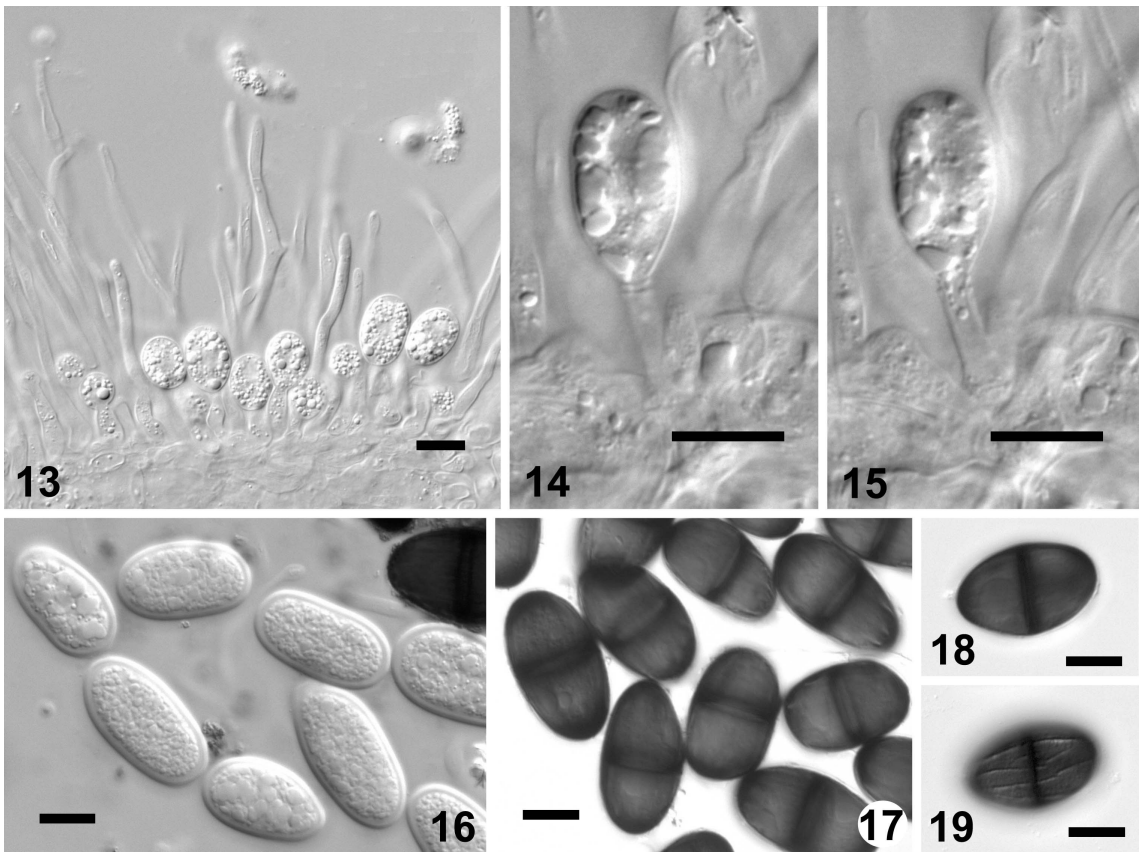
Conidiomata formed on pine needles in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. Paraphyses hyaline, cylindrical, septate, ends rounded, up 105 µm long, 3–4 µm wide arising amongst the

conidiogenous cells. Conidiogenous cells hyaline, smooth, cylindrical, slightly swollen at the base, holoblastic, proliferating percurrently to form one or two annelations, or proliferating at the same level giving rise to periclinal thickenings. Conidia ovoid, apex broadly rounded, base rounded or truncate, widest in the middle or upper third, thick-walled, initially hyaline and aseptate and remaining so for a long time, becoming one-septate and dark walled only some time after release from the conidiomata, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (15.5–)16.0–23.5(–24.5) × (10.0–)10.5–13.0(–14.5) µm, 95 % confidence limits = 19.8–20.5 × 11.4–11.7 µm ($\bar{x}$ ± S.D. = 20.2 ± 1.9 × 11.5 ± 0.8 µm, l/w ratio = 1.8 ± 0.1).

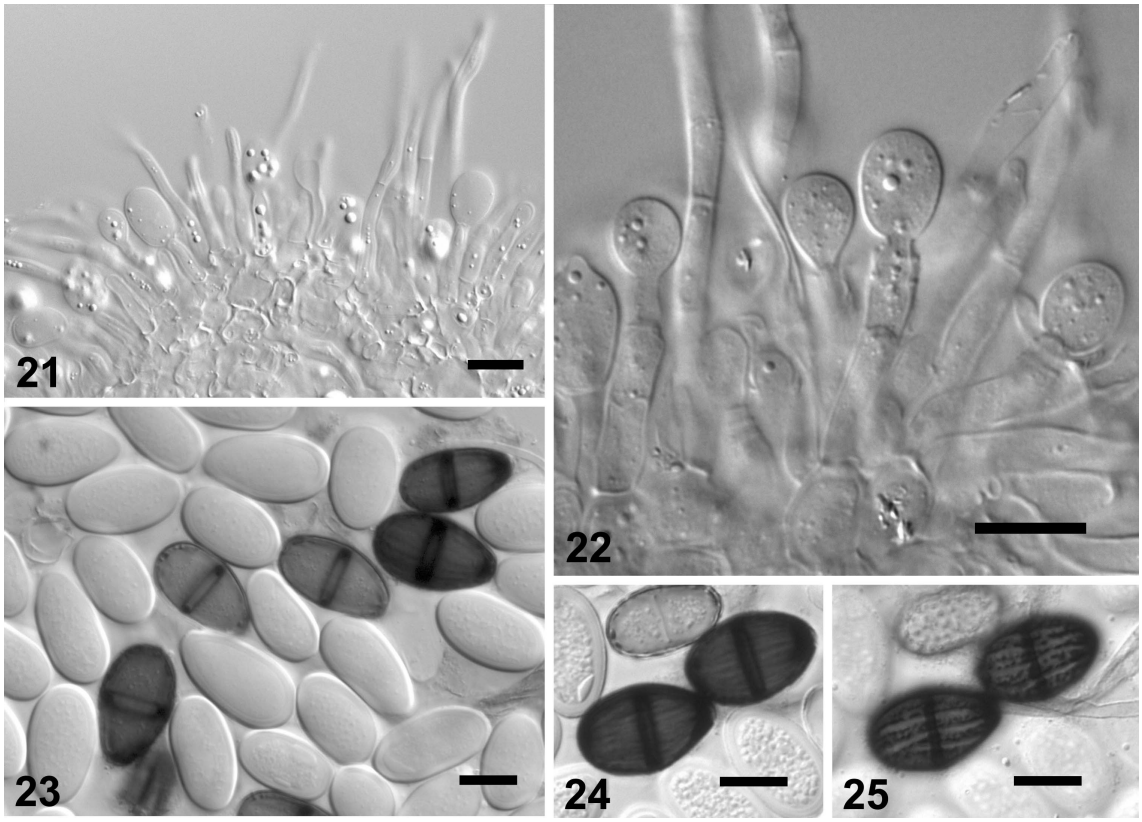
Etymology: Named for the small size of conidia.

Holotype: Culture ex-holotype CBS 456.78. Other cultures examined CBS 494.78.

Note: This species can be distinguished from *L. theobromae* and *L. pseudotheobromae* on its smaller conidia.



Figures 13–19. *Lasiodiplodia pseudotheobromae*. 13. Conidiogenous layer with paraphyses. 14, 15. Percurrently proliferating conidiogenous cell at two different focal planes. 16. Hyaline, aseptate conidia. 17. Septate, dark-walled conidia. 18, 19. Mature condium at two focal planes to show striations. Scale bars = 10 µm.

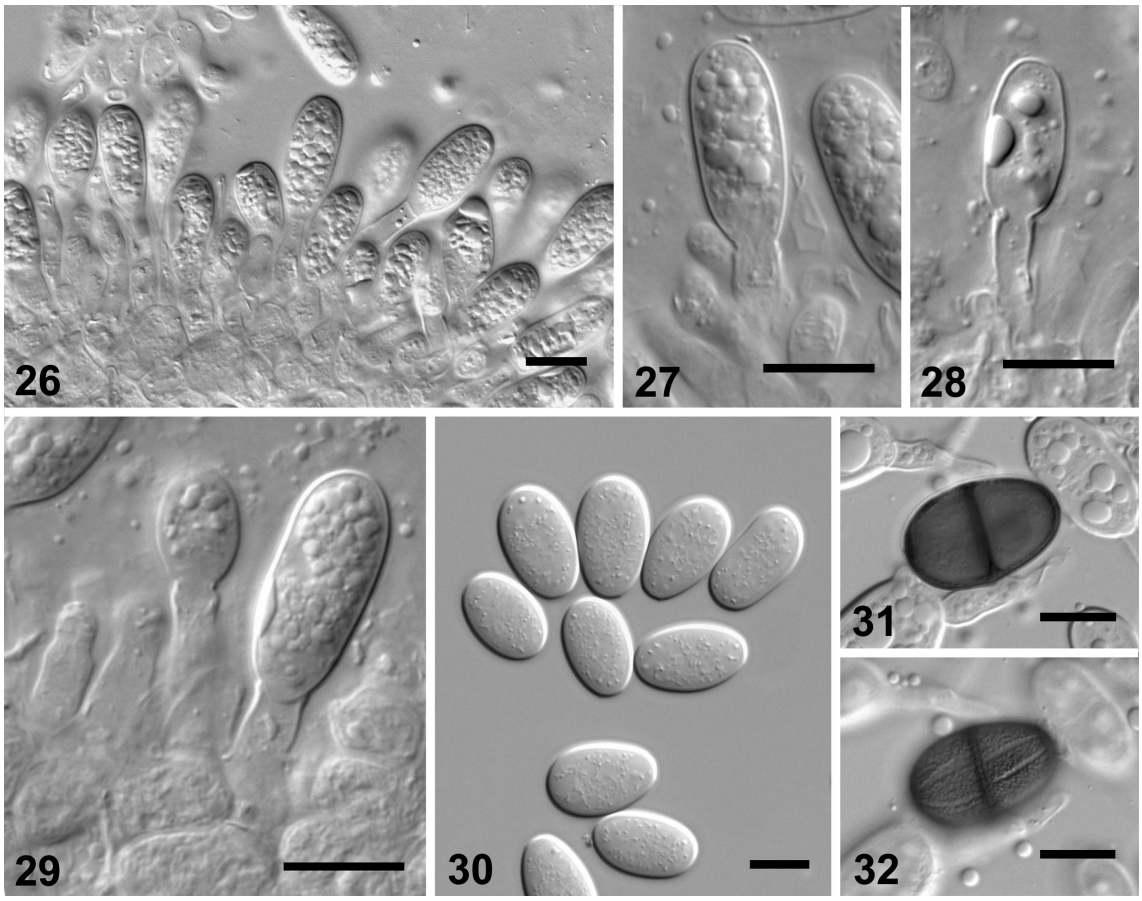


FIGURES 21–25. *Lasiodiplodia parva* 21. Conidiogenous layer with paraphyses. 22. Percurrently proliferating conidiogenous cells. 23. Hyaline, aseptate conidia and dark-walled, septate conidia. 24, 25. Mature condia at two focal planes to show striations. Scale bars = 10 µm.

Diplodia phoenicum (Sacc.) H.S. Fawc. & Klotz, *Bulletin of the California Agricultural Experimental Station* 522: 8 (1932). FIGS 26–32

Conidiomata formed on pine needles in culture pycnidial, multiloculate, dark brown to black, immersed in the host becoming erumpent when mature. Paraphyses not seen. Conidiogenous cells hyaline, smooth, cylindrical, swollen at the base, holoblastic, proliferating percurrently to form one or two annelations, or proliferating at the same level giving rise to periclinal thickenings. Conidia ovoid to ellipsoid, apex and base broadly rounded, widest in the middle to upper third, thick-walled, initially hyaline and aseptate, becoming dark brown and one-septate some time after discharge from the pycnidia, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (14.5–)17.0–21.0(–24.0) × (9.0–)10.0–12.5(–14.0) µm, 95 % confidence limits = 18.6–19.5 × 11.2–11.8 µm ($\bar{x}$ ± S.D. = 19.1 ± 1.7 × 11.5 ± 1.1 µm, l/w ratio = 1.7± 0.2).

Cultures examined: JL 515, JL 574.



FIGURES 26–32. *Diplodia phoenicum*. 26. Conidiogenous layer. 27–29. Percurrently proliferating conidiogenous cells. 28. Periclinal thickenings. 30. Hyaline, aseptate conidia. 31, 32. Mature condia at two focal planes to show striations. Scale bars = 10 µm.

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CHAPTER

10

Unpublished

Molecular systematics of *Botryosphaeria* and allied genera in *Botryosphaeriaceae*

ABSTRACT

The family *Botryosphaeriaceae* is a family of bitunicate ascomycetes which includes a large number of important plant pathogens. The phylogenetic relationships between members of this family is examined using nuclear large- and small-subunit ribosomal DNA, ITS, EF1-α and β-tubulin sequence data. The current circumscription of *Botryosphaeria* includes taxa with morphologically conserved teleomorphs but whose anamorphs are very diverse. Species in *Botryosphaeria s.l.* segregated into well-supported monophyletic clades. Each of these clades represents distinct genera and is discussed in view of the molecular data and morphological characters. The genus *Melanops* is reinstated to accommodate species previously included in *Botryosphaeria.* The type species *M. tulasnei* (=*B. melanops*) is studied and a neotype designated. The position of the *Botryosphaeriaceae* within the higher classification of Ascomycetes is also discussed.

***Key words*:** *Botryosphaeriaceae*, Ascomycetes, rDNA, EF1-α, β-tubulin, phylogeny, systematics

INTRODUCTION

The Loculoascomycetes are a group of fungi that encompass more than 6000 species including many economically important plant pathogens (Hawksworth *et al*., 1995; Lumbsch, 2000). Loculoascomycetes were originally proposed to be a separate group of ascomycetes on the basis of ontogeny of the ascostromatic ascoma (Nannfeldt, 1932), centrum development and on the ascus-type present in this group of fungi (Luttrell, 1951, 1955, 1973).

In these fungi ascoma development starts before dikaryotisation. A locule is formed in which ascogonia develop (ascolocular development), while other fungi start their ascoma formation after the beginning of the production of ascogonia (ascohymenial development). While the other filamentous ascomycetes usually have thin-walled asci with a single functional wall layer (unitunicate asci), the Loculoascomycetes have thick-walled asci with two separable wall layers (bitunicate asci). The thick inner layer of the ascus wall extends out of the rigid outer wall layer and then the ascospores shoot out of the ascus. In most other filamentous ascomycetes, hyaline, aseptate ascospores predominate while among the Loculoascomycetes, pigmented spores with

both transverse and longitudinal septation are common (Luttrell, 1951, 1955, 1973; Eriksson, 1981; Sivanesan, 1984; Barr, 1976, 1987; Lumbsch, 2000).

Given these derived morphological features, Loculoascomycetes were generally believed to form a monophyletic group and were recognised in several classifications as a supraordinal taxon. Loculoascomycete systematists disagree, however, about naming, ranking and subdividing the group (Nannfeldt, 1932; Luttrell, 1951, 1955, 1973; Eriksson, 1981; Hawksworth *et al*., 1995; Sivanesan, 1984; Barr, 1976, 1979, 1983, 1987).

The class Loculoascomycetes is still accepted by many mycologists, but unlike morphological studies, molecular studies have shown that it is paraphyletic. Nuclear rDNA sequence data did not support the monophyly of the Loculoascomycetes (Berbee, 1996; Silva-Hanlin and Hanlin, 1999; Spatafora *et al.;* 1995; Winka *et al.*, 1998; Lindemuth *et al*., 2001).

The Loculoascomycetes are currently placed in two classes, the Chaetothyriomycetes and Dothideomycetes (Eriksson and Winka, 1997; Eriksson 2006). The Chaetothyriomycetes appear to be more closely related to the Lecanoromycetes (Winka *et al.,* 1998) or Eurotiomycetes (Berbee, 1996; Liu *et al.*, 1999; Silva-Hanlin and Hanlin, 1999; Lumbsch *et al.*, 2000).

Phylogenetic analyses based on sequence data of the RPB2 gene (Liu *et al.*, 1999) and the nuclear LSU rRNA gene (Lumbsch *et al.*, 2000) showed that the Loculoascomycetes are not monophyletic and also placed the Chaetothyriomycetes as sister-group to the Eurotiomycetes. However, a sister-group relationship of the Chaetothyriomycetes and Dothideomycetes, and hence a monophyly of the Loculoascomycetes cannot be rejected with the current molecular data (Liu *et al.*, 1999; Lumbsch *et al.*, 2000). Moreover, Liu and Hall (2004) in a phylogenetic analysis of the RPB2 gene have shown that the Loculoascomycetes (Chaetothyriomycetes and Dothideomycetes) are monophyletic.

The position of the genus *Botryosphaeria* within the higher classification of Ascomycetes (family and order levels) has been the subject of many taxonomic rearrangements since it was introduced in 1863. This topic has been reviewed in detail elsewhere (Denman *et al.*, 2000; Crous *et al*., 2006). The ordinal placement of the genus was and still is the subject of much debate among fungal taxonomists.

Within Loculoascomycetes, fruiting body form and development have traditionally been key characters for defining orders. Luttrel (1951) defined three types of centrum development, the *Dothidea* Fr., the *Pleospora* Rabenh., and the *Elsinöe* Racib. types. The *Pleosporales* Luttrel ex M.E. Barr is a large order whose members produce pseudoparaphyses, as well as setose or glabrous, mostly unilocular pseudothecia. On

the contrary, pseudoparaphyses are lacking in the *Dothideales* Lindau, even though remnants of interthecial tissue can be found in some genera (Sivanesan, 1984), generating most of the confusion about the taxonomic placement of several fungi, as is the case of *Botryosphaeria*.

Barr (1972) treated *Guignardia* Viala & Ravaz as a synonym of *Botryosphaeria* and placed both in the *Dothideales* (*Dothioraceae* Theiss. & Sydow). Luttrel (1955, 1973) acknowledged that *Botryosphaeria* species have a centrum typical of the *Pleosporales*, and so placed the genus in that order. This was later supported by Barr (1987) who accepted *Botryosphaeria* and *Guignardia* (=*Discochora* Höhn) in the *Botryosphaeriaceae* Theiss. & P. Syd. (*Pleosporales*).

According to von Arx and Müller (1975) and von Arx (1981, 1987) the orders proposed by Luttrel (1955, 1973) and Barr (1972, 1987) comprised a collection of unrelated genera and the taxonomic characters used to separate the orders overlapped. Moreover, von Arx and Müller (1975) did not support the placement of closely related genera like *Guignardia* and *Botryosphaeria* in different orders, the *Dothideales* and *Pleosporales* respectively (Luttrel, 1973). Von Arx and Müller (1975) placed all bitunicate ascomycetes in a single order *Dothideales*, comprising two suborders and 34 families including the family *Botryosphaeriaceae*.

To complicate this issue further, Sivanesan (1984) placed *Guignardia* and *Botryosphaeria* in the *Dothideaceae* (*Dothideales*), whereas Hawksworth *et al*. (1995) listed *Botryosphaeria* under the *Botryosphaeriaceae* and *Guignardia* under the *Mycosphaerellaceae* Lindau, both in the *Dothideales*.

Currently it is widely accepted that the genus *Botryosphaeria* belongs in the family *Botryosphaeriaceae* erected by Theissen and Sydow (1918). Eriksson (2006) accepted the family *Botryosphaeriaceae* in the Dothideomycetes but listed it as a family *incertae sedis* due to the lack of support for its ordinal placement. He also accepts the closely related genus *Guignardia* in the family *Mycosphaerellaceae*.

Detailed descriptions of the family *Botryosphaeriaceae* have been presented by several authors (von Arx and Müller, 1954, 1975; Hawksworth *et al*., 1995; Eriksson, 1981; Sivanesan, 1984; Barr, 1987). Members of the family are biotrophic, necrotrophic or saprobic, especially on woody plants. The *Botryosphaeriaceae* are separated primarily on the basis of their large, ovoid to oblong usually hyaline, aseptate ascospores. Although this main seem an inadequate basis for recognition of a family, it is a very unusual spore type among Loculoascomycetes (Luttrel, 1973; Eriksson, 1981; Sivanesan, 1984; Barr, 1987). The coarse hyphae and cells of the peridium, thick-walled, wide usually clavate asci, and the coelomycetous anamorphs when present, are also major features of this family (Barr, 1987). Characters that are

used to differentiate genera are essentially based on morphological features of the ascospores (Barr 1987, 1989; Hsieh and Chen, 1994).

Despite recent phylogenetic analyses based on nuclear and mitochondrial rDNA sequences (Winka *et al.*, 1998; Silva-Hanlin and Hanlin, 1999; Liew *et al*., 2000; Lumbsch *et al.*, 2000; Berbee, 1996, 2001; Tehler *et al*., 2000, 2003; Lutzoni *et al*., 2004) as well as sequences of protein-coding genes like RPB2 (Liu *et al*., 1999; Lutzoni *et al*., 2004) the ordinal position of *Botryosphaeriaceae* remains ambiguous. In some analyses the *Botryosphaeria* species representative of the family cluster with the *Dothideales*, while in others they cluster with the *Pleosporales*. In all cases, however, the placement of the genus in each of the orders lacked statistical support. The only exception is the work of Liu and Hall (2004), where the phylogenetic analysis of RPB2 sequences from ascomycetes places *Botryosphaeria* in the *Pleosporales* with statistically significant support.

The genus *Botryosphaeria* is characterised essentially by stromatic ascomata, uniloculate or multiloculate, with a thick pseudoparenchymatic wall; asci clavate or cylindric-clavate, bitunicate developing on a broad basal hymenial layer. Ascospores are typically hyaline, thin-walled, smooth, one-celled sometimes 1–2 septate, and may become brown with age. Pseudoparaphyses are thin-walled, hyaline, frequently septate, often constricted at the septa, usually disintegrating when the asci mature (von Arx and Müller, 1954; Shoemaker, 1964; Eriksson, 1981; Sivanesan, 1984; Denman *et al.* 2000).

As currently circumscribed the genus *Botryosphaeria* seems to be composed of a heterogeneous assembly of taxa, as exemplified by the morphologically and phylogenetically distinct anamorph genera linked to it (Crous *et al*., 2006). The description of new species with dark-brown and septate ascospores (Luque *et al*., 2005; Phillips *et al*., 2005a) similar to those reported for *Dothidotthia* Höhn. (Barr, 1987, 1989) further widened the circumscription of *Botryosphaeria* and raised some questions regarding the relationships between these two genera. Moreover, Crous *et al*. (2006) identified several phylogenetic lineages within *Botryosphaeria* on the basis of phylogenetic analysis of partial LSU ribosomal DNA gene sequences.

In this study we performed phylogenetic analyses of several species of botryosphaeriaceous fungi with the objective of answering the following questions:

i) is the genus *Botryosphaeria* monophyletic?

ii) which morphological characters are phylogenetically informative for delimiting *Botryosphaeria* and other genera in *Botryosphaeriaceae*?

iii) what are the phylogenetic affinities of the family to other ascomycetes?

TABLE 1. List of *Botryosphaeriaceae* species used in this study.

Species	Accession numbers				
	SSU rDNA	LSU rDNA	ITS	EF1-α	β-tubulin
Botryosphaeria dothidea CBS 115476	ND	*AY928047*	*AY236949*	*AY236898*	*AY236927*
Botryosphaeria dothidea CBS 110300	ND	*AY928048*	*AY640253*	*AY640256*	ND
Botryosphaeria dothidea CBS 110302	ND	ND	*AY259092*	*AY573218*	ND
Botryosphaeria corticis CBS 119047	ND	ND	*DQ299245*	ND	ND
Botryosphaeria corticis CBS 119048	ND	ND	*DQ299246*	ND	ND
Neofusicoccum luteum CBS 110299	ND	*AY928043*	*AY259091*	*AY573217*	*DQ458848*
Neofusicoccum luteum CAP 037	ND	ND	ND	ND	ND
Neofusicoccum australe CBS 119046	ND	ND	*DQ299244*	ND	ND
Neofusicoccum australe CAP 189	ND	ND	ND	ND	ND
Neofusicoccum ribis CBS 121.26	ND	ND	*AF241177*	*AY236879*	*AY236908*
Neofusicoccum ribis CBS 115475	ND	*AY928044*	*AY236935*	*AY236877*	*AY236906*
Neofusicoccum parvum CMW 9081	ND	*AY928045*	*AY236943*	*AY236888*	*AY236917*
Neofusicoccum parvum CBS 110301	ND	*AY928046*	*AY259098*	*AY573221*	ND
Neofusicoccum mangiferum CBS 118531	—	+	—	—	—
Neofusicoccum mangiferum CBS 118532	—	+	—	—	—
Neofusicoccum arbuti CBS 116131	—	+	—	—	—
Neofusicoccum arbuti CBS 117090	—	+	—	—	—
Neofusicoccum andinum CBS 117453	—	+	—	—	—
Neoscytalidium dimidiatum CBS 204.33	*AY307355*	—	—	—	—
Neoscytalidium dimidiatum CBS 251.49	—	+	—	—	—
Neoscytalidium dimidiatum CBS 499.66	—	+	—	—	—
Neosccytalidium hyalinum CBS 145.78	*AF258606*	*AY213628*	—	—	—
"Botryosphaeria" mamane CBS 117444	—	+	—	—	—
Macrophomina phaseolina CPC 11070	—	+	—	—	—
Tiarosporella phaseolina CBS 227.33	—	+	—	—	—
Dothidotthia sarmentorum IMI 63581b	ND	*AY928052*	*AY573212*	*AY573235*	ND
Dothidotthia sarmentorum CBS 115038	ND	ND	*AY573206*	*AY573223*	ND
Dothidotthia iberica CBS 113188	ND	ND	*AY573198*	ND	ND
Dothidotthia iberica CBS 115041	ND	*AY928053*	*AY573202*	*AY573222*	ND
Dothidotthia viticola CBS 117006	ND	ND	*AY905555*	*AY905562*	ND
Dothidotthia viticola CBS 117009	ND	ND	*AY905554*	*AY905559*	ND
Dothiorella coryli CBS 242.51	ND	ND	ND	ND	ND
Dothiorella sp. CAP 187	ND	ND	ND	ND	ND
Dothiorella sp. CAA 005	ND	ND	ND	ND	ND
"Botryosphaeria" rhodina CBS 124.13	ND	*AY928054*	*DQ458890*	*DQ458875*	*DQ458858*
"Botryosphaeria" rhodina CBS 164.96	ND	ND	*AY640255*	*AY640258*	ND
"Botryosphaeria" rhodina CAA 006	ND	ND	*DQ458891*	*DQ458876*	*DQ458859*
Lasiodiplodia pseudotheobromae CBS 447.62	ND	ND	ND	ND	ND
Lasiodiplodia pseudotheobromae CBS 116459	ND	ND	ND	ND	ND
Lasiodiplodia parva CBS 356.59	ND	ND	ND	ND	ND
Lasiodiplodia parva CBS 494.78	ND	ND	ND	ND	ND
Diplodia phoenicum JL 515	ND	ND	ND	ND	ND
Diplodia phoenicum JL 574	ND	ND	ND	ND	ND
"Botryosphaeria" subglobosa CBS 448.91	—	+	—	—	—
Diplodia cupressi CBS 168.87	ND	ND	*DQ458893*	*DQ458878*	*DQ458861*
Diplodia cupressi CBS 261.85	ND	ND	*DQ458894*	*DQ458879*	*DQ458862*
"Botryosphaeria" stevensii CBS 230.30	ND	ND	*DQ458886*	*DQ458869*	*DQ458849*
"Botryosphaeria" stevensii CBS 112553	ND	*AY928049*	*AY259093*	*AY573219*	*DQ458850*
"Botryosphaeria" corticola CBS 112549	ND	*AY928051*	*AY259100*	*AY573227*	*DQ458853*
"Botryosphaeria" corticola CBS 112546	ND	ND	*AY259090*	ND	ND
"Botryosphaeria" obtusa CBS 112555	ND	*AY928050*	*AY259094*	*AY573220*	*DQ458856*
"Botryosphaeria" obtusa CBS 119049	ND	ND	*DQ458889*	*DQ458874*	*DQ458857*
"Botryosphaeria" tsugae CBS 418.64	ND	ND	*DQ458888*	*DQ458873*	*DQ458855*
Diplodia scrobiculata CBS 109944	ND	ND	*DQ458899*	*DQ458884*	*DQ458867*
Diplodia scrobiculata CBS 113423	ND	ND	*DQ458900*	*DQ458885*	*DQ458868*
Diplodia pinea A CBS 393.84	ND	ND	*DQ458895*	*DQ458880*	*DQ458863*
Diplodia pinea A CBS 109727	ND	ND	*DQ458897*	*DQ458882*	*DQ458865*
Diplodia pinea C CBS 109725	ND	ND	*DQ458896*	*DQ458881*	*DQ458864*
Diplodia pinea C CBS 109943	ND	ND	*DQ458898*	*DQ458883*	*DQ458866*
Lasiodiplodia gonubiensis CBS 115812	ND	ND	*DQ458892*	*DQ458877*	*DQ458860*
Lasiodiplodia crassispora CBS 110492	ND	ND	ND	ND	ND
Lasiodiplodia venezuelensis CBS 118739	—	+	—	—	—
Lasiodiplodia rubropurpurea CBS 118740	—	+	—	—	—
"Sphaeropsis" visci CBS 218.25	—	+	—	—	—

Pseudofusicoccum stromaticum CBS 117448	—	+	—	—	—
Pseudofusicoccum stromaticum CBS 117449	—	+	—	—	—
Melanops tulasnei CBS 116805	ND	ND	ND	ND	ND
Melanops tulasnei CBS 116806	ND	ND	ND	ND	ND
"Botryosphaeria quercuum" CBS 118.39	—	+	—	—	—
Guignardia bidwellii CBS 111645	ND	ND	ND	ND	ND
Guignardia bidwellii CBS 237.48	—	+	—	—	—
Guignardia philoprina CBS 447.68	ND	ND	ND	ND	ND
Guignardia colombia CPC 10978	—	+	—	—	—
Guignardia citricarpa CBS 102374	ND	ND	ND	ND	ND
Saccharata proteae CBS 115206	—	+	—	—	—

[1]Acronyms of culture collections: CAA – Artur Alves, Universidade de Aveiro, Portugal; CAP – A.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; CPC – Pedro Crous working collection housed at CBS; JL – J. Luque, IRTA, Barcelona, Spain.
[2]Sequence numbers in italics were retrieved from the GenBank public database. + – sequences kindly provided by Dr. Pedro Crous. ND – sequences obtained in this study and not deposited in GenBank yet.

MATERIALS AND METHODS

Isolates and type material

The fungal strains used in this study are listed in TABLE 1. Cultures were maintained on half-strength Difco potato dextrose agar or oatmeal agar.

Single-conidial or ascospore isolates were prepared according to the methods described by Alves *et al.* (2004). Additional isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA isolation

Fungal cultures were grown in potato dextrose broth for 5 days at approximately 25 ºC. Genomic DNA was extracted from fresh mycelium as published elsewhere (Alves *et al*., 2004).

PCR amplification and sequencing

PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania). The PCR reaction mixtures contained 1 × PCR buffer (PCR buffer without $MgCl_2$:PCR buffer with $(NH_4)_2SO_4$; 1:1 v:v), 5 % DMSO, 3 mM $MgCl_2$, 200 μM of each nucleotide, 15 pmol of each primer, 1 U of *Taq* polymerase, and 50–100 ng of template DNA. Each reaction volume was made up to 50 μl with sterile water. Negative controls with sterile water instead of the template DNA were used in every PCR reaction. All PCR primers were synthesized by MWG Biotech AG (Ebersberg, Germany).

A portion of the nuclear ribosomal SSU gene was amplified with primers NS1 and NS4 (White *et al*., 1990). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 45 s at 94 ºC, 45 s at 48 ºC,

and 1½ min at 72 ºC, and a final extension period of 10 min at 72 ºC. The nucleotide sequence of the SSU region was determined using the above primers along with the internal sequencing primers NS2 and NS3 (White *et al*., 1990).

Part of the nuclear rRNA cluster comprising the ITS region plus the D1/D2 variable domains of the ribosomal LSU gene was amplified using the primers ITS1 (White *et al*., 1990) and NL4 (O'Donnell, 1993) as described by Alves *et al*. (2005). Nucleotide sequences of the ITS and D1/D2 regions were determined as described previously (Alves *et al*., 2004, 2005) using the primers ITS4 (White *et al*., 1990) and NL1 (O'Donnell, 1993) as internal sequencing primers.

The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify and sequence part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene respectively. The amplification conditions for EF1-α and β-tubulin regions were as follows: initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 55 ºC, and 1½ min at 72 ºC, and a final extension period of 10 min at 72 ºC.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. The cycle sequencing procedure has already been described elsewhere (Alves *et al*., 2004). The sequences were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences.

Phylogenetic analysis

The sequences 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 phylogenetic analysis using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses were carried out using PAUP* version 4.0b10 (Swofford, 2003) for Neighbour-joining (NJ) and Maximum-parsimony (MP) analysis, and Mr

Bayes v3.0b4 (Ronquist and Huelsenbeck, 2003) for the Bayesian analysis. Trees were visualised with TreeView (Page, 1996).

For distance analysis (NJ), the datasets were analysed under different nucleotide substitution models including K2P (Kimura, 1980), HKY85 (Hasegawa *et al*., 1985), JC (Jukes and Cantor, 1969), GTR (Rodriguez *et al*., 1990) and also the uncorrected "p" distance. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Maximum-parsimony analysis was performed using the heuristic search option with 1000 simple taxa additions and nearest neighbor interchanges (NNI) 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 by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo (MCMC) method were performed. 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. Four MCMC chains were run simultaneously, starting from random trees, for 10^6 generations. Trees were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated from the remaining 9000 trees. The analysis was repeated three times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis.

The validity of the incongruence length difference (ILD) test (= partition homogeneity test in PAUP*) for determining whether multiple datasets should be combined or not has recently been questioned (Cunningham, 1997; Barker and Lutzoni, 2002). In this study we adopted the method of assessing combinability of datasets by comparing highly supported clades among trees generated from different datasets to detect conflict. High support typically refers to bootstrap support values ≥ 70 % and Bayesian posterior probabilities ≥ 95% (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different datasets, this suggests the genes share similar phylogenetic histories and phylogenetic resolution and support could ultimately be increased by combining the datasets.

TABLE 2. List of Ascomycetes species used in this study.

Classification[1]	Species	Accession numbers	
class			
order		SSU rDNA	LSU rDNA
Saccharomycetes			
Saccharomycetales	*Saccharomyces cerevisiae*	Z75578	AJ508581
	Candida albicans	AB013586	AF156537
Pezizomycetes			
Pezizales	*Peziza vesiculosa*	U53384	AF378367
	Sarcosphaera crassa	AY544712	AY544668
	Morchella esculenta	U42642	AJ698466
	Tuber melanosporum	L37001	AF435821
	Wilcoxina mikolae	U62014	AF127119
Chaetothyriomycetes			
Chaetothyriales	*Capronia semiimersa*	AY554291	AB100616
	Exophiala jeanselmei	X80705	AB100666
	Ceramothyrium carniolicum	AF346418	AY004339
Verrucariales	*Dermatocarpon miniatum*	AF412409	AY584644
Eurotiomycetes			
Eurotiales	*Aspergillus fumigatus*	AB008401	AF433093
	Penicillium expansum	DQ266450	U15483
	Emericella nidulans	U77377	U29859
Arachnomycetales	*Arachnomyces minimus*	AJ315167	AB075350
Onygenales	*Onygena equina*	U45442	AB075356
	Coccidioides immitis	M55627	AB232897
	Aphanoascus mephitalis	AB015779	AB040693+AY176725
Dothideomycetes			
Capnodiales	*Capnodium citri*	AY016340	AY004337
	Raciborskiomyces longisetosum	AY016351	AY016367
Myriangiales	*Myriangium duriaei*	AY016347	AY016365
Dothideales	*Dothidea sambuci*	AY544722	AY544681
	Dothidea ribesia	AY016343	AY016360
	Aureobasidium pullulans	M55639	AF050239
	Delphiniella strobiligena	AY016341	AY016358
	Discosphaerina fagi	AY016342	AY016359
	Stylodothis puccinioides	AY016353	AY004342
	Sydowia polyspora	AY544718	AY544675
	Hortaea werneckii	Y18693	AB079586
	Piedraia hortae	AY016349	AY016366
	Mycosphaerella punctiformis	AY490775	AY490776
	Mycosphaerella lateralis	AY251101	AF309583
Hysteriales	*Hysteropatella clavispora*	AF164359	AY541493
Pleosporales	*Pleospora herbarum*	U05201	AF382386
	Alternaria alternata	U05194	AB100675
	Cochliobolus heterostrophus	AY544727	AY544645
	Setosphaeria monoceras	AY016352	AY016368
	Ampelomyces quisqualis	AY293782	AY293793
	Phaeosphaeria avenaria	AY544725	AY544684
	Didymella cucurbitacearum	AY293779	AY293792
	Westerdykella cylindrica	AY016355	AY004343
	Preussia terricola	AY544726	AY544686
	Letendraea helminthicola	AY016345	AY016362
	Bimuria novae-zelandiae	AY016338	AY016356
	Pleomassaria siparia	AF164373	AY004341
	Byssothecium circinans	AY016339	AY016357
	Lojkania enalia	AF053730	AY016363
Leotiomycetes			
Helotiales	*Ascocoryne cilichnium*	AY789393	AY789394
	Neofabraea malicorticis	AY544706	AY544662
Erysiphales	*Blumeria graminis*	L26253	AB022376
	Golovinomyces orontii	AB033483	AB077697
	Phyllactinia moricola	AB033481	AB022376
Rhytismatales	*Coccomyces dentatus*	AY544701	AY544657
Sordariomycetes			
Sordariales	*Sordaria fimicola*	X69851	AY780079
	Neurospora crassa	AY046271	AF286411+M38154

	Chaetomium globosum	AB048285	AY545729+U47825
	Cercophora septentrionalis	U32400	U47823
Hypocreales	*Hypocrea jecorina*	AF548102	AY281097
	Trichoderma viride	AF525230	AY283548
Xylariales	*Xylaria hypoxylon*	AY544692	AY544648
	Daldinia concentrica	U32402	U47828
Microascales	*Microascus cirrosus*	M89994	AF275539
	Petriella setifera	U43908	AY882377
Halosphaeriales	*Halosphaeria appendiculata*	U46872	U46885
	Aniptodera chesapeakensis	AF050483	U46882
Diaporthales	*Diaporthe phaseolorum*	AY779278	AY346279
	Endothia gyrosa	L42443	AY194114
	Cryphonectria havanensis	L42440	AF408339
Ophiostomatales	*Ophiostoma piceae*	AB007663	AF234837
	Ambrosiella tingens	AF282870	AY282871
	Fragosphaeria purpurea	AF096176	AF096191

[1] classification according to Eriksson (2006).

RESULTS

Phylogeny of Botryosphaeriaceae

Partial nucleotide sequences of the SSU ribosomal DNA (1134 bp), the D1/D2 variable domains of the LSU ribosomal DNA (614 bp), the ITS region (500–600 bp), β-tubulin (approx. 400 bp) and EF1-α genes (approx. 300 bp) were determined for several isolates of *Botryosphaeria* species as well as three *Guignardia* species. The remaining sequences used in the analyses were retrieved from GenBank (TABLES 1 and 2). Sequences of the five genes were aligned and analysed separately by NJ, MP and Bayesian analysis, and the resulting trees were compared. No major conflicts were detected between single gene phylogenies indicating that the datasets could be combined.

The individual as well as combined SSU+LSU sequences of *Botryosphaeria* and *Guignardia* species were aligned with a set of sequences representing several orders in the Dothideomycetes, as well as two Sordariomycetes sequences (TABLE 2) that were selected as outgroup taxa (*Neurospora crassa* Shear & B.O. Dodge and *Xylaria hypoxylon* (L.) Grev.).

LSU rDNA phylogeny

The alignment of the LSU sequences consisted of 93 taxa and contained 660 characters including coded alignment gaps. Indels were coded separately and added to the end of the alignment as characters 632–660.

Of these 660 characters 378 were constant and 64 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 218 parsimony informative characters resulted in 1000 equally parsimonious trees with TL = 937, CI = 0.462, RI = 0.770 and HI = 0.538.

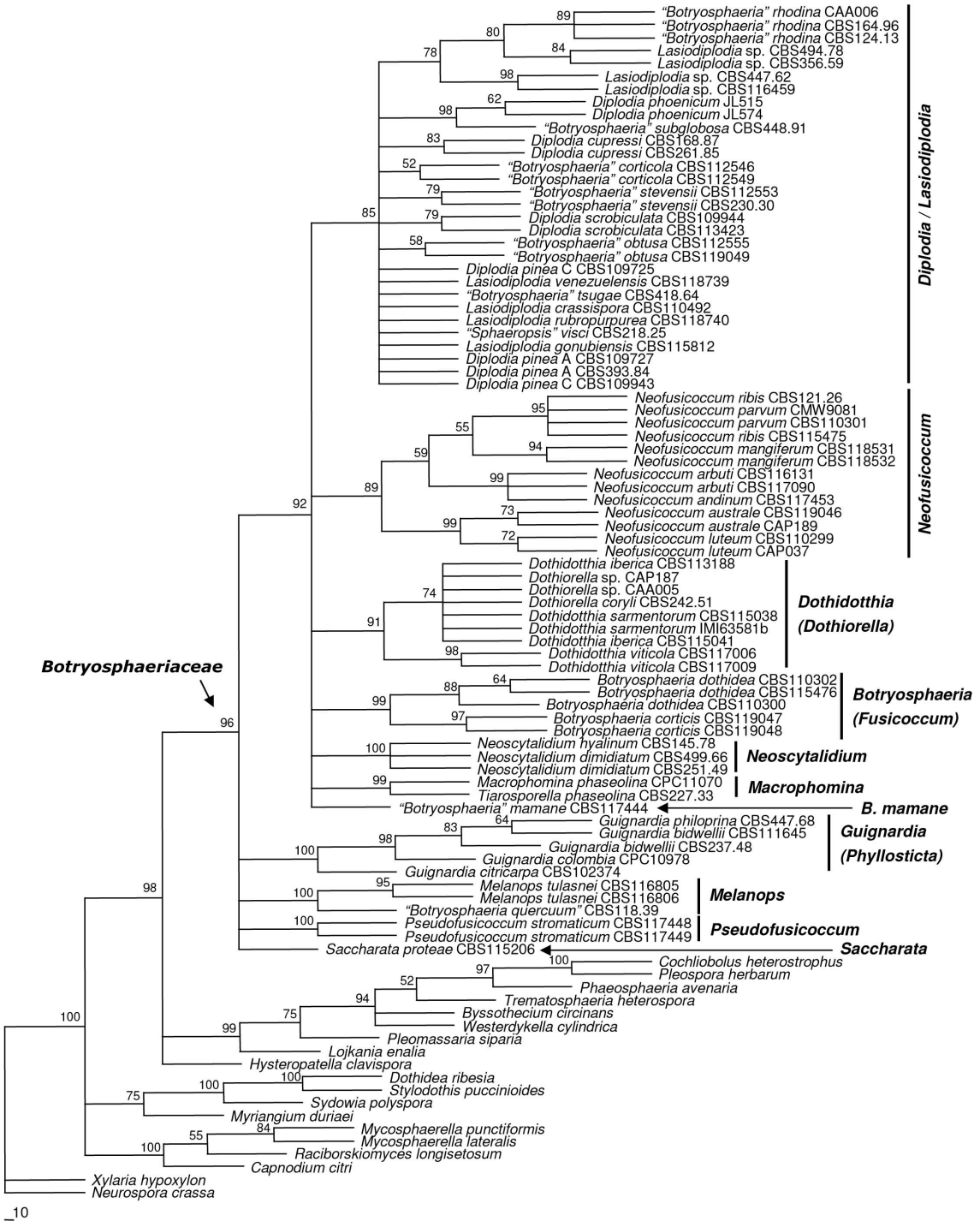


FIGURE 1. Neighbour-joining bootstrap consensus tree obtained from the analysis of LSU rDNA sequence data. Bootstrap support values from 1000 replicates are shown above the branches. The tree was rooted to *N. crassa* and *X. hypoxylon*.

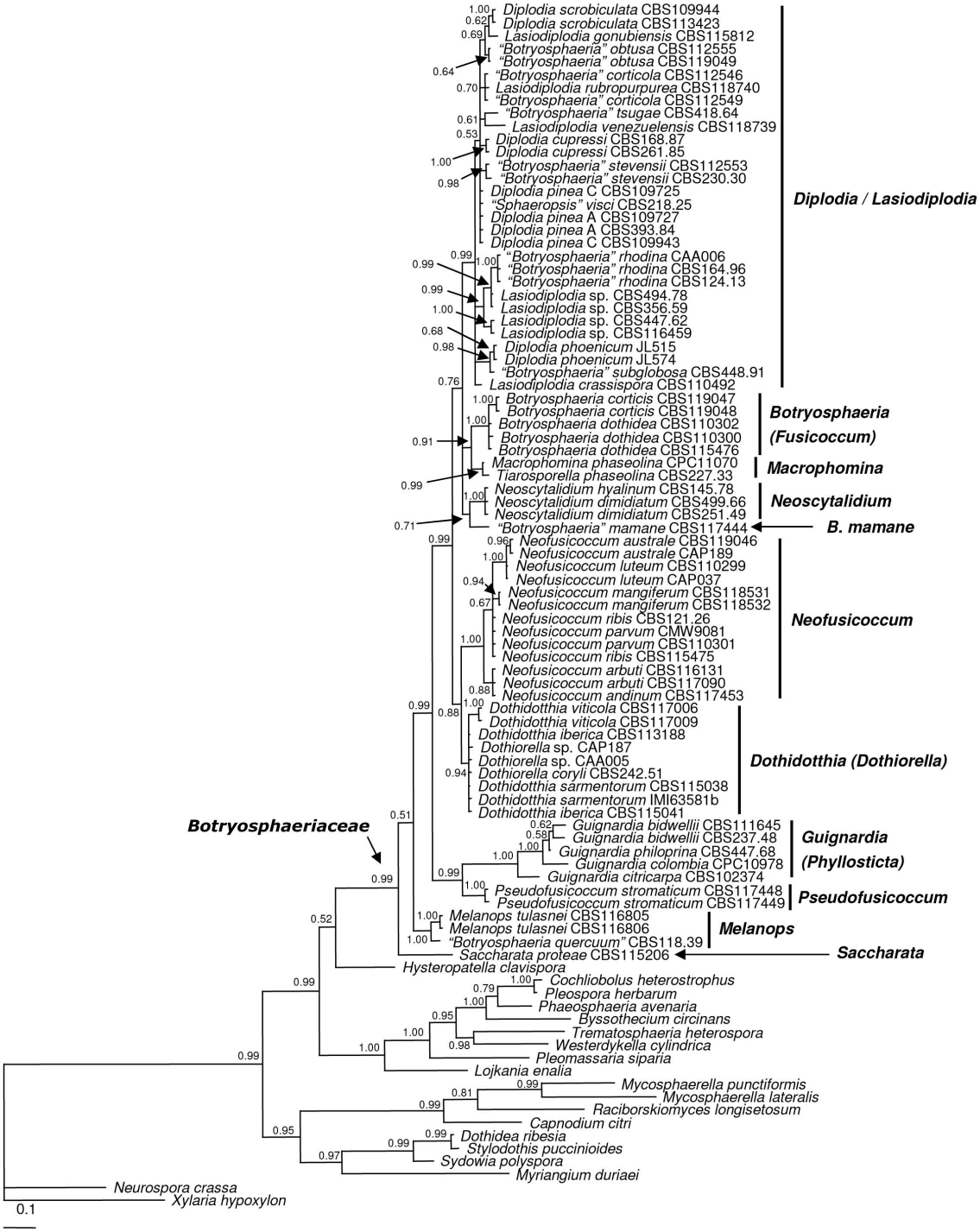


FIGURE 2. Bayesian tree resulting from analysis of LSU rDNA sequence data. The numbers above the branches indicate posterior percentages from Bayesian analysis, consisting of 10^6 Markov Chain Monte Carlo generations (GTR+Γ+G model), with a burn-in of 10^3 generations. The tree was rooted to *N. crassa* and *X. hypoxylon*.

The phylograms generated by the Neighbour-joining analyses were similar under different models (K2P, HKY85, JC, and GTR) and thus only the results for the HKY85 model are presented (FIG. 1). Distance analysis under the HKY85 model resulted in a single tree with TL = 952, CI = 0.455, RI = 0.763 and HI = 0.545. This tree is topologically identical to the trees obtained in the MP analysis, but with slightly higher bootstrap values.

The LSU dataset was subjected to a Bayesian analysis and the 50 % majority-rule consensus tree of 10 000 trees sampled is presented in FIG. 2. The topology of this tree differs from the NJ and MP in the internal branching order of some clades.

All three analyses identified 11 clades within the botryosphaeriaceous fungi that correspond to the same 11 clades identified by Crous *et al*. (2006), which are here identified as follows: Clade 1 ("*Botryosphaeria*" species with *Diplodia* anamorphs), Clade 2 ("*Botryosphaeria*" species with *Neofusicoccum* anamorphs), Clade 3 ("*Botryosphaeria*" species with *Dothiorella* anamorphs), Clade 4 (*Botryosphaeria* species with *Fusicoccum* anamorphs), Clade 5 (*Neoscytalidium* species), Clade 6 (*Macrophomina phaseolina*), Clade 7 ("*Botryosphaeria*" *mamane*), Clade 8 (*Guignardia* species with *Phyllosticta* anamorphs), Clade 9 ("*Botryosphaeria*" *melanops*), Clade 10 (*Pseudofusicoccum* species) and Clade 11 (*Saccharata proteae*). All receive high bootstrap (85–100 %) and posterior probabilities (0.94–1.00) support. The botryosphaericeous clades form a larger monophyletic clade with high bootstrap (96 % in NJ) and posterior probabilities (0.99) support which corresponds to the family *Botryosphaeriaceae*. Of these 11 clades, six (clades 1–5, and 7) represent species that have previously been included in *Botryosphaeria sensu lato*.

SSU+LSU rDNA phylogeny

The combined SSU+LSU alignment consisted of 72 taxa and contained 1719 characters including coded alignment gaps (1049 characters for SSU and 625 characters for LSU). Indels were coded separately and added to the end of the alignment as characters 1675–1719. Of these 1719 characters 1320 were constant while 131 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 358 parsimony informative characters resulted in 36 equally parsimonious trees with TL = 1244, CI = 0.535, RI = 0.781 and HI = 0.465.

The phylograms generated by the Neighbour-joining analyses were similar under different models (K2P, HKY85, JC, and GTR) and thus only the results for the HKY85 model are presented (FIG. 3). Distance analysis under the HKY85 model resulted in a single tree with TL = 1248, CI = 0.533, RI = 0.779 and HI = 0.467. This tree is

topologically identical to the trees obtained in the MP analysis, but with slightly higher bootstrap values.

The 50 % majority-rule consensus tree of 10 000 trees sampled during the Bayesian analysis is presented in FIG. 4. Although the overall topology of this tree is similar to the NJ and MP trees the position of some clades differs.

All three analyses identified seven clades within the botryosphaeriaceous fungi. Of these clades, six correspond to species identified as members of the genus *Botryosphaeria* and are here identified as follows: Clade 1 ("*Botryosphaeria*" species with *Diplodia* anamorphs), Clade 2 (*Botryosphaeria* species with *Fusicoccum* anamorphs), Clade 3 ("*Botryosphaeria*" species with *Dothiorella* anamorphs), Clade 4 ("*Botryosphaeria*" species with *Neofusicoccum* anamorphs), Clade 5 (*Neoscytalidium* species) and Clade 6 ("*Botryosphaeria*" *melanops*). The seventh clade corresponds to the genus *Guignardia*. These clades all received high bootstrap (96 %–100 %) and posterior probabilities (1.00) support. The botryosphaericeous clades form a larger monophyletic clade with high bootstrap (90 % in MP, 98 % in NJ) and posterior probabilities (1.00) support which corresponds to the family *Botryosphaeriaceae*. In NJ and MP analyses the *Guignardia* species form the basal clade within the *Botryosphaeriaceae*, while in the Bayesian phylogeny the basal clade corresponds to *B. melanops*.

Multi-gene phylogeny

For the multi-gene dataset (SSU, LSU, ITS, β-tubulin and EF1-α) analysis of the family *Botryosphaeriaceae*, the three *Guignardia* species were used as outgroup taxa. The alignment of 53 isolates, including the three outgroup species, consisted of 1134 characters for the SSU region, 669 characters for the ITS region, 614 characters for LSU region, 401 characters for the EF1-α region, and 504 characters for the β-tubulin region, including alignment gaps (data not shown). Indels were coded separately and added to the end of the alignment as characters 3327–3577. In the analyses, alignment gaps were treated as missing data.

The combined dataset contained 3577 characters, of which 888 were parsimony informative, 165 were variable and parsimony uninformative and 2524 were constant. Maximum parsimony analysis of the combined dataset resulted in 12 equal, most parsimonious trees (tree length = 2032 steps, CI = 0.656, RI = 0.894, RC = 0.613, HI = 0.315).

The phylograms generated by the Neighbour-joining analyses were similar under different models (K2P, HKY85, JC, and GTR) and thus only the results for the HKY85 model are presented (FIG. 5). Distance analysis under the HKY85 model resulted in a

single tree with TL = 2048, CI = 0.680, RI = 0.891, RC = 0.606 and HI = 0.320. This tree is topologically identical to the trees obtained in the MP analysis, but with slightly higher bootstrap values. This tree is identical to the trees obtained in the MP analysis, but with slightly higher bootstrap values.

The 50 % majority-rule consensus tree of 10 000 trees sampled during the Bayesian analysis is presented in FIG. 6. Although the overall topology of this tree is similar to the NJ and MP trees the position of some clades differs.

In these analyses six clades were identified within the the family *Botryosphaeriaceae*. Of these clades, five correspond to species identified as members of the genus *Botryosphaeria* and are here identified as follows: Clade 1 ("*Botryosphaeria*" species with *Diplodia*/*Lasiodiplodia* anamorphs), Clade 2 (*Botryosphaeria* species with *Fusicoccum* anamorphs), Clade 3 ("*Botryosphaeria*" species with *Dothiorella* anamorphs), Clade 4 ("*Botryosphaeria*" species with *Neofusicoccum* anamorphs), and Clade 5 ("*Botryosphaeria*" *melanops*). The sixth clade corresponds to the genus *Guignardia*. These clades all received high bootstrap (99 %–100 %) and posterior probabilities (1.00) support. Clade 1 is further subdivided into two clades, one corresponding to the anamorphic genus *Diplodia* (92 % bootstrap support; 1.00 posterior probabilities) and the second corresponding to the anamorphic genus *Lasiodiplodia* (100 % bootstrap support; 1.00 posterior probabilities). Within each of the above clades species are readily identified and all receive high bootstrap and posterior probabilities support.

SSU+LSU rDNA phylogeny of Ascomycetes

In order to evaluate the phylogenetic affinities of the family *Botryosphaeriaceae* to other Ascomycetes, a smaller number of sequences representing the overall diversity of the family were selected and aligned with a set of ascomycete sequences from a large number of families and orders. For these analyses sequences of *S. cerevisiae* Meyen ex E.C. Hansen and *C. albicans* (C.P. Robin) Berkhout (Saccharomycetes, *Saccharomycetales*) were selected as outgroups for rooting the trees. The alignment consisted of 83 taxa and contained 1748 characters including coded alignment gaps (974 characters for SSU and 605 characters for LSU). Indels were coded separately and added to the end of the alignment as characters 1584–1748. Of these 1748 characters 856 were constant and 231 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 661 parsimony informative characters resulted in 8 equally parsimonious trees with TL = 3924, CI = 0.305, RI = 0.692 and HI = 0.695.

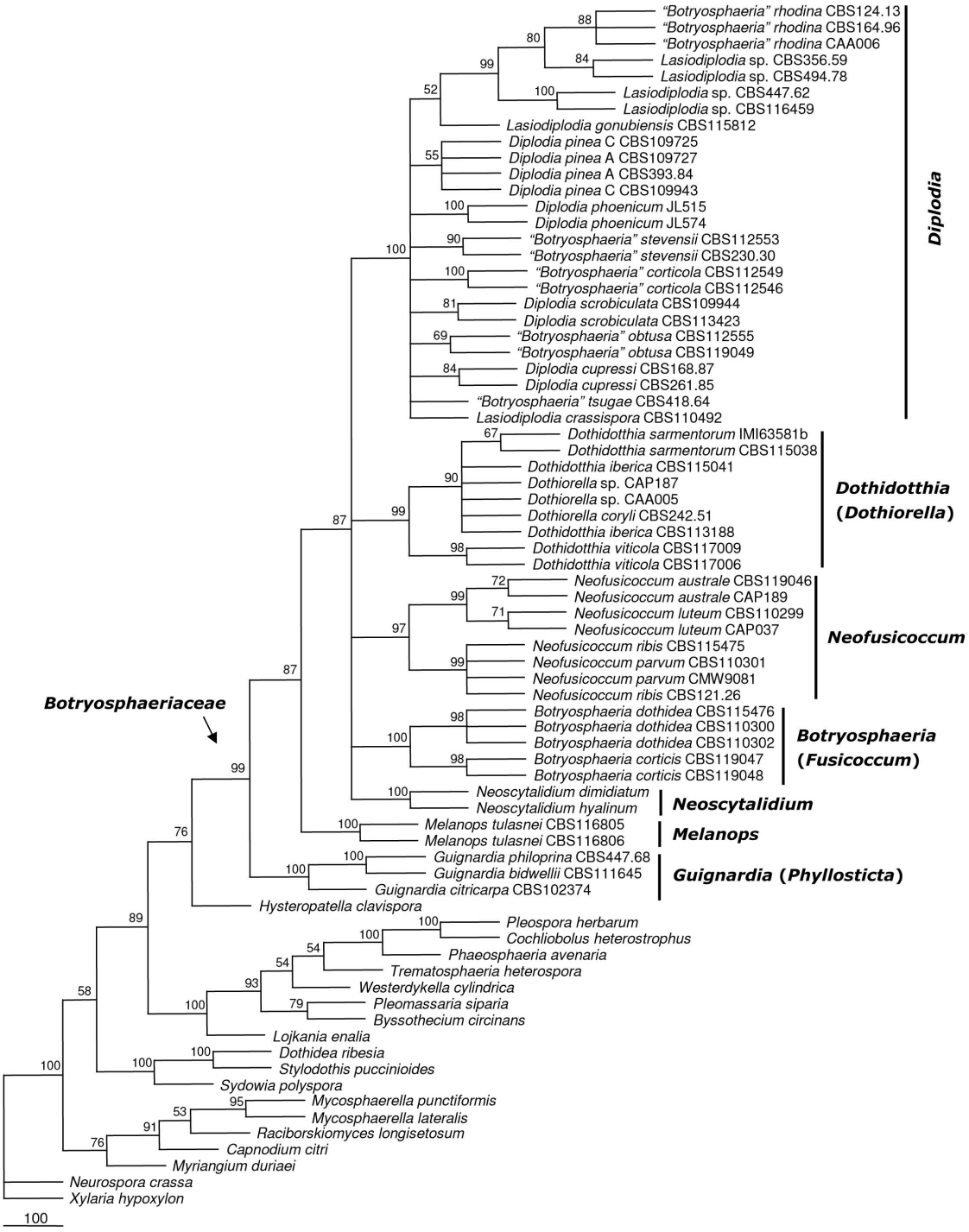


FIGURE 3. Neighbour-joining bootstrap consensus tree obtained from the analysis of combined SSU rDNA and LSU rDNA sequence data. Bootstrap support values from 1000 replicates are shown above the branches. The tree was rooted to *N. crassa* and *X. hypoxylon*.

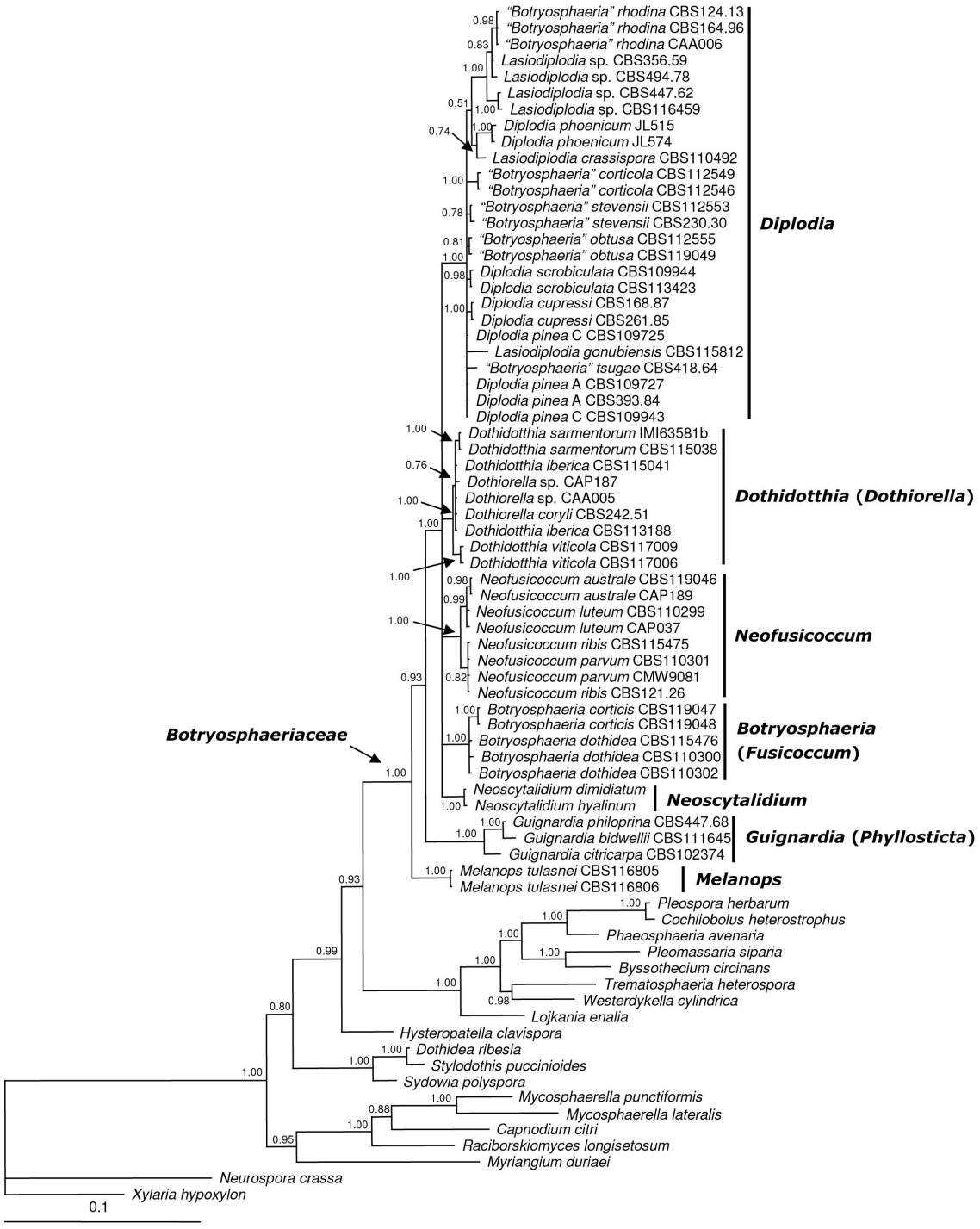


FIGURE 4. Bayesian tree resulting from analysis of combined SSU rDNA and LSU rDNA sequence data. The numbers above the branches indicate posterior percentages from Bayesian analysis, consisting of 10^6 Markov Chain Monte Carlo generations (GTR+Γ+G model), with a burn-in of 10^3 generations. The tree was rooted to *N. crassa* and *X. hypoxylon*.

The Neighbour-joining analysis generated similar phylograms under different models (K2P, HKY85, JC, and GTR). The results obtained for the HKY85 model are presented (FIG. 7). Distance analysis under the HKY85 model resulted in a single tree with TL = 3946, CI = 0.347, RI = 0.690 and HI = 0.653.

The 50 % majority-rule consensus tree of 10 000 trees sampled during the Bayesian analysis is presented in FIG. 8. The topology of the trees generated by NJ, MP and Bayesian analysis is identical in terms of the terminal clades identified. However, the trees differed considerably regarding the internal branching order.

The NJ, MP and Bayesian analyses of this dataset identified a large number of clades that correspond to known ascomycete orders. With few exceptions, these clades (orders) receive high bootstrap as well as posterior probabilities support. The members of the family *Botryosphaeriaceae* group in a highly supported monophyletic clade (90 % bootstrap support in MP and 97 % in NJ; 1.00 posterior probabilities in Bayesian analysis).

This family however received no support for its placement in any of the orders analysed including the *Pleosporales* and *Dothideales*, although it is clearly more closely related to the *Pleosporales* than to the *Dothideales*. Curiously, in both NJ and Bayesian phylogenies the closest relative to the family is a member of the *Hysteriales*. This close relationship received high statistical support in NJ analysis (74 % bootstrap support) as well as in the Bayesian analysis (1.00 posterior probabilities). In MP analysis the family forms a clade with members of the Pleosporales, but with low bootstrap support value (57 %).

DISCUSSION

Systematics of the genus Botryosphaeria

The phylogenetic analyses presented in this work show that the genus *Botryosphaeria* as currently circumscribed is paraphyletic. According to the phylogenetic analyses several monophyletic clades are recognized within species currently identified as members of the genus *Botryosphaeria*. These clades all correlate well with morphological features. With few exceptions, sexual states (teleomorphs) are extremely conserved in morphological terms. Thus the characters most phylogenetically informative and useful for delimiting genera in the *Botryosphaeriaceae* are based largely on the morphology of the anamorphs. The correct taxonomic placement of the members of each of these clades is discussed in detail below.

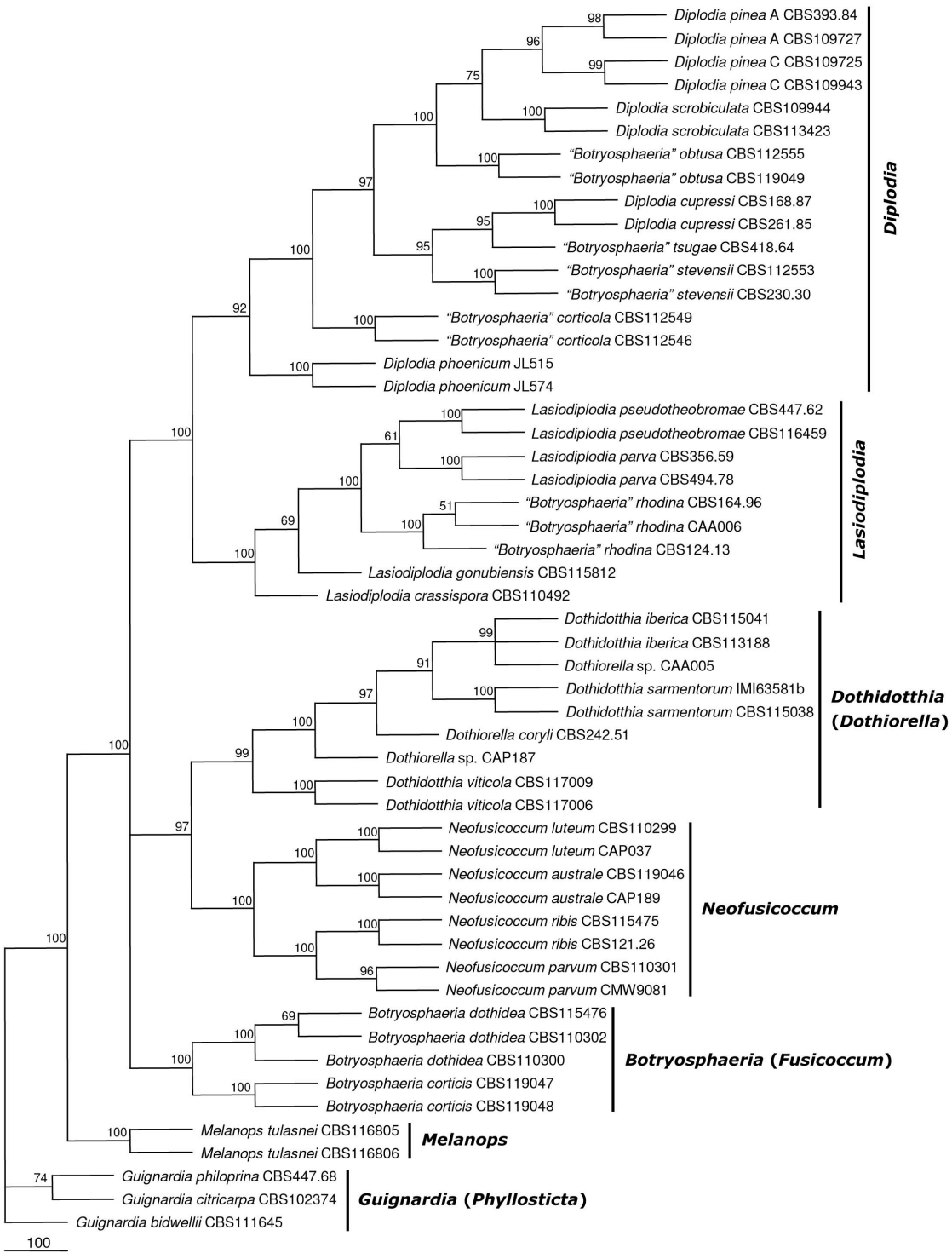


FIGURE 5. Neighbour-joining bootstrap consensus tree obtained from the analysis of combined SSU, LSU, ITS, EF1-α and β-tubulin sequences of some *Botryosphaeriaceae* species. Bootstrap support values from 1000 replicates are shown above the branches. The tree was rooted to *G. philoprina, G. bidwellii and G. citricarpa.*

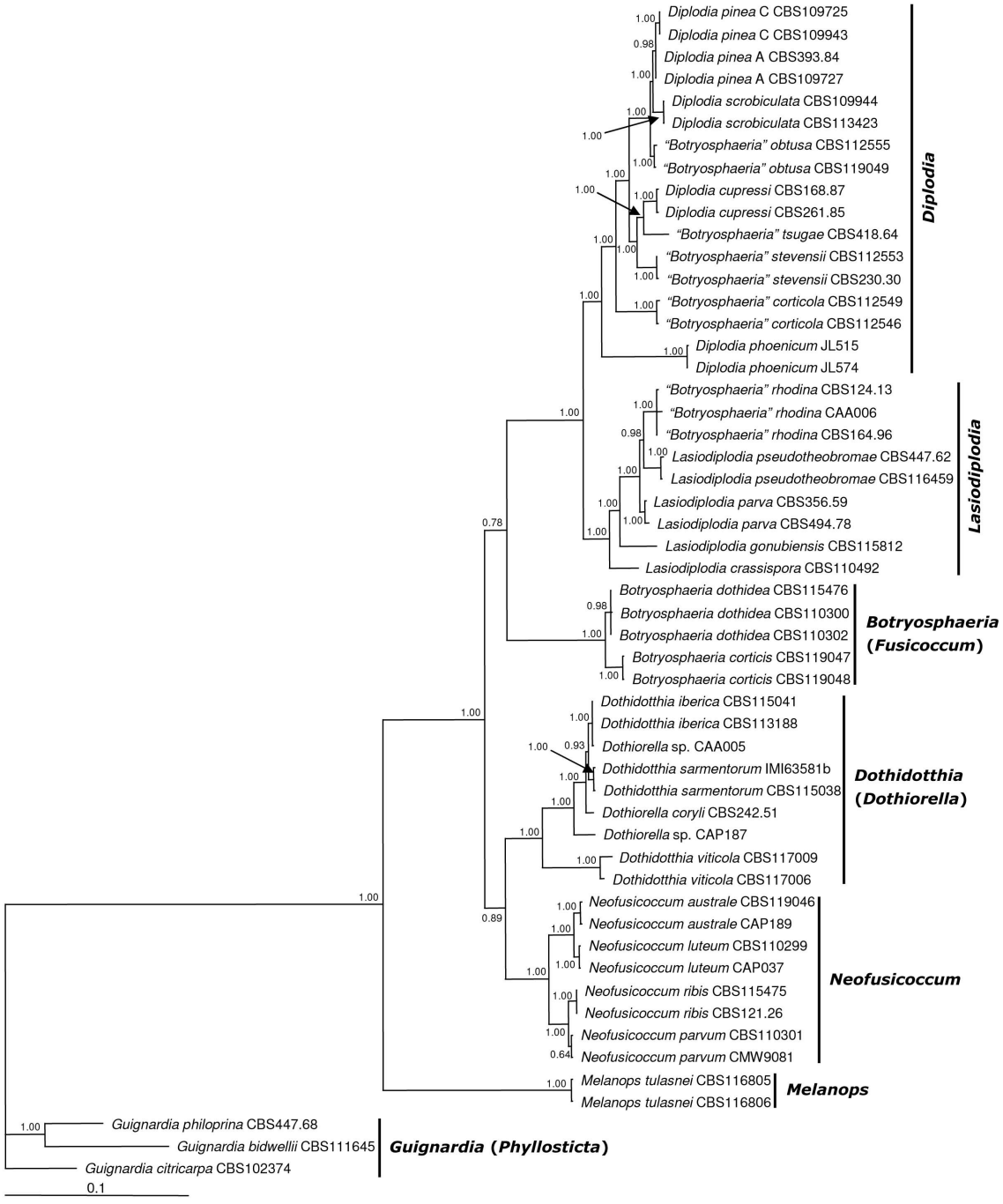


FIGURE 6. Bayesian tree resulting from analysis of combined SSU, LSU, ITS, EF1-α and β-tubulin sequences of some *Botryosphaeriaceae* species. The numbers above the branches indicate posterior percentages from Bayesian analysis, consisting of 10^6 Markov Chain Monte Carlo generations (GTR+Γ+G model), with a burn-in of 10^3 generations. The tree was rooted to *G. philoprina, G. bidwellii and G. citricarpa.*

***Botryosphaeria* clade**

This clade represents the genus *Botryosphaeria* as described by Cesati and De Notaris (1863) and is composed of two species only, namely *B. dothidea* the type species of the genus and the closely related species *B. corticis*. These species are characterised by thin-walled, hyaline and aseptate ascospores.

The anamorphs of *Botryosphaeria* species belong in *Fusicoccum* as typified by *F. aesculi* (Slippers *et al*., 2004a). Conidia are fusiform, hyaline, unicellular, rarely forming a septum before germination, but sometimes become dark walled and septate with age (Slippers *et al*., 2004a; Phillips *et al*., 2005b). Phillips *et al*. (2005b) reported that conidia of *Fusicoccum aesculi* can become pigmented, ovoid, ellipsoid or fusiform, 1–2-septate, similar to those observed by Barber *et al*. (2005) for *F. aesculi*.

Phylogenetic analysis of the LSU gene (Crous *et al*., 2006) supported *B. dothidea* and its anamorph *F. aesculi* as a distinct phylogenetic lineage within the *Botryosphaeriaceae* separate from a large group of "*Botryosphaeria*" species with *Fusicoccum*-like anamorphs, such as *B. ribis* and *B. lutea* among others. This subdivision of the *Fusicoccum* species into two groups had been noted in previous studies involving phylogenetic analysis of the ITS and EF1-α gene regions (Zhou and Stanosz, 2001b; Zhou *et al*., 2001; Slippers *et al*., 2004a, b; Farr *et al*., 2005; Barber *et al*., 2005; Chapters 2 to 6) as well as the mitochondrial SSU rDNA gene (Zhou and Stanosz, 2001a). In the present work, LSU and combined SSU+LSU phylogenies also supported this split into two groups with the inclusion of *B. corticis* in the clade formed by *B. dothidea* and its anamorph.

***Neofusicoccum* clade**

As stated previously phylogenetic analysis of the ITS, EF1-α, and mt SSU rDNA gene regions showed that *Fusicoccum* species formed two distinct clades. Analysis of the LSU region (Crous *et al*., 2006; this work), combined SSU+LSU regions and multi-gene phylogenies (this work) support the split of these species into two distinct genera.

The teleomorphs in this group are virtually indistinguishable from *Botryosphaeria sensu stricto*. Thus, the features that distinguish this group from the previous are based on morphological characters of the anamorphs.

The genus *Neofusicoccum* was introduced by Crous *et al*. (2006) to accommodate the *Fusicoccum*-like anamorphs of this large group of *Botryosphaeria*-like species. Although the anamorph resembles *Fusicoccum* there are some morphological differences between both genera. *Fusicoccum* has conidia that are more fusoid than the conidia of *Neofusicoccum* which tend to be more ellipsoid. The most characteristic

features that distinguish between these genera are found in their synanamorphs. The *Dichomera*-like synanamorphs in this clade are characterised by having globose to pyriform conidia, while the older, brown conidia in *Fusicoccum* are obovoid, ellipsoid or fusiform, never globose or subglobose (Phillips *et al*. 2005b; Barber *et al*. 2005; Crous *et al*., 2006).

Although the teleomorphic state is known and well characterized for some of the species, no name was applied to it. In agreement with the vision of a single name for species of fungi (Rossman and Samuels, 2005), Crous *et al*. (2006) decided to introduce a single generic name, namely for the anamorph (which occurs with a *Dichomera*-like synanamorph), which is the more informative morphological state

***Diplodia*/*Lasiodiplodia* clade**

This is a very large and extremely diverse group whose taxonomy is particularly complicated by several issues regarding both the anamorph and the teleomorph states. The anamorphs were in the past included in several different genera namely *Macrophoma*, *Sphaeropsis*, *Lasiodiplodia* and *Diplodia* (Denman *et al*., 2000).

Sutton (1980) reduced *Macrophoma* (Sacc.) Berl. & Voglino to synonymy under *Sphaeropsis*. Denman *et al*. (2000) regarded *Sphaeropsis* and *Lasiodiplodia* as synonyms of *Diplodia*. These three genera were distinguished essentially on the basis of conidial characters (coloration, septation and wall ornamentation) that develop with age (Sutton, 1980; Denman *et al*., 2000). Percurrent proliferation seen in conidiogenous cells was considered a feature that separated *Sphaeropsis* from *Diplodia* (Sutton, 1980). However, *Diplodia* species also produce percurrent proliferation in conidiogenous cells (Denman *et al*., 2000; Alves *et al.*, 2004). *Lasiodiplodia* was separated by the presence of paraphyses in conidiomata and conidial striations (Denman *et al*., 2000).

The synonymy of *Sphaeropsis* under *Diplodia* has been accepted by most authors (e.g. de Wet *et al*., 2003; Burgess *et al*., 2004a, b; Pavlic *et al*., 2004) and is supported by phylogenetic analyses of multiple gene regions like ITS, EF1-α and β-tubulin (e.g. Zhou and Stanosz, 2001b; Slippers *et al*., 2004a; Pavlic *et al*., 2004; Chapters 2,3,7,9). Nevertheless, Xiao and Rogers (2004) recently described a new species in *Sphaeropsis* (*S. pyriputrescens* Xiao & J.D. Rogers) thus preferring to keep *Sphaeropsis* as a separate genus. The genus *Lasiodiplodia* is still retained by some authors due to morphological (presence of paraphyses and conidial striations) and phylogenetic distinctions (Pavlic *et al*., 2004, Burgess *et al*., 2006; see Chapter 9).

The phylogenetic analysis of LSU and combined SSU+LSU sequence data were unable to discriminate between the anamorph genera and showed that species in

Sphaeropsis, *Lasiodiplodia* and *Diplodia* form a monophyletic and well supported clade. This clade had been previously identified by Crous *et al*. (2006) but it was not fully resolved and received no bootstrap support in the LSU phylogeny. Phylogenetic analyses of combined multi-gene sequence data (SSU, LSU, ITS, EF-1α and β-tubulin) further support the synonymy of *Sphaeropsis* under *Diplodia.* In contrast, these multi-gene phylogenies do not support the synonymy between *Lasiodiplodia* and *Diplodia* (Chapter 9; this work).

Conidial striations were shown to be unsuitable for discrimination at the genus level and the only feature that separates both genera is the presence of paraphyses (Chapter 9). Thus, the genera *Lasiodiplodia* and *Diplodia* although closely related should be maintained as separate in view of the current morphological and molecular data.

The sexual state (teleomorph) is known for several species, but the correct name to be used for the teleomorph still needs to be resolved. Hyaline and aseptate ascospores seem to be frequent, however in some species like *B. stevensii, B. quercuum* and *B. corticola* ascospores can become dark and septate with age (Shoemaker, 1964; Sivanesan, 1984; Alves *et al*., 2004). Also, in *B. tsugae* ascospores initially hyaline are reported to become pale brown at maturity (Funk, 1964; Sivanesan, 1984). *Botryosphaeria rhodina* (Cooke) von Arx which has hyaline asepate ascospores is generally regarded as the teleomorph of *L. theobromae* (von Arx, 1981). However, the connection between both states has not been absolutely proved (Sivanesan, 1984).

Botryosphaeria subglobosa (C. Booth) von Arx & E. Müller whose anamorph is described as *Sphaeropis subglobosa* Cooke has ascospores that are hyaline and aseptate for a long time becoming brown and one septate after discharge (Punithalingam, 1969; von Arx and Müller, 1975; Sivanesan, 1984). This species had previously been described under the genus *Neodeightonia* (Punithalingam, 1969). There is only one isolate of this species available and this isolate is not ex-type and has not been associated with authentic material. However, LSU rDNA phylogenies (Crous *et al*., 2006; this work) place this isolate within the *Diplodia* clade and show that it is closely related to *D. phoenicum*.

Another interesting species although poorly known are *B. laricis* (Whem.) von Arx & E. Müller and *Botryosphaeria visci* (Kalchbr.) von Arx & E. Müller. *Botryosphaeria laricis* apparently has brown ascospores with hyaline, generally evanescent apical appendages (von Arx and Müller, 1954; Sivanesan, 1984). The anamorph of this species is described as a form of *Macrophoma sapinea* (Smerlis, 1970) and is most likely a *Diplodia* sp.. *Botryosphaeria visci* (anamorph *Spaheropsis visci* (Fr.) Sacc.) has ascospores which are hyaline at first becoming dark violet brown (von Arx and Müller,

1954; Sivanesan, 1984). However, in the absence of authentic cultures for these species it is impossible to establish their correct taxonomic placement.

Denman *et al.* (2000) listed several synonyms of *Botryosphaeria*, many of which have *Diplodia* or *Diplodia*-like anamorphs, and could thus potentially be available for this clade. As stated by Crous *et al*. (2006), this issue can only be resolved once the type specimen has been re-examined, an epitype recollected, and an ex-epitype sequence generated.

***Pseudofusicoccum* clade**

Mohali *et al*. (2006) described a new *Fusicoccum* species occurring on *Eucalyptus* and *Acacia* spp. in Venezuela, *Fusicoccum stromaticum* Mohali, Slippers & M. J. Wingf.. The species was distinguished from other species of *Fusicoccum* based on its unusually large conidiomata, ability to grow at 35 ºC, and thick-walled conidia (Mohali *et al*., 2006).

Recently, Crous *et al*. (2006) re-examined strains of this species and found that conidia were encased in a persistent mucous sheath, a feature which is absent in other species of *Fusicoccum*. On the basis of this morphological character and LSU sequence data, Crous *et al*. (2006) proposed the new genus *Pseudofusicoccum* Mohali, Slippers & M.J. Wingf. to accomodate *Pseudofusicoccum stromaticum* Mohali, Slippers & M.J. Wingf..

***Neoscytalidium* clade**

The taxonomic history of *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko was explained in detail by Crous *et al*. (2006). Recently, this species had been transferred to *Fusicoccum* as *F. dimidiatum* (Penz.) D.F. Farr (Farr *et al*., 2005). Crous *et al*. (2006) on the basis of LSU sequence data showed that this fungus is a genus in its own right within the *Botryosphaeriaceae* and that the ex-type strain of *Scytalidium*, *S. lignicola* Pesante, (CBS 233.57) is not a member of the *Botryosphaeriaceae*. So, Crous *et al*. (2006) introduced the new genus *Neoscytalidium* Crous & Slippers to accommodate this hyphomycete fungus, as *Neoscytalidium dimidiatum* (Nattrass) Crous & Slippers. *Hendersonula toruloidea* Natrass is recognized as the coelomycete synanamorph of this species (Sutton and Dyko, 1989; Crous *et al*., 2006).

This species is characterized by conidia occurring in arthric chains in aerial mycelium, powdery to the touch, disarticulating, cylindrical-truncate, oblong-obtuse to doliiform, dark brown, thick-walled, 0–2-septate (Crous *et al*., 2006).

Slippers *et al*. (2005) made the new combination *Fusicoccum mangiferum* (Syd. & P. Syd.) Johnson, Slippers & M.J. Wingf. (= *N. mangiferae* = *H. toruloidea*). The

synonymy of *H. toruloidea* (which has a *Neoscytalidium* synanamorph) with *F. mangiferum* (which does not have a *Neoscytalidium* synanamorph) was rejected by Crous *et al*. (2006) who showed that *F. mangiferum* is a member of the genus *Neofusicoccum*.

In this work we present phylogenetic analyses of the LSU and combined SSU+LSU genes that support the view of Crous *et al*. (2006). In both analyses, *Neoscytalidium* forms a well supported clade within the *Botryosphaeriaceae*. The species *Scytalidium hyalinum* C.K. Campb. & J.L. Mulder is also shown to be a member of the genus *Neoscytalidium*. Although there has been considerable debate in the literature as to whether *S. dimidiatum* and *S. hyalinum* are the same or distinct species, some morphological and genetic characteristics (such as mycelium pigmentation, intronic insertions in the 18S gene, and the A-G polymorphism), apparently do differentiate these species (Machouart-Dubach *et al*., 2001; Machouart *et al*., 2004). In contrast, Roeijmans *et al*. (1997) considered *S. hyalinum* to be a synonym of *S. dimidiatum* after conducting molecular taxonomy studies using ARDRA. Indeed, most authors agree that *S. hyalinum* may be a melanin-deficient cultural mutant of *S. dimidiatum* (Sutton and Dyko, 1989; Roeijmans *et al*., 1997).

***Dothidotthia* clade**

Barr (1987, 1989) introduced the genus *Dothidotthia* Höhn. for a group of *Botryosphaeria*-like species whose ascospores are one to three septate and various shades of brown.

Phillips *et al.* (2005a) and Luque *et al.* (2005) broadened the concept of *Botryosphaeria* to include species with brown, one-septate ascospores, namely *B. sarmentorum*, *B. iberica*, and *B. viticola*. In so doing they included species that would otherwise be placed in *Dothidotthia*. Their reasons for doing this were based on the fact that ITS and EF1-α phylogenies placed such species within the boundaries of *Botryosphaeria*. This raised the possibility that *Dothidotthia* could be a later synonym of *Botryosphaeria*, or alternatively that *Botryosphaeria* was paraphyletic.

The phylogenies based on partial sequences of the LSU gene (Crous *et al.*, 2006) revealed that *Botryosphaeria* sensu lato is composed of several lineages, and this is supported by the phylogenies generated in this work. The species described by Phillips *et al.* (2005a) and Luque *et al.* (2005) conform to the morphological description of the genus *Dothidotthia* as typified by *D. aspera* (Ellis & Everh.) Barr (Barr, 1989). Thus, it is likely that these with dark, one-septate ascospores will have to be transferred to the genus *Dothidotthia*. However, any final decision should be based on studies of type material to clarify the status of *Dothidotthia*.

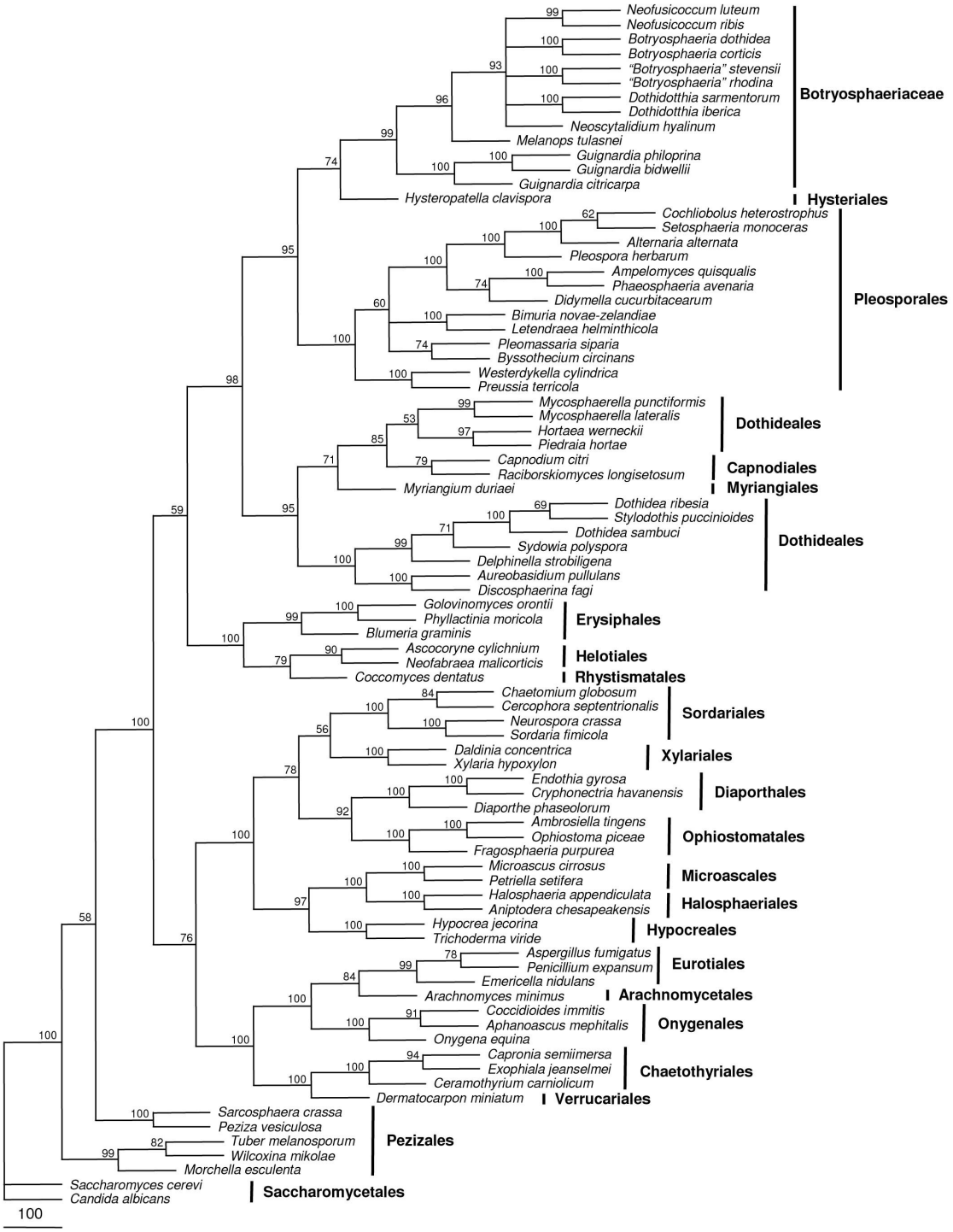


FIGURE 7. Neighbour-joining bootstrap consensus tree obtained from the analysis of combined SSU rDNA and LSU rDNA sequences of Ascomycetes. Bootstrap support values from 1000 replicates are shown above the branches. The tree was rooted to *S. cerevisiae* and *C. albicans*.

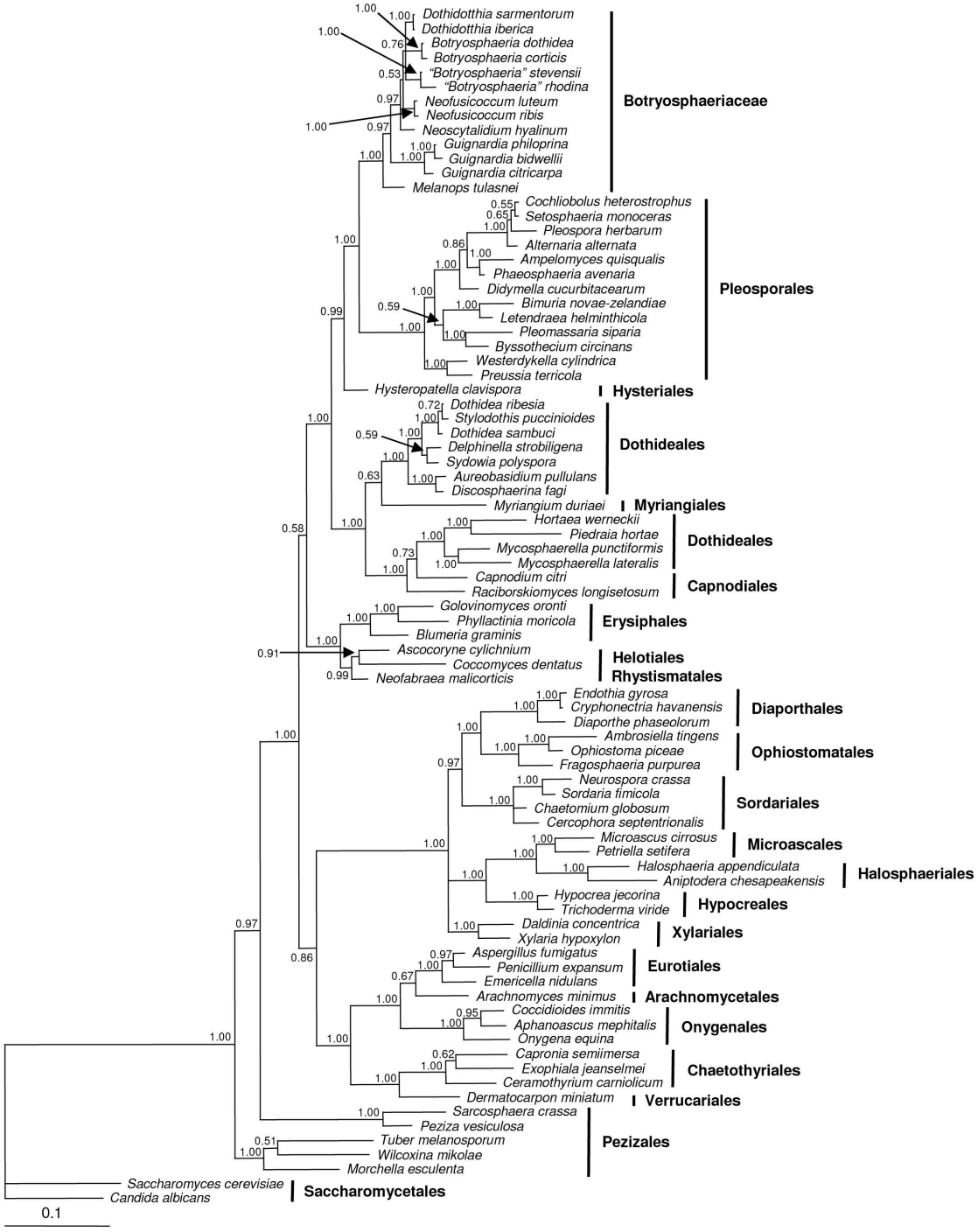


FIGURE 8. Bayesian tree resulting from analysis of combined SSU rDNA and LSU rDNA sequences of Ascomycetes. The numbers above the branches indicate posterior percentages from Bayesian analysis, consisting of 10^6 Markov Chain Monte Carlo generations (GTR+Γ+G model), with a burn-in of 10^3 generations. The tree was rooted to *S. cerevisiae* and *C. albicans*.

Phillips *et al.* (2005a) showed that the anamorph of these species reside in the genus *Dothiorella* Sacc. This genus was reduced to synonymy under *Diplodia* by Crous and Palm (1999), who due to the lack of molecular data and cultures used a wider morphological concept for the genus *Diplodia*. However, as shown by Phillips *et al.* (2005a) in *Dothiorella* conidia become brown and one-septate at an early stage in their development, often before they are released from the conidiogenous cell, while in *Diplodia* conidia are hyaline and aseptate but sometimes they become brown and one-septate after a period of aging. The *Diplodia*-like anamorphs with brown septate conidia reported for *Dothidotthia* species (Barr, 1987, 1989) are thus better accommodated in *Dothiorella*.

In contrast to other genera in *Botryosphaeriaceae*, morphological differences between *Dothidotthia* species are more apparent in the teleomorphs while the anamorphs are morphologically similar and dimensions of the conidia overlap considerably (Phillips *et al.*, 2005a; Luque *et al.*, 2005).

The Index of Fungi lists ten species in *Dothidotthia* and Barr (1989) gives detailed descriptions of six species. However, no cultures of any of these species are available and thus its correct taxonomic position cannot be established.

***Melanops* clade**

The taxonomy of *Botryosphaeria melanops* and its anamorph, *Fusicoccum advenum* (Sacc.) Died., was reviewed recently by Phillips and Pennycook (2005) and because the type specimen could not be located, a neotype was designated. However, in the absence of cultures, the phylogenetic relationship of this species to other species of *Botryosphaeria* could not be established. In this work we included isolates of a *Botryosphaeria* species with characteristics of *B. melanops*, isolated from dead branches of *Quercus robur* collected in Munich, Germany and this appears to be suitable as an epitype (see below).

Ascomata of the collection studied here were indistinguishable from those of *Dothidea melanops* Tul. as described by Tulasne and Tulasne (1863) and the neotype and other specimens examined by Phillips and Pennycook (2005). Asci were also similar. Ascospores were rhomboid, widest in the middle and tapered to the ends, and measured (32.0–)39.2–45.0(–53.5) × (14.0–)15.4–16.7(–19.1) µm (mean ± S.D. = 42.1 ± 5.5 × 16.1 ± 1.2 µm).

Irrespective of whether cultures were derived from single ascospores or single conidia, the resulting colonies were the same. Colonies developed slowly (< 1 mm per day at 25 ºC) on PDA. Colony margins were wavy and the mycelium, initially hyaline, became darker with age. Cultures rarely formed pycnidia on PDA, even after

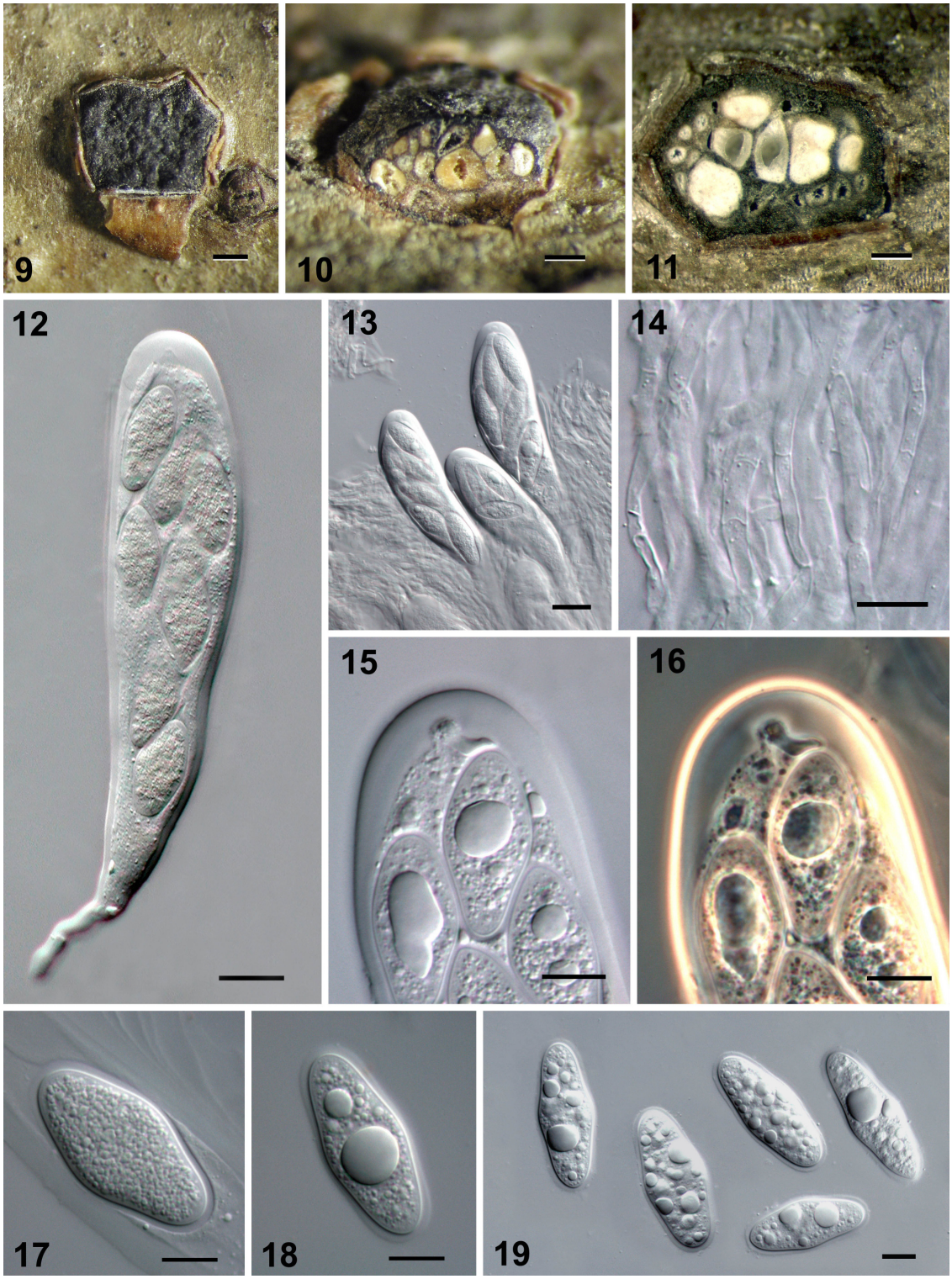
incubation at 25 ºC for more than three months. On WA or PDA, to which pieces of autoclaved *Q. ilex* twigs were added, pycnidia started to form on the twigs after 2 weeks. They gradually became dark brown to black and after 4–6 weeks of incubation at 20–25 ºC, cirrhi of hyaline conidia exuded from the ostioles, or sometimes the conidia oozed out and collected as wet droplets.

In culture, the conidiomata were superficial, multiloculate and up to 2 mm diameter. Conidiophores arose from the inner wall of the locules and were branched at the base. Conidiogenous cells were cylindrical, holoblastic, 2.5–4.0 µm diameter and slightly wider at the base, up to 40 µm long, producing a single conidium at the tip, proliferating percurrently to form 2–3 indistinct annelations, rarely proliferating at the same level to form minute periclinal thickenings. Paraphyses, 2–3 µm wide, were abundant and formed between the conidiogenous cells. They were cylindrical, filiform, sparingly branched at the base, and aseptate. Conidia produced in culture were hyaline, aseptate, thin-walled, fusiform, widest in the middle, smooth with granular contents, (44.2–)51.0–53.0(–58.0) × (8.7–)10.1–10.5(–12.4) µm (mean ± S.D. of 50 conidia = 52.0 ± 3.6 × 10.3 ± 0.9 µm; L/W ratio = 5.1). These characters of the anamorph conform to those of *Fusicoccum advenum* (Shear and Davidson, 1936; Phillips and Pennycook, 2005).

In phylogenetic analyses the cultures identified as *B. melanops* formed a clade at the base of the *Botryosphaeriaceae* family. This corresponds to the clade identified by Crous *et al*. (2006) as "*Botryosphaeria quercuum*" (anamorph *Diplodia*-like). In fact, in the LSU tree the sequence from the culture "*Botryosphaeria quercuum*" CBS 118.39 used by Crous *et al*. (2006) forms a highly supported clade with the two cultures identified as *B. melanops*.

Von Arx and Müller (1954) regarded *B. melanops* as a synonym of *B. quercuum* (see Phillips and Pennycook, 2005). However, their synonymy was based on a study of herbarium specimens of the teleomorph and no anamorph characters were taken into account. The anamorph of *B. melanops* is a *Fusicoccum*-like species, albeit with very large conidia (Shoemaker, 1964; Phillips and Pennycook, 2005). Although *B. quercuum* has not been studied in detail, its anamorph is a *Diplodia* species (Shoemaker, 1964). Thus, it can be concluded that *B. melanops* is not a synonym of *B. quercuum*.

Taking into account the phylogenetic data presented here the cultures identified as *B. melanops* cannot be considered as members of the genus *Botryosphaeria*. Also, its anamorph although morphologically resembling a *Fusicoccum* species is clearly not a member of this anamorphic genus.



Figures. 9–19. *Melanops tulasnei* 9. Ascoma erumpent through host bark. 10. Vertical section through an ascoma showing several locules at different levels. 11. Horizontal section through an ascoma showing several locules. 12. Ascus with ascospores. 13. Asci with ascospores amongst pseudoparaphyses. 14. Pseudoparaphyses. 15, 16. Ascus tip with apical chamber. 17–19. Ascospores. Bars: 9–11 = 250 µm, 12, 13 = 20 µm, 14–19 = 10µm.

It is thus proposed that the genus *Melanops* Nitschke ex Fuckel should be reinstated to accommodate *B. melanops* under the name *Melanops tulasnei* Nitschke. We have chosen not to provide a name for the anamorph since the teleomorph is well known and well characterised. The most distinctive features that define this species are the large conidia and, to a lesser extent, the large and characteristically-shaped ascospores.

The Index of Fungi lists 53 species that have been described under *Melanops*. All these species will have to be re-examined to determine their correct position. To facilitate further research on this genus, an epitype specimen with associated cultures of *M. tulasnei* (type species of the genus) is designated below.

TAXONOMY

Melanops Nitschke ex Fuckel, *Jahrbuch des Nassauischen Vereins für Naturkunde*, Wiesbaden **23–24** (Symb. Mycol.): 225 (1870).

Anamorph: *Fusicoccum*-like but the conidia have a persistent mucous sheath.

Ascomata pseudothecial, multiloculate, immersed, partially erumpent at maturity, black, subglobose, thick-walled, wall composed of thick-walled textura angularis. Pseudoparaphyses septate. Asci bitunicate, stipitate, clavate, eight-spored. Ascospores hyaline, aseptate, thin-walled, ellipsoid to rhomboid, with a persistent mucous sheath.

Melanops tulasnei Nitschke, in Fuckel, *Jahrb. Nassauischen Vereins Naturk.* **23–24**: 225, 1870 ('1869–70'), nom. nov. FIGS. 9–19

≡ *Dothidea melanops* Tul., *Compt. Rend. Hebd. Séances Acad. Sci.* **42**: 705, 1856, nom. nud.

≡ *Dothidea melanops* Tul., *Ann. Sci. Nat. Bot.*, 4^{e} Sér., **5**: 116, 1856.

= *Botryosphaeria melanops* (Tul.) G. Winter, *Rabenh. Krypt.-Fl.* Ed. 2, **1**(2): 800. 1886 ('1887').

Misapplied name:

Botryosphaeria advena sensu Sacc., *Michelia* **1**(1): 42, 1877, non (Ces.) Ces. & De Not., 1863 [fide Winter, 1886; Traverso, 1907].

Anamorph: "*Fusicoccum" advenum* (Sacc.) Died., *Krypt. Fl. Brandenburg* **9**: 314. 1912 ('1915'). FIGS. 20–27

≡ *Dothiorella advena* Sacc., *Michelia* **2**(8): 620, 1882.

= *Fusicoccum testudo* Höhn., *Ann. Mycol.* **1**(5): 399, 1903 [fide Diedicke, 1915; Shear & Davidson, 1936].

= *Dothiorella melanops* Traverso, *Fl. Ital. Crypt., Fungi* **2**(2): 409, 1907 [microconidial state].

Misapplied name:

Sphaeria quercina sensu Fr., Scler. Suec. Exs. Nº 143, 1821, non Pers. : Fr., 1794 [fide Tulasne & Tulasne, 1865; Shear & Davidson, 1936].

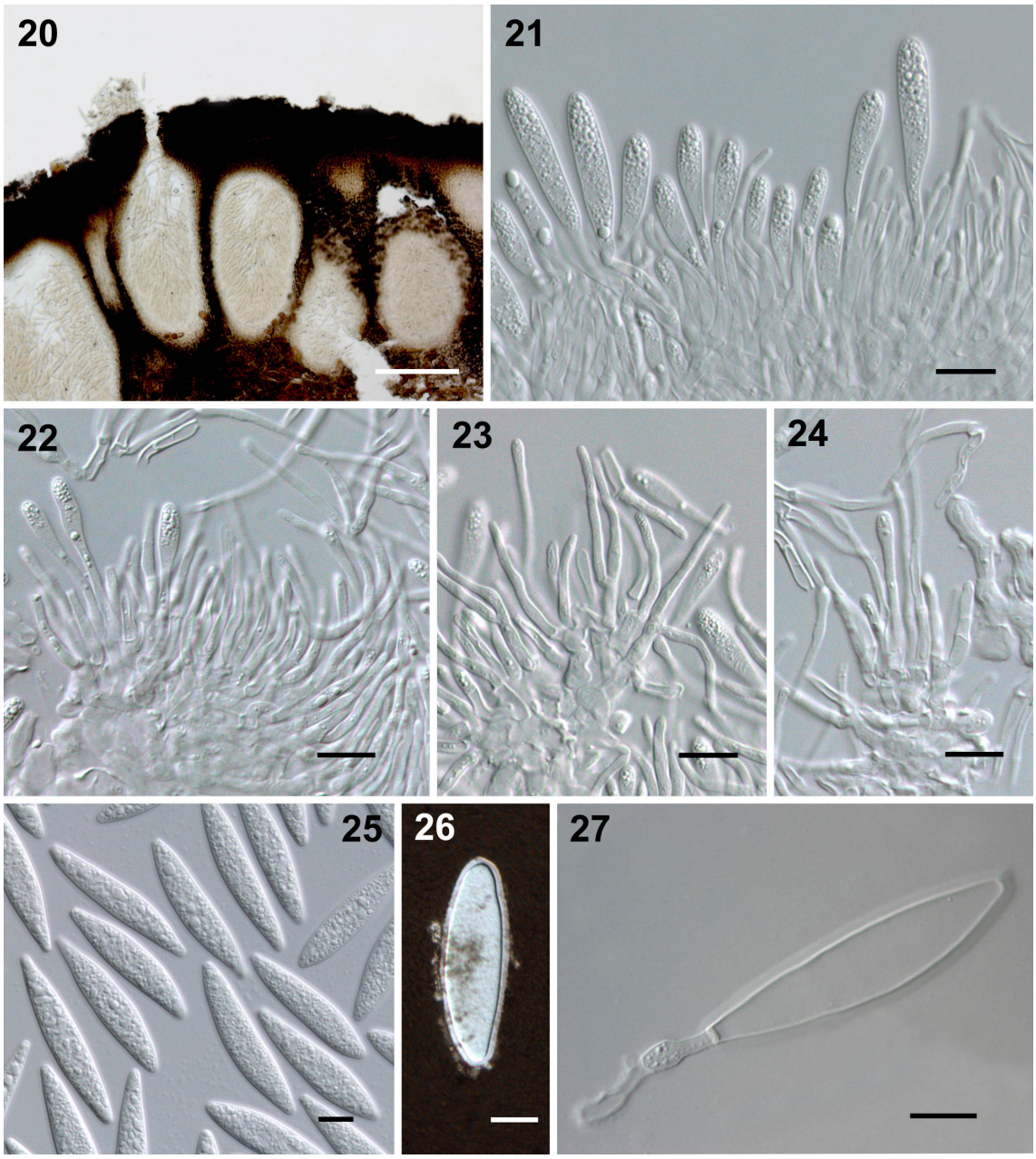
Ascomata up to 2 mm diameter, pseudothecial, initially immersed, partially erumpent at maturity, black, multilocular, thick walled, wall composed of pseudoparenchymata, outer layers composed of thick-walled, brown *textura angularis*, becoming progressively thinner-walled and paler towards the loculi, individual locules 150–300 µm diameter. *Ostioles* circular and central on each locule, papillate. *Pseudoparaphyses* 3–4 µm diam., thin-walled, hyaline, frequently septate, constricted at the septum. *Asci* 150–240 × 30–48 µm, clavate, stipitate, bitunicate with a well developed apical chamber, eight-spored, irregularly biseriate. *Ascospores* (30–)37.5–40.9(–47.0) × (13.0–)15.1–16.3(–19.0) µm, with a persistent mucous sheath, rhomboid, widest in the middle, narrowing abruptly from the middle then tapering gradually to the apices, thin walled, smooth, hyaline, aseptate but becoming pale brown and one- or two-septate with age.

Conidiomata indistinguishable from ascomata and often formed in the same stroma. *Paraphyses* filiform, arising between the conidiogenous cells. *Conidiogenous cells* cylindrical, hyaline, unbranched, discrete, formed from the inner wall of the conidioma, forming a single conidium at the tip and proliferating percurrently to form one or two annellations, or proliferating at the same level giving rise to periclinal thickenings. *Conidia* (37.2–)45.0–46.8(–53) × (7.2–)9.1–9.7(–12.1) µm, hyaline, aseptate, fusiform, widest in the middle, apex acute, base truncate with a minute marginal frill, with a persistent mucous sheath.

Neotype: In PAD, labelled as *Botryosphaeria advena* (misapplied name), on *Quercus*, Altichiero, Italy, June 1876, Herbarium Mycologicum P. A. Saccardo, Padova (designated by Phillips and Pennycook, 2005).

Epitype: LISE 95179. GERMANY. BAVARIA. Munich, English Garden, on dead twigs of *Quercus robur* L., 8 July 2004, *A.J.L. Phillips*. Cultures ex-epitype CBS 116805, CBS 116806, CBS 116807, CBS 116808.

Cardinal temperatures for growth: min 5 ºC, opt 20–25 ºC, max below 35 ºC.



Figures. 20–27. "*Fusicoccum" advenum* 20. Sectioned conidioma. 21. Conidiogenous layer. 22. Conidiogenous cells and paraphyses. 23. Paraphyses. 24. Conidiogenous cells and paraphyses. 25. Conidia. 26. Conidium with mucous sheath. 27. Conidium attached to a conidiogenous cell. Bars: 20 = 200 µm, 21–26 = 10 µm, 27 = 5 µm.

"*Botryosphaeria" mamane* clade

The correct taxonomic placement of this species is currently elusive. Gardner (1997) applied the name *B. mamane* Gardner to a fungus causing witches'-brooms on *Sophora chrysophylla* in Hawaii. The anamorphic state of the fungus produced conidia that agreed closely with those described for *F. aesculi* (teleomorph *B. dothidea*) except for their larger dimensions (Gardner, 1997). Phylogenetic analysis of the ITS region revealed a close and highly supported relationship between *B. mamane*, *B. dothidea*, and *B. corticis* (Zhou and Stanosz, 2001b; Alves *et al*., 2004; Slippers *et al*., 2004a; Farr *et al*., 2005; Barber *et al*., 2005). This relationship was also supported by ISSR fingerprinting (Zhou *et al*., 2001) although the similarity between *B. mamane* and the other two species was low.

The two known ex-type cultures of *B. mamane* are no longer extant (G. Stanosz pers. comm.). Recent attempts to re-collect and isolate *B. mamane* from the type location were unsuccessful (Crous *et al*., 2006). In phylogenetic analysis of the LSU rDNA gene (Crous *et al*., 2006; this study) an isolate identified as *B. mamane* appears to be distantly related to *B. dothidea* and *B. corticis* forming a separate clade. This culture however has the same ITS sequence as that of the original ex-type strain (Crous *et al*., 2006).

To resolve the taxonomic status of *B. mamane*, authentic cultures need to be obtained and studied. Sequencing of the SSU rDNA gene could help clarify its position in the family *Botryosphaeriaceae*.

***Macrophomina phaseolina* clade**

Macrophomina phaseolina is the type species of the genus *Macrophomina* Petr., and is also the name given to the coelomycete synanamorph of *Rhizoctonia bataticola* (Taubenh.) E.J. Butler. The taxonomic problems surrounding this species have been discussed in detail by Crous *et al*. (2006) who showed also that this fungus is a member of the *Botryosphaeriaceae* on the basis of LSU rDNA sequence data. The authors supplied a detailed morphological description of the fungus.

In the LSU phylogeny presented here this fungus forms a separate and highly supported clade within family *Botryosphaeriaceae*. At present the teleomorphic state of *M. phaseolina* is unknown.

***Guignardia* clade**

The genus *Guignardia* Viala & Ravaz has in the past been regarded as a synonym of *Botryosphaeria* (Barr, 1972). However, *Guignardia* is easily distinguished from *Botryosphaeria* in having smaller ascospores with persistent mucilaginous caps on the

apices, and anamorphs in *Phyllosticta* Pers. (van der Aa, 1973; Sivanesan, 1984, Hanlin, 1990; van der Aa and Vanev, 2002).

The status of *Guignardia* as a separate genus within the *Botryosphaeriaceae* is supported by phylogenetic analysis of the ITS region (Zhou and Stanosz, 2001b) and mt SSU rDNA gene (Zhou and Stanosz, 2001a). This is further supported in the present work as *Guignardia* (*Phyllosticta*) species form a separate and well supported clade in LSU phylogenies as well as in the combined SSU+LSU and multi-gene phylogenetic analyses.

***Saccharata* clade**

Denman *et al*. (1999) studied the species *Phyllachora proteae* Wakef., placed it in *Botryosphaeria*, and also established a cultural link with its anamorph, "*Fusicoccum*" *proteae* Denman & Crous, which also forms a *Diplodia*-like synanamorph in culture. On the basis of ITS DNA sequence data, Denman *et al*. (2000) later showed that it clustered outside of the typical *Botryosphaeria* clades which accommodated *Fusicoccum* and *Diplodia* anamorphs. Given its unilocular ascomata, the presence of a clypeus, and its unusual *Fusicoccum*- and *Diplodia*-like synanamorphs, Crous *et al*. (2004) established a new genus, *Saccharata* Denman & Crous to accommodate *S. proteae* (Wakef.) Denman & Crous. The LSU phylogeny obtained in the present study as well as that of Crous *et al*. (2006) support *Saccharata* as a distinct genus within the *Botryosphaeriaceae*. Crous *et al*. (2006) hypothesized that the genus could lie outside of this family. However, phylogenies of the LSU gene presented here clearly support this genus as a member of the family *Botryosphaeriaceae*.

The family Botryosphaeriaceae within Ascomycetes classification

The family *Botryosphaeriaceae* is currently accepted as a member of the class Dothideomycetes (Eriksson, 2006). However, its ordinal affiliation within this class still remains equivocal. Some authors consider that the family should be placed in the order *Dothideales* (e.g. Sivanesan, 1984; Hawksworth *et al*., 1995) while others accept it as a member of the *Pleosporales* (e.g. Luttrel, 1973; Barr, 1987).

Despite several attempts to clarify this taxonomic issue the phylogenies resulting from nucleotide sequence analysis of nuclear rDNA, mitochondrial rDNA genes, as well as protein coding genes were unable to unequivocally place the family within each of these orders (Winka *et al*., 1998; Liu *et al*, 1999; Silva-Hanlin and Hanlin, 1999; Liew *et al*., 2000; Lumbsch *et al*., 2000; Berbee, 1996, 2001; Tehler *et al*., 2000, 2003; Lutzoni *et al*., 2004).

These previous studies had a poor taxon sampling that was probably not representative of the family *Botryosphaeriaceae*. In this work we performed phylogenetic analyses on combined nuclear SSU and LSU rDNA partial sequences for a large set of ascomycete species representing the majority of orders within this group of fungi. Also, a more representative sampling of the family *Botryosphaeriaceae* as currently known was used.

In all phylogenetic analyses presented here (NJ, MP and Bayesian) the family *Botryosphaeriaceae* formed a monophyletic clade highly supported by bootstrap analyses and Bayesian posterior probabilities (Figs. 1–4). Hawksworth *et al*. (1995) and Eriksson (2006) accepted *Guignardia* under the family *Mycosphaerellaceae*. However, in morphological terms *Guignardia* is closely related to *Botryosphaeria* and some authors place the genus within the *Botryosphaeriaceae* (von Arx and Müller, 1975; Barr, 1987). These two genera have similar hyaline, aseptate ascospores, which is an unusual feature amongst the Loculoascomycetes (Luttrel, 1973; Eriksson, 1981; Sivanesan, 1984; Barr, 1987). In fact, due to the morphological resemblance both genera have in the past been considered synonyms (Barr, 1972). As can be seen from the phylogenies presented here as well as in other works (Silva-Hanlin and Hanlin, 1999; Crous *et al*., 2006) the genus *Guignardia* is clearly a member of the *Botryosphaeriaceae*. The genus *Guignardia* is well separated from the members of *Mycosphaerellaceae* in the current phylogenetic analysis.

The genus *Mycosphaerella* Johanson has usually been accommodated in the *Dothideales* (Barr, 1972, 1987; Sivanesan, 1984; Hawksworth *et al*., 1995). Recently (Kirk *et al*,. 2001) *Pseudosphaeriales* suborder *Mycosphaerellineae* Nannf. was raised to ordinal rank as *Mycosphaerellales* (Nannf.) P.F. Cannon. This was not accepted by Eriksson *et al*. (2002) who stated that *Mycosphaerella* is closely related to other members of *Dothideales*. However, the inclusion of *Mycosphaerella* in the *Dothideales* is not supported by the phylogenetic analyses presented here as the genus forms a clade separated from members of that order like *Dothidea*. The family *Mycosphaerellaceae* is currently listed by Eriksson (2006) as a member of the Dothideomycetes but with no ordinal placement.

The *Mycosphaerella* species used in this study always grouped closely to *Hortaea werneckii* (Horta) Nishim. & Miyaji and *Piedraia hortae* Fonseca & Leão. The first species, for which no teleomorph is known, clustered within the *Dothideales* in some previous analysis. *Piedraia* is the single genus in the family *Piedraiaceae* Viégas ex Cif., Bat. & S. Camposa which clustered with members of the *Myriangiales* and *Capnodiales* in a SSU+LSU phylogenetic analysis (Lumbsch and Lindemuth, 2001), but the exact position of the genus and family is uncertain (Eriksson *et al*., 2001).

The genus *Botryosphaeria* as representative of the family *Botryosphaeriaceae* has been considered a member of the *Dothideales* due mainly to the fact that as other members in the order mature fruiting bodies in *Botryosphaeria* species do not contain pseudoparaphyses (Barr, 1972; Sivanesan, 1984). However, the combined SSU+LSU phylogenies presented do not support the inclusion of the *Botryosphaeriaceae* in the order *Dothideales*.

The finding that immature fruiting bodies of *Botryosphaeria* do have pseudoparaphyses led Barr (1987) to place the genus and family within the *Pleosporales*. This feature was also found to occur in *Guignardia* (Janex-Favre *et al*., 1996). In the MP analysis presented here the family *Botryosphaeriaceae* clustered with the members of the *Pleosporales* but with no bootstrap support. In NJ and Bayesian phylogenies the closest relative to the family is *Hysteropatella clavispora*, a member of the *Hysteriales*. This close relationship receives high statistical support in NJ analysis (74 % bootstrap support) as well as in the Bayesian analysis (1.00 posterior probability). A wider sampling of members of this order needs to be studied in order to further verify this close relationship.

The ordinal placement of the *Botryosphaeriaceae* could not be determined conclusively in previous studies. Berbee (1996) suggested that *Botryosphaeria* could very well have affinities to both the *Pleosporales* and the *Dothideales*, which would concur with it having a combination of both pleosporareous and dothideaceous morphological features.

In the present work the ordinal placement of these botryosphaeriaceous fungi could not be definitively established. Although these fungi are phylogenetically more closely related to the *Pleosporales* than to the *Dothideales* the *Botryosphaeriaceae* do not fit in any of the orders. In fact, phylogenetic analyses suggest that their closest relatives reside in the *Hysteriales*. However, members of the *Botryosphaeriaceae,* which produce pseudothecia are distinctively different from the *Hysteriales*, which have apothecioid ascomata (Barr, 1987; Liew *et al*., 2000).

Given the current knowledge based on morphological and molecular data it seems reasonable that in order to establish a natural classification of the family *Botryosphaeriaceae*, a new order *Botryosphaeriales* should be introduced to accommodate these botryospheriaceous ascomycetes. This subject is currently being addressed in the AFTOL project (Assembling the Fungal Tree of Life – http://ocid.nacse.org/research/aftol/).

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CHAPTER 11

General Discussion

The genus *Botryosphaeria* encompasses a cosmopolitan group of fungi that are well known as pathogens of a wide range of plants, particularly woody hosts (Barr, 1987). The study of any disease caused by *Botryosphaeria* and its anamorphs must be underpinned by a reliable and definitive taxonomy of the species that comprise this genus. This work may be weakened or lead to erroneous conclusions if it is based on incorrect taxonomic assumptions.

Over the last 10 years the genus has received considerable attention, several new species have been described and the phylogenetic relationships between species have been resolved. Also, the status of some species has been clarified, which has helped to guide studies on its pathology.

Virtually since it was first described the taxonomy of the genus *Botryosphaeria* and its anamorphs has been very confused. Initial classification schemes placed substantial emphasis on morphological characters of the teleomorph. Ascomatal morphology as well as ascospore colour, size, septation, and shape were considered taxonomically informative characters. A good example is the revision of the genus made by von Arx and Müller (1954). These authors examined 183 species (only 31 were species of *Botryosphaeria*) reducing them to 11 species, nine of which they described as new. On the basis essentially of ascospore size, von Arx and Müller (1954) synonymized 108 species under *B. quercuum* and 24 species under *B. dothidea*.

This very broad species concept applied by von Arx and Müller (1954) was not widely accepted and is nowadays considered too conservative. In fact, it was shown that *B. quercuum* and *B. dothidea* represent a complex of numerous distinct species (Shoemaker, 1964; Slippers *et al*., 2004). The species name *B. dothidea* and some of its synonyms proposed by von Arx and Müller (1954) like *B. ribis* and *B. berengeriana* have been inconsistently used in the literature resulting in considerable confusion amongst pathologists.

Regarding the teleomorph state there are few morphological features that can be used to distinguish between *Botryosphaeria* species, essentially ascus and ascospore dimensions, and colour and septation of ascospores. There are also some difficulties in using these characters to delimit species. The teleomorph structures are not frequently found in nature, and only rarely have been induced in artificial culture. For example, Maas and Uecker (1984) were unable to induce ascomata formation in culture by pairing isolates of *B. dothidea* (*B. ribis*?) from blackberry. In the same way, Michailides and Morgan (1992) were unsuccessful when they tried to induce the sexual

state of *B. dothidea* both *in vitro* and *in vivo*. Taylor (1958) reported the formation of reproductive structures when certain isolates of *B. corticis* where paired. He also found perithecia in cultures of a *Botryosphaeria* species causing stem blight of blueberry. Witcher and Clayton (1963) stated that perithecia of *B. dothidea* (*B. ribis*?) occasionally developed in cultures of isolates from blueberry and other hosts. These authors found perithecia in only 2 of 110 paired cultures. Single ascospore cultures of *B. subglobosa* were reported to produce ascomata in culture (Punithalingam, 1969).

Asci and ascospores of *Botryosphaeria* species develop and mature slowly, and as a consequence specimens are often collected and studied in an immature state (Slippers *et al*., 2004). Thus, it is likely that important characters like colour, size and septation of ascospores are sometimes not detected. Herbarium specimens of some *Botryosphaeria* species are represented by immature collections as is the case of the type species *B. dothidea* (Slippers *et al*., 2004).

In order to overcome these problems and facilitate future studies it is imperative to select epitype specimens and provide cultures linked to these specimens that can be used for comparative purposes. Slippers *et al*. (2004a) epitypified the type species *B. dothidea* which clarified the confusion surrounding this name. In the present study we have also provided epitype specimens and ex-type cultures for *B. corticis* (Chapter 6) and *Melanops tulasnei* (Chapter 10). Nevertheless, many more species like *B. stevensii*, *B. obtusa*, and *B. rhodina* (to mention only a few) are in need of epitypification in order to clarify their taxonomic status and facilitate pathological studies.

Because of the constraints associated with the morphological features of the teleomorphs it has been recognised that the anamorphs of *Botryosphaeria* species are more useful in discriminating between species. The asexual states or anamorphs are frequently found in nature and provided that the right conditions exist (light, temperature, suitable culture medium) these are easily induced in artificial culture.

Taxonomy of *Botryosphaeria* anamorphs has also been complicated. These have been associated with several different Coelomycete form genera. However, in recent years and particularly with the use of phylogenetic analysis considerable progress has been made with clarifying the taxonomy and phylogenetic relationships of the anamorph genera associated with *Botryosphaeria* (e.g. Crous and Palm, 1999; Denman *et al*., 2000; Crous *et al*, 2006; Chapters 3, 9 and 10).

The anamorphs display a wide range of morphologies that separate the species and define the genera. Amongst the characters of the anamorphs only the conidia are consistently used to distinguish taxa. Conidiomata are frequently indistinguishable from ascomata and their morphologies are not useful to delimit species or even genus

boundaries. Also, the mode of conidiogenesis has not proven useful for species discrimination. Conidia are morphologically diverse and possess a range of characters like size, shape, colour, septation, wall thickness and ornamentation, which can be used to differentiate species. Despite the value of conidial morphology, some features exhibit extensive plasticity and there is some overlap particularly between closely related species.

Some cultural characteristics like the production of pigments, the colour of mycelium, and the cardinal temperatures for growth have been used to characterise species, but these characters have a reduced taxonomic value. Also, in the past the ability to infect a specific host was considered important which led to a proliferation of species names described almost solely on the basis of host association. It is now evident that host association is of little or no taxonomic value because most *Botryosphaeria* species have wide host ranges.

The more commonly used species concepts are the Morphological Species Concept (MSC), Biological Species Concept (BSC), and Phylogenetic Species Concept (PSC). The MSC is clearly the dominant fungal species concept. With few exceptions, all the described fungi are diagnosed by morphological characters. The great strength of MSC is that it has been applied widely so that comparisons can be made among existing taxa, between new and existing taxa and also comparisons with type specimens. The weakness is that species as defined by MSC often comprise more than one species when BSC or PSC are applied (Harrington and Rizzo, 1999; Taylor *et al.*, 2000).

Species in *Botryosphaeria* have been traditionally defined on the basis of morphological species concepts in which discontinuities of one or more sets of morphological characters demarcate taxa. In fungi, BSC has been used to identify groups of mating compatible individuals, which have been equated with species (e.g. Dettman *et al*., 2003). However, mating tests are impossible to apply to many fungi that are morphologically asexual. Others, like *Botryosphaeria*, although sexual have not been induced to mate in culture thus hindering the application of the BSC. Recently, with the introduction of DNA sequence analysis and multiple gene genealogies the PSC has been widely applied in the description of new species and also unravelled cryptic speciation in *Botryosphaeria* (e.g. Smith *et al.*, 2001; Phillips *et al*., 2002; Denman *et al*., 2003; Slippers *et al*., 2004a, b, c; van Niekerk *et al*., 2004; Farr *et al.*, 2005; this work).

In the present study we favoured the application of the PSC (Harrington and Rizzo, 1999; Taylor *et al*., 2000) to circumscribe *Botryosphaeria* species. Harrington and Rizzo (1999) advocate a type of PSC that diagnoses species as "... the smallest aggregation of populations with a common lineage that share unique, diagnosable

phenotypic characters". This, however, can be particularly difficult because in fungi genetic differentiation seems to precede morphological differentiation. So, it is not expected that recently genetically isolated species will show immediate phenotypic differences, although over time they should (Taylor *et al*., 2000).

Once phylogenetic species are identified using gene genealogies, an effort has to be made in order to re-evaluate the characters used to distinguish species or identify new ones whose taxonomic significance may have been disregarded.

Nucleotide sequence data combined with morphological characteristics has been successfully applied in the identification and description of *Botryosphaeria* species. Many studies have relied on the use of ITS rDNA phylogenies only (e.g. Jacobs and Rehner, 1998; Denman *et al*., 2000, 2003; Zhou and Stanosz, 2001; Smith and Stanosz, 2001; Smith *et al.*, 2001; Phillips *et al*., 2002; Pavlic *et al*., 2004; Chapters 2 and 6).

Despite the vast contribute that ITS phylogenies gave to the taxonomy of the genus, the use of this single gene can underestimate the true diversity of species. When dealing with closely related or cryptic species (e.g. *B. ribis*/*B. parva*, *B. lutea/B. australis*) it is crucial to obtain information from multiple gene sequences. In this regard, introns of protein coding genes like EF1-α and β-tubulin have been successfully applied (e.g. de Wet *et al.*, 2003; Slippers *et al*., 2004a, b, c; van Niekerk *et al*., 2004; Burgess *et al.*, 2005; Farr *et al*., 2005; Chapters 3, 4, 7 and 9).

As discussed previously the identification of *Botryosphaeria* species can be difficult to accomplish if based solely on the morphological features. This is especially evident when dealing with closely related species. The use of DNA sequencing (ITS, EF1-α and β-tubulin) overcomes these problems but sequencing large amounts of isolates is very laborious and expensive. Thus, PCR-based methods like ARDRA (Chapter 5) and MSP- or rep-PCR (Chapter 8) are excellent alternatives for routine identification of large sets of isolates.

Ideally, the taxonomic placement of *Botryosphaeria* at all levels from genus through class should reflect its natural phylogeny. However, it is now evident that the genus has become a heterogeneous assembly of taxa and as currently circumscribed it is clearly artificial. Phylogenetic analyses (Crous *et al*., 2006; Chapter 10) have shown that the genus *Botryosphaeria sensu lato* is composed of several lineages that represent separate natural phylogenetic groups. These groups correspond to different genera that are better defined on the basis of morphological features of the anamorphs.

Botryosphaeria has been considered a very large genus comprising more than 200 species. However, according to phylogenetic analyses, *Botryosphaeria sensu stricto*

includes only two species, namely *B. dothidea* (the type species) and *B. corticis*. Curiously, species that have in the past been considered as synonyms of *B. dothidea* as is the case of *B. ribis* are now deemed to belong in a separate genus.

These recent phylogenetic analyses represent a great advance towards a natural classification of the botryosphaericeous group of fungi. It is hoped that the outcome of this classification will in the future facilitate the work of plant pathologists studying diseases caused by these fungi.

Despite these recent advances in the systematics of the family *Botryosphaeriaceae* the ordinal position of the family within the higher classification of Ascomycetes still remains unclear. In the present work (Chapter 10), it was shown that although the family *Botryosphaeriaceae* shares morphological characters with both orders *Dothideales* and *Pleosporales* it cannot be considered as a member of any of those orders. According to the data presented it is conceivable that this family might represent a separate order with intermediate morphological characters between the *Dothideales* and *Pleosporales*.

Future work

- In the last years the taxonomy and pathology of the *Fusicoccum* group (*Fusicoccum*, *Neofusicoccum* and *Pseudofusicoccum*) has been studied intensively. Several new species have been described and the phylogenetic relationships between species analysed. In comparison, the *Diplodia* group has received little attention and several taxonomic problems remain. The teleomorph-anamorph connection between *B. rhodina* and *L. theobromae* must be definitively elucidated. Also, there are some species complexes that should be resolved. Some results presented in this work suggest that *B. stevensii* and *B. obtusa* probably represent complexes of species. This is further supported by differences reported on the pathology of these species, particularly of *B. obtusa*.

- Several botryosphaeriaceous fungi are important plant-pathogens some of them having a latent endophytic stage during which the host does not develop any symptoms. In order to facilitate diagnosis of diseases caused by these fungi and implement efficient control measures it is imperative to develop fast, accurate and sensitive diagnostic methods. Taking advantage of the availability

of a large amount of DNA sequence data for many species it is possible to develop species-specific primers to be used in PCR-based diagnostic tests.

- Another issue that needs to be investigated is the mating behaviour of *Botryosphaeria* species. Attempts to induce the sexual state (teleomorph) in artificial culture are almost invariably unsuccessful. The reports regarding the production of ascomata in culture are scarce. So, virtually nothing is known about the genetics of mating systems in *Botryosphaeria*, and whether species use different mating strategies: homothallism, heterothallism or pseudohomothallism. According to Punithalingam (1969) single ascospore cultures of *B. subglobosa* produce both ascomata and pycnidia indicating that the fungus is homothallic. Also, Witcher and Clayton (1963) occasionally found ascomata in paired cultures of *B. dothidea* (*B. ribis*?), but they could not determine whether the fruiting bodies had resulted from a cross or from a single isolate.

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