1

Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*

Abstract: *Botryosphaeria dothidea* is one of the most commonly reported species in a genus of important pathogens of woody plants. This taxon is generally accepted to represent a species complex, and hence its identity remains unclear. Previous studies have either treated *B. dothidea* as the valid name for *B. ribis* and *B. berengeriana*, or argued for them to be separate entities. To add to the confusion, no ex-type cultures are available for either *B. dothidea* or *B. ribis*. The aim of the present study, therefore, was to recollect and characterize these fungi, and designate a set of reference cultures that can be used in future studies. To this end morphological, cultural, and multi-allelic DNA sequence data sets from the rDNA (ITS 1, 5.8S, and ITS 2), β-tubulin and EF1-α genes were used to fully characterize these species. *Botryosphaeria dothidea* was found to be distinct from *B. ribis*, while *B. berengeriana* was retained as synonym of the former name. Furthermore, *Fusicoccum aesculi* is accepted as anamorph of *B. dothidea*, while the anamorph of *B. ribis* is newly described as *F. ribis* sp. nov. *Botryosphaeria ribis* could be distinguished from *B. parva* based on β-tubulin and EF1-α sequence data. A combined phylogeny of the three gene regions used in this study also showed that the genus *Botryosphaeria* represents two distinct phylogenetic assemblages that correspond to species with *Diplodia* and *Fusicoccum* anamorphs.

INTRODUCTION

Botryosphaeria Ces. & De Not. was described in 1863 (Cesati and De Notaris 1863). Cesati and De Notaris (1863) first included 12 species in the genus, but did not provide detailed morphological descriptions of the species. De Notaris (1863) added another 4 species, including *B. berengeriana* De Not., for which he provided detailed descriptions and sketches. Saccardo (1877) amended the initial generic descriptions of Cesati and De Notaris to exclude hypocreaceous species, which he transferred to two new genera, *Gibberella* and *Lisea*. Von Arx and Müller (1954, 1975), who did an extensive revision and key (respectively) of the genus, cite this amendment as part of the generic description.

Cesati and De Notaris (1863) did not select a type species for the genus. Barr (1972) rejected propositions that either *B. quercuum* (Schwein.) Sacc. or *B. berengeriana* be designated as the lectotype species of the genus because these species were not part of the original description of the genus. Barr (1972) therefore designated *B. dothidea* (Moug.:Fr.) Ces. & De Not. (= *Sphaeria dothidea* Moug.:Fr.), one of the original species included by Cesati and De Notaris (1863), as the lectotype species of the genus.

Despite obvious similarities between specimens, early researchers tended to describe new *Botryosphaeria* species, where these fungi occurred on different hosts (Cesati and De Notaris 1863, De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928). Von Arx and Müller (1954), however, synonymized many of these species under *B. quercuum* and *B. dothidea*, based on teleomorph herbarium material. Many researchers did not accept the extensive synonymies of von Arx and Müller (1954). For example, *B. dothidea* and *B. ribis* Grossenb. & Duggar have been viewed as distinct species by many due to differences in anamorph morphology (Punithalingam and Holliday 1973, Morgan-Jones and White 1987, Rayachhetry et al 1996, Smith and Stanosz 2001, Zhou and Stanosz 2001a, b), while others treated them as synonyms *sensu* von Arx and Müller (Witcher and Clayton 1963, Barr 1972, English et al 1975, Spiers 1977, Maas and Uecker 1984, Pennycook and Samuels 1985, Brown and Britton 1986, Smith et al 1994). A further basis for confusion is that von Arx and Müller (1975) considered *B. berengeriana*, which they had synonymized earlier with *B. dothidea* (von Arx and Müller 1954), as one of the most common species of the genus. According to von Arx (1987), the name *B. dothidea*

should be restricted to isolates pathogenic to roses, while he considered *B. berengeriana* (including *B. ribis*) as polyphagous. Currently, the name *B. berengeriana* is not commonly used, except in Japan (Sassa et al 1998, Ogata et al 2000).

The *Botryosphaeria* teleomorph is seldom seen in culture, whereas the anamorphs are common. Species differences are manifested in the anamorph, while there is considerable overlapping in the continuous characters of the teleomorph, such as spore sizes. For these reasons, anamorph characters are often considered important to identify species in this genus (Shoemaker 1964, Pennycook and Samuels 1985). Denman et al (2000) recorded 18 anamorph genera that have been linked to *Botryosphaeria*, with *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Cda., *Lasiodiplodia* Ellis & Everh., and *Macrophoma* (Sacc.) Berl. & Voglino the most common. Of these, *Macrophoma* has been synonymized with *Sphaeropsis* Sacc. (Sutton 1980). Crous and Palm (1999) also showed that *Botryodiplodia* is a *nomen dubium* and that the type specimen of *Dothiorella* is best accommodated in *Diplodia*. The use of anamorph characters is also complicated by the overlapping characteristics between species and the effect of aging on conidium morphology (Pennycook and Samuels 1985, Jacobs and Rehner 1998, Smith and Stanosz 2001).

Phylogenetic studies, using both morphological and molecular data, have contributed significantly to *Botryosphaeria* taxonomy. Recent studies have used a combination of morphological and DNA sequence, RAPD or ISSR data to study relations among species and to define *Botryosphaeria* spp. (Jacobs and Rehner 1998, Denman et al 1999, Smith et al 2001, Smith and Stanosz 2001, Zhou et al 2001, Zhou and Stanosz 2001a). One of the main conclusions drawn from these studies is that *Botryosphaeria* spp. can be separated into two groups, namely those with dark-conidial diplodia-like anamorphs, and those with hyaline-conidial fusicoccum-like anamorphs. Denman et al (2000) revised the generic taxonomy of the anamorphs that have been linked to *Botryosphaeria* and concluded that those with hyaline conidia be included in *Fusicoccum* and those with conidia that are dark and opaque when mature be included in *Diplodia*. These findings were supported by Zhou and Stanosz (2001a), who referred the two anamorph genera to section *Hyala* and section *Brunnea*. Contrary to these studies, Zhou and Stanosz (2001b) found that these groups were not supported by partial mitochondrial (mt) SSU sequence data. These authors suggested that these contradictions might have been due to lack of resolution using this part the mitochondrial rDNA gene region. Alternatively, that it might have arisen through

hybridization or horizontal gene transfer before the separation of the two groups mentioned above.

Despite their considerable contribution to *Botryosphaeria* taxonomy, single gene phylogenies and other molecular data have not resolved some long-standing taxonomic controversies. The morphological species *B. dothidea* is paraphyletic and divided into two clades based on molecular data (Jacobs and Rehner 1998, Denman et al 1999, Denman et al 2000, Smith et al 2001, Smith and Stanosz 2001, Zhou et al 2001, Zhou and Stanosz 2001a, b). These clades are regarded in some of the above studies as representing *B. dothidea* and *B. ribis*, raising a question regarding their synonymy. Furthermore, while rDNA sequence data and RAPD marker data could not distinguish *B. parva* Pennycook & Samuels and *B. ribis* (Smith and Stanosz 2001, Zhou and Stanosz 2001a), ISSR markers showed that they were distinct (Zhou et al 2001).

It is evident that the commonly encountered and economically important genus *Botryosphaeria* continues in taxonomic disarray. The objective of the present study is to test morphologically based hypotheses with data derived from multiple gene sequences. The need to use multiple gene phylogenies to distinguish closely related species has been emphasized before (O'Donnell and Cigelnik 1997, Taylor et al 2000). For this reason, rRNA [spanning the internal transcribed spacer region one (ITS 1), 5.8S gene and ITS 2 regions] sequence data were used in this study together with data from the partial β-tubulin and translation elongation factor 1-α (EF1-α) gene sequences to determine the phylogenetic relationships of *B. dothidea*, *B. ribis*, and *B. parva*.

MATERIALS AND METHODS

Isolates and type material.--Thirty-one isolates representing nine *Botryosphaeria* spp. were used in this study (TABLE I). In an attempt to obtain representative specimens and isolates of *B. dothidea*, fresh material was collected from southern Switzerland and northern Italy in October 2000. This is the same time of year, area, and included the same hosts upon which Cesati and De Notaris based the original descriptions. Isolations were made from ascomata or pycnidia on dead or dying twigs of various hardwood species (TABLE I). Similarly, isolations were made from twigs of *Ribes* spp. showing symptoms of cane die-back from Ithaca, New York state. This is the host genus and area from which the original material for the description of *B. ribis* was collected by

Grossenbacher and Duggar. Ex-type isolates of *B. parva* Pennycook & Samuels were obtained from the International Collection of Microorganisms from Plants (ICMP), Landcare Research New Zealand Ltd., Auckland, New Zealand. Other isolates of representative *Botryosphaeria* spp. were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and the Culture Collection of the Tree Pathology Co-operative Programme (CMW), FABI, University of Pretoria, South Africa (TABLE I). *Botryosphaeria dothidea*, *B. ribis* and *B. parva* were compared based on morphological and molecular data. Other common *Botryosphaeria* spp. were used in molecular comparisons only.

Initial identification of the isolates was achieved based on conidial morphology. Isolates were grown on 2% water agar (WA; Biolab agar, Midrand, Johannesburg, S.A.) with sterilized pine needles, or halved twigs of *Malus* sp., *Eucalyptus* sp. or *Populus* sp. as substratum, at 25 C under near-UV light, to induce sporulation. Cultures were maintained on malt and yeast extract agar (MYA; 2% malt extract, 0.2% yeast extract and 1.5% agar; Biolab, Midrand, Johannesburg, S.A.) at 25 C and stored on this medium at 4 C. Colony morphology, color (Rayner 1970), and growth rates between 10 and 30 C, were determined on potato dextrose agar (PDA; 0.4% potato extract, 2% dextrose, 1.5% agar, Biolab, Midrand, Johannesburg, S.A.).

Type material or other representative specimens and cultures of *B. dothidea*, *B. ribis, B. parva* and *B. berengeriana* were obtained from various herbaria, including CUP, PDD, RO, S. Ascomata or pycnidia were mounted in lactophenol. Sections of herbarium, freshly collected and *in vitro* ascomata and pycnidia were cut with an American Optical Freezing Microtome or by hand. Morphological observations and measurements were made with a light microscope, an Axiocam digital camera and accompanying software (Carl Zeiss, Germany).

DNA isolation and amplification.--A modification of the phenol:chloroform DNA extraction method of Raeder and Broda (1985) was used to isolate DNA from the fungal isolates as described in Smith et al (2001). The primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al 1990) were used to amplify part of the nuclear rRNA operon in PCR reactions. The amplified region included the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene. A part of the β-

tubulin gene region was amplified by use of the primers Bt2a (5' GGTAACCAAATCG GTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass and Donaldson 1995). Amplification of part of the EF1-α was done with the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTGAAGGA ACCCTTACC) (Carbone et al 1999). PCR reaction mixtures contained final concentrations of: 2.5 Units *Taq* DNA polymerase (Roche Molecular Biochemicals, Almeda, California, USA), 1X Buffer & $MgCl_2$ mixture (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP and 0.15 µM of each primer and made up to a final volume of 50 µL with water. During the PCR reaction, the DNA was first denatured at 94 C for 2 min, followed by 40 cycles of denaturation (94 C for 30 s), annealing (55 C for 45 s) and elongation (72 C for 1½ min) and ended with a final elongation step at 72 C for 5 min. Amplification of the EF1-α region was problematic for some species. In these cases amplifications were done by replacing the *Taq* polymerase with Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California, USA) with the same reaction concentrations as above, and PCR cycle conditions as indicated by the supplier. PCR amplicons were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Size estimates were made using 100bp or λ standard size markers.

DNA sequencing and analysis.--PCR products were cleaned using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, California, USA). Both strands of the amplicons were sequenced using the same primers that were used for the initial amplification. Reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied BioSystems, Foster City, California, USA) as indicated by the manufacturer and run on an ABI PRISM 377 autosequencer (Perkin-Elmer Applied BioSystems, Foster City, CA).

To compare the *B. dothidea*, *B. ribis, B. parva* and other *Botryosphaeria* isolates used in this study, with those from previous studies, 22 ITS rDNA sequences from GenBank were included in the analyses (TABLE I) (Jacobs and Rehner 1998, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001a). BLAST searches were used to find any other related sequences from GenBank, not referred to in these studies. Trees were rooted to sequence data of an isolate of *Guignardia philoprina* (Berk. &

M.A. Curtis) Aa, which was previously described in the genus *Botryosphaeria* before it was placed in the closely related genus, *Guignardia* Viala & Ravaz. Despite the close relationship between these last named genera, unambiguous alignment of outgroup sequence with that of the ingroup was not possible for all parts of intron and ITS regions.

ITS rDNA sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, California, USA) and manually aligned by inserting gaps. Phylogenetic analyses based on parsimony were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999). Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were found using heuristic searches, and including only informative characters in stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch supports, using 1000 bootstrap replicates (Felsenstein 1985), and estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were also determined in PAUP. Decay analyses of the branch nodes were determined using Autodecay (Eriksson 1998). Phylogenetic hypotheses were also tested using distance analyses with the Neighbor Joining algorithm and an uncorrected p-factor in PAUP.

Statistical congruence between the ITS rDNA, β-tubulin, and EF1-α sequence data sets was tested using partition homogeneity tests (Farris et al 1995, Huelsenbeck et al 1996) in PAUP. These tests revealed that the data were combinable. The data sets were subsequently analyzed together. Repetitive minisatellite regions in the intron of the EF1-α were coded to represent a single, rather than multiple evolutionary events.

RESULTS

Morphological characteristics and typification.--The published description of *S. dothidea* (Fries 1823) refers to a fungus from fallen twigs of a *Fraxinus* sp. However, the herbarium specimen of *S. dothidea* in the Fries collection, collected by Mougeot, which has been cited as the type material (von Arx and Müller 1954), contains a sample with thorns that appears to be a *Rosa* sp. This sample can, thus, not be the holotype. No

type specimen of *S. dothidea* on *Fraxinus* by Mougeot could be located in other herbaria that might have such a collection (BM, BR, K, LILLE, LIP, NCY, STR). Given that the holotype could not be located, the only remaining *S. dothidea* sample in the Fries herbarium is designated here as the neotype representing *B. dothidea*. This material is, however, not definitive of the species, because the specimens are immature and contain no spores.

In order to clarify taxonomic confusion surrounding *B. dothidea,* this taxon is epitypified here. An epitype is designated to complement the neotype and other authentic specimens, as well as their descriptions, which represent *B. dothidea*. The epitype also allowed isolation of cultures. To find an epitype, three samples were collected during the present study from a nearby locality (the border between Switzerland and Italy) to that of some of the collections of Cesati and De Notaris (TABLE II). The specimens were collected from *Fraxinus* sp., *Prunus* sp. and *Ostrya* sp. These samples contained ascomata that conformed to descriptions of *B. dothidea* by Fries (1823) and Cesati and De Notaris (1863). Thus, one of these samples (PREM57372, on *Prunus* sp.) is designated as the epitype specimen.

A taxonomic description of *B. dothidea* (FIGS. 1-7) based on the epitype material and cultures made from it, is given below. Cultures obtained from these samples produced an anamorph that matches descriptions of *F. aesculi* Corda by Pennycook and Samuels (1985) and Crous and Palm (1999).

A specimen labeled as *B. berengeriana* was obtained from the collection of De Notaris (RO). This specimen carries the signature of De Notaris and is from the same host (*Rhamnus frangula*) referred to in the original description (De Notaris 1863). It is likely that this is the material used by De Notaris for that description, or at least is similar to it. Ascomata on this material, as well as the original description of *B. berengeriana*, were not distinguishable from those of the lectotype or epitype specimens of *B. dothidea* (TABLE II).

Grossenbacher and Duggar (1911) described, but did not typify, *Botryosphaeria ribis*. Three collections used by Grossenbacher and Duggar for this description were located in CUP. We have designated one of these as lectotype for *B. ribis*. This material of *B. ribis* is mature and well preserved, and provides ample substance to characterize this species. This lectotype of *B. ribis* also contains a well-developed and preserved anamorph.

Specimens of *Ribes* sp. canes with die-back, that were collected on our behalf from the same geographical area as the original type material (New York state, USA), contained pycnidia of a *Fusicoccum* sp. and a *Diplodia* sp. The former species corresponds to the anamorph on the type material and to the original description of an anamorph associated with *B. ribis*. Isolates from this *Fusicoccum* sp. were used in cultural, morphological and molecular studies. In this paper, a description of *B. ribis* and its anamorph, based on this material and the lectotype specimens, is given to accompany the molecular characterization of this taxon (FIGS. 8-14).

Isolates residing in the clades that represent *B. dothidea* and *B. ribis* have similar ascomata, ascospores, conidial morphology, and cultural characteristics. However, these species can be distinguished from each other using average dimensions of these features, especially in culture (taxonomic description, key, FIGS. 1-14).

Both the type and corresponding ex-type cultures for *B. parva* are well preserved and representative of the description of this taxon. Using this material, *B. parva* and *B. dothidea*, as described here, were clearly distinguishable based on morphological features (key). There is no consistent morphological distinction between *B. ribis* and *B. parva*, other than some variation in septation in discharged and aged spores (key, FIGS. 8-15) (TABLE III).

Phylogenetic sequence analyses.--The ITS data set consisted of 563 characters after alignment, 418 uninformative characters were excluded and 145 parsimony-informative characters were used in the analyses. These data contained significant phylogenetic signal ($P < 0.01$; g1 = -0.644) (Hillis and Huelsenbeck 1992). After heuristic searches in PAUP, 71 most parsimonious trees of 325 steps were retained (CI = 0.757; RI = 0.933). (FIG. 16).

A partition homogeneity test of the full data set, combining ITS-rDNA, β-tubulin and EF1-α, indicated that the data sets could be combined (P value = 0.32). The combined data set consisted of 1344 characters after alignment (TreeBASE S861, M1396). A total of 968 characters were excluded, including 954 uninformative characters and 14 minisatellite characters from the EF1-α intron region that were coded to represent one evolutionary event. Using the 390 parsimony-informative characters (significant phylogenetic signal [<0.01; g1 = -0.851] [Hillis and Huelsenbeck 1992]), 10

most parsimonious trees of 858 steps were retained after heuristic searches in PAUP (CI = 0.795; RI = 0.91) (FIG. 17).

For both data sets, the same clades were identified by parsimony and distance analyses. The analyses of both datasets showed that the branch supports separating the main clades, which are identified as *B. lutea* A.J.L. Phillips, *B. eucalyptorum* Crous, H. Smith & M.J. Wingf., *B. dothidea*, *B. quercuum*, *B. stevensii* Shoemaker, *B. obtusa* (Schw.) Shoemaker and *B. rhodina* (Cooke) von Arx, were well supported (>d4/91% bootstrap) (FIGS. 16, 17). ITS rDNA sequences of *B. mamane* Gardner and *B. corticis* (Demaree & Wilcox) von Arx & Müller are significantly distinct, but were more closely related to each other than to any other species used in the analysis. These two species grouped in a sister clade to *B. dothidea*. *Botryosphaeria ribis* and *B. parva* could not be distinguished based on ITS rDNA data (FIG. 16), but were clearly separated in the combined datasets (FIG. 17).

Botryosphaeria spp. were divided into two main clades in the combined dataset. These correspond to the fusicoccum-like and diplodia-like anamorph types, respectively (FIG. 17). In the clade with *Fusicoccum* conidia, *B. dothidea* was clearly distinct, with the strongest support (d53/100% bootstrap in analyses of combined datasets) of all the clades. Similarly, among *Botryosphaeria* spp. with *Diplodia* anamorphs, the distinction of *B. rhodina* was strongly supported (d63/100% bootstrap). *Botryosphaeria rhodina* did not group with other isolates having *Diplodia* anamorphs in the rDNA dataset when using parsimony, but the branch separating these taxa was weakly supported (d2/60% bootstrap) (FIG. 16). Distance analysis of this rDNA dataset (tree not shown), however, also placed this species amongst other *Botryosphaeria* spp. with dark-spored (*Diplodia*) anamorphs, as we found in the analysis of the combined dataset.

TAXONOMY

There are a number of published descriptions pertaining to the type material and other authentic specimens of *B. dothidea*, *B. ribis*, and their anamorphs (Fries 1823, Cesati and De Notaris 1863, De Notaris 1863, Winter 1886, Saccardo 1877, Grossenbacher and Duggar 1911, von Arx and Müller 1954, Punithalingam and Holliday 1973, Sutton 1980, Pennycook and Samuels 1985, Crous and Palm 1999). Due to the confusion regarding the use of these names in the descriptions, revised descriptions based on the type material and fresh collections made as part of this study are provided here. The

morphological description of *B. parva*, which is also considered in this study, is not repeated here as this would be redundant and would not add substantially to the original description provided by Pennycook and Samuels (1985).

Botryosphaeria dothidea (Moug.: Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital. 1:212. 1863. FIGS. 1-7

= *Sphaeria dothidea* Moug.: Fr. in Fries, Syst. Mycol. 2:423. 1823.

= *Botryosphaeria berengeriana* De Not., Sfer. Ital. 82. 1863 [1864].

Anamorph. ***Fusicoccum aesculi*** Corda in Sturm, Deutschl. Fl., Abth. 3, 2:111. 1829.

Ascostroma erumpent through the bark, 200--500 µm diam. *Ascomata* pseudothecial, forming a botryose aggregate of up to 100, sometimes solitary, globose with a central ostiole, ¼ to ½ emergent, rarely imbedded, papillate or not, brown to black; pseudothecial wall comprising 5--15 layers of *textura angularis*, outer region of dark brown or brown cells, inner region of 2--4 layers of hyaline cells lining the locule. *Asci* bitunicate, clavate, 63--125 x 16--20 µm, 8-spored, between numerous filiform, septate, rarely branched towards the tip, pseudoparaphyses, 2--4 µm wide. *Ascospores* fusoid to ovoid, sometimes with tapered ends giving a spindle shaped appearance, (17--) 19--24(--32) x (6--)7--8(--10) µm (average of 102 ascospores 22.7 x 7.8 µm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* pycnidial (anamorph structures were present only on the sample from *Ostrya* sp.), shape indistinguishable from the ascomata. *Conidia* narrowly fusiform or irregularly fusiform, base subtruncate to bluntly rounded, (17--)18--20(--22) x 4--5 µm (average of 35 conidia 19.6 x 4.8 µm, l/w 4.1), hyaline, unicellular, rarely forming a septum before germination, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 6--20 x 2--5 µm, proliferating percurrently with 1--2 proliferations and periclinical thickening. *Spermatia* unicellular, hyaline, allantoid to rod-shaped, 3--6 x 1.5--2 µm. *Spermatophores* hyaline, cylindrical to subcylindrical, 4--10 x 1--2 µm.

Cultural characteristics. Colonies olivaceous buff (21'''d), becoming olivaceous grey (21'''''i) to violaceous black (65''''k), with a sparse to moderately dense, appressed mycelial mat, occasional columns of aerial mycelium reaching the lid, margin smooth appearing crenulate as the colony darkens with age. Optimum temperature for growth 25(--30) C, colony reaching a 50 mm radius on PDA after 4 d at 25 C in the dark. *Pycnidia* (formed on WA on sterilized twigs of *Malus* sp., *Eucalyptus* sp., *Populus* sp., or needles of *Pinus* sp. within 7--14 d) superficial, globose, mostly solitary and

covered by mycelium. *Conidia* produced in culture similar to those formed in nature, but regularly shaped, longer and appearing more narrowly fusiform, (20--)23--27(--30) x 4--5(--6) µm (average of 102 conidia 24.7 x 4.9 µm, l/w 5).

Specimens examined. FRANCE. *Rosa* sp., 1823, *Fries ex Mougeot* (NEOTYPE designated here, *Sphaeria dothidea*, herbarium S). SWITZERLAND. TICINO: Crocifisso, *Prunus* sp., October 2000, *B. Slippers* (EPITYPE designated here, PREM57372, culture CMW8000); *Ostrya* sp., October 2000, *B. Slippers* (PREM57373, culture CMW7999); Molinizza, *Fraxinus* sp., October 2000, *B. Slippers* (PREM57374, culture CMW7780). ITALY. Pusiano, *Populus* sp., 31 October 1846, *Cesati et De Notaris*; Pusiano, *Fraxinus* sp., 1846, *Cesati et De Notaris*; Locality unknown, *Rhamnus frangula*, 1863, *De Notaris*. GERMANY. Pr. Jever, *Fraxinus* sp., *Koch ex Cesati et De Notaris* (Rabenhorst. Herb. Mycol. 750, herbarium RO); Pr. Dreisen, *Robinia pseudoacacia*, *Lasch ex Cesati et De Notaris* (Rabenhorst. Herb. Mycol. 1330, RO).

Botryosphaeria ribis Grossenb. & Duggar, Tech. Bull. N.Y. Agric. Exp. St. 18:128. 1911. FIGS. 8-14

Anamorph. ***Fusicoccum ribis*** Slippers, Crous, M.J. Wingf., sp. nov.

Ascomata aggregata 5--50-ni, pseudoperitheciales, botryosa, globosa ostiolo centrali, papillata vel non, brunnea vel nigra, 175--250 µm, pariete pseudothecii 5--15 stratis texturae angularis composita, stratis exterioribus atrobrunneis vel nigris, cum 2--4 stratis cellularum hyalinarum cavitatem saepientibus. Pycnidia in stromate eisdem quibus ascomatis, et illis simillimis, vel singularia, in surculis juvenibus hospitis inclusa. Conidia unicellularia, fusiformia, interdum irregulariter fusiformia, basin subtruncata vel obtuse rotundata, hyalina, granularia, superficiebus levibus, raro cum aetate septata, (16--)19--23(--24) x 5--6(--7) µm. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 6--22 x 2--5 µm, percurrenter cum 1--2 proliferationibus prolificentes. Pycnidia (in vitro in 'WA' in surculis sterilifactis specierum generum *Mali, Eucalypti, Populi* que, vel foliis *Pini* intra dies 7--14 facta) superficialia, globosa, plerumque solitaria vel bini ad quaterni aggregata mycelio tecta. Conidia a fructificationibus istis, illis in vivo factis similia sed breviora, late fusiformia vel ovoidea, forma regulariores, semel vel bis septata, aetate pallide brunnea, post emissionem (15--)16--20 x 5--6(--7) µm.

Ascostroma erumpent through the bark, pulvinate, 100--400 µm in diam. *Ascomata* pseudothecial, forming botryose aggregate of 5--50 structures, globose with central ostiole, papillate or not, brown to black, 175--250 µm, pseudothecial wall comprising 5--15 layers of *textura angularis*, outer region of dark brown or brown cells, inner region 2--4 layers of hyaline cells lining the locule. *Asci* bitunicate, clavate, 80--120 x 17--20 µm, 8-spored, between numerous filiform, septate, rarely branched towards the tip, pseudoparaphyses, 2--4 µm wide. *Ascospores* fusoid to ellipsoid, often round at the ends then broadly ellipsoidal, (14--)18--23(27) x 6--8(--10) µm (average of 80 ascospores 20.5 x 7.1 µm, l/w 2.9), hyaline, unicellular, smooth with granular contents, biseriate in the ascus. *Pycnidia* in same stromata as ascomata and morphologically indistinguishable from them, or solitary and imbedded in young host shoots. *Conidia* fusiform, sometimes irregularly fusiform, base subtruncate to blunt, (16--)19--23(--24) x 5--6(--7) µm (average of 90 conidia 20.8 x 5.5 µm, l/w 3.8), hyaline, unicellular, rarely septate with age, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 6--22 x 2--5 µm, proliferating percurrently with 1--2 proliferations with periclinical thickening. Spermatia not seen.

Cultural characteristics. Colonies white to olivaceous buff (21'''d), becoming olivaceous grey (21'''''I) to violaceous black (65''''k), sectors often becoming rapidly darker and remaining darker than the rest of the culture, with very thick, felty mycelial mat from the surface to the lid, and smooth margin, but those of darker sections appearing lacinate. Optimum temperature for growth 25 C, colony reaching 65 mm radius on PDA after 4 d at 25 C in the dark. *Pycnidia* (formed on WA on sterilized twigs of apple (*Malus* sp.), *Eucalyptus* sp., *Populus* sp., or needles of *Pinus* sp. within 7--14 d) superficial, globose, mostly solitary or in aggregates of 2--4 and covered by mycelium. *Conidia* similar to those formed on the host, but shorter, broadly fusiform to ovoid and more regular in shape, occasionally 1--2 septate and light brown upon aging after discharge, (15--)16--20 x 5--6(--7) µm (average of 85 conidia 17.2 x 5.5 µm, l/w 3.1).

Specimens examined. USA. NEW YORK: Geneva, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [LECTOTYPE of teleomorph CUP-A-(F.Col. 3408)]; Milton, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [CUP-A-(F.Col. 3407)]; Milton, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [CUP-A-(F.Col. 3409)]; Ithaca, *Ribes* sp., 2000, *G. Hudler* (HOLOTYPE of anamorph,

PREM57368 culture CMW7772); Ithaca, *Ribes* sp., 2000, *G. Hudler* (PREM57369, culture CMW7773).

DISCUSSION

In this study, *B. dothidea*, *B. ribis* and *B. parva* are distinguished from each other and characterized based on morphological features. Epitype material is identified to complement lectotype and syntype material of *B. dothidea,* the type species of *Botryosphaeria*. Ex-type cultures from designated type specimens and other representative specimens has made it possible to confirm the identity of the groups of isolates that represent these taxa, through sequence data derived in this and other studies.

The type specimen of *B. dothidea* needed careful re-examination to clarify confusion regarding its name. In the original description by Mougeot (in Fries 1823, as *Sphaeria dothidea*), no specimen was designated as type, but reference was made to a collection from fallen branches of *Fraxinus* sp. This material appears to be lost, as the only material under this name from the Fries herbarium (which has been viewed as the type before; annotated by AJL Phillips and JA von Arx with the sample) contains only material from what appears to be a *Rosa* sp. The holotype material from *Fraxinus* could also not be located in other herbaria that house collections of Mougeot. Given that no type material exists, a neotype was designated here for the remaining *S. dothidea* sample from the Fries collection (Greuter et al 2000 ICBN articles 9.6 and 9.11). This material is, however, immature as noted by other researchers (von Arx and Müller 1954; note by AJL Phillips with the sample), and thus does not bear characteristics that would make it possible to clearly define the name.

As part of the description of *B. dothidea* by Cesati and De Notaris (1863), two additional specimens, one from *Fraxinus* sp. collected by Koch, and one from *Robinia pseudoacacia* collected by Lasch, were cited. The type sheet bearing *B. dothidea* samples collected by Koch and Lasch also contains samples from *Populus* sp. (the inscription indicating the host species is not clear) and *Fraxinus* sp. collected by them. All materials are, however, also immature, as is true for the collection from Fries' herbarium, or poor, and might even contain fruiting structures of more than one fungal species. This is not surprising, as spore morphology was not a critical characteristic used in descriptions of different species by either Fries (1823) or Cesati and De Notaris

(1863). Johnson (1992) also made reference to the immature and degraded state of the material, but reported seeing one ascospore.

The type and other early specimens of *B. dothidea* mentioned in this study are not sufficient to characterize this species. This is due to the poor state of development of structures on specimens, poor preservation of characters, and overlapping that exists in the morphological characteristics of *Botryosphaeria* spp. For these reasons, an epitype sample has been selected. Care has been taken with the selection of this epitype and the accompanying description to consider all aspects of the protologue, as well as to preserve the current usage of the name *B. dothidea* (Greuter et al 2000 ICBN article 9.7 and recommendation 9A). The epitype was selected from the same hosts and areas that the Cesati and De Notaris (1863) collections were made. Live cultures from this sample are deposited in culture collections (CMW, CBS).

We accept the synonymy of *B. berengeriana* and *B. dothidea* by von Arx and Müller (1954). In reviewing the original description, sketches and holotype material of *B. berengeriana*, and comparing these with the epitype of *B. dothidea*, no morphological or other reason could be found to resurrect *B. berengeriana*. The original description (De Notaris 1863) is very detailed and the ascomata on *Rhamnus frangula*, relatively well preserved. The original separation of this species from *B. dothidea* was most likely due to the variation in host and some variation in ascocarp morphology. Currently, however, we know that some *Botryosphaeria* spp. are not host specific. Furthermore, von Arx and Müller (1954) noted, and it is confirmed in this study, that variation can exist in the ascomatal and pycnidial morphology (e.g. size, aggregation and imbedding in tissue) of one *Botryosphaeria* sp. on different parts of one plant and between hosts.

Fusicoccum aesculi is generally accepted as the anamorph of *B. dothidea sensu* von Arx and Müller (1954). The separation of *B. dothidea* into at least two species, raises the question of the true identity of the anamorph of *B. dothidea*. The anamorph of the epitype of *B. dothidea* was thus studied *in vivo* and *in vitro*. The characteristics of the conidia and other morphological structures are in accordance with the amended description of *F. aesculi* (Crous and Palm 1999). The designation of *F. aesculi* as the anamorph of *B. dothidea*, as defined in this study, is thus accepted.

The type specimen of *B. ribis* is well preserved, providing ample material to define this taxon. There are, however, no cultures linked to this material. Freshly collected material from *Ribes* sp. in Ithaca, New York, the same host and area from

which the lectotype of *B. ribis* was collected, contained only anamorph structures and conidia and could thus not be designated as epitype of the teleomorph (Greuter et al 2000 ICBN article 59.2). The structures on this material were, however, morphologically identical to anamorph structures and conidia on the lectotype specimen. The isolates from this freshly collected material are thus accepted as representing *B. ribis*.

Two cultural forms of *B. ribis* were described by Grossenbacher and Duggar (1911). The chromagena variant produced a reddish pigment when grown on starch media in diffuse daylight, while the achromagena variant did not. One isolate from *Ribes* sp. (CMW7054) was designated as a chromagena variant and produced a pigment similar to that described above, but not regularly, perhaps due to the age of the isolate as suggested by Witcher and Clayton (1963). No other *B. ribis* isolates used in this study produced such a pigment under artificial light or daylight and they would thus all be classified as achromagena variants. This characteristic was initially believed to relate to pathogenicity (Grossenbacher and Duggar 1911, Stevens and Jenkins 1924), but this notion was later rejected (Witcher and Clayton 1963). These designations are no longer used and are viewed as representative of intraspecific variation.

Grossenbacher and Duggar (1911) described a 'simple or *Macrophoma* stylosporic form' and a 'compound stylosporic or *Dothiorella* form' associated with *B. ribis*. These forms were separated based on whether the pycnidia were solitary, pycnidial and imbedded (on young succulent shoots) or botryose, stromatic and erumpent (on older more woody material). The former structures were also reported to have slightly smaller conidia. Re-examination of the material, however, revealed that the spores of these two morphological forms are of the same average dimension. *Botryosphaeria* spp. are known to display variation in the morphology of conidiomata on different parts or developmental stages of the same host (von Arx and Müller 1954, Phillips et al 2002). We, therefore, view these two forms described by Grossenbacher and Duggar (1911) as representing different characteristics of the same species.

The anamorph of *B. ribis* was not named in the description by Grossenbacher and Duggar (1911). These authors argued that the anamorph of *B. ribis* was not *Dothiorella ribis* (Fuckel) Sacc. or *D. ribicola* Ellis & Barthol., but did not provide a name for it. The morphological and molecular data provided in this and other studies (Morgan-Jones and White 1987, Rayachhetry et al 1996, Denman et al 2000), however, show that the anamorph of *B. ribis* is a species of *Fusicoccum*. We have chosen to provide the name

F. ribis for this element of the holomorph. Lectotype material of the teleomorph also contains anamorph structures. Freshly collected material is, however, designated here as holotype for the anamorph name, to also allow characterization of living isolates and so preserve all features of this taxon. Although it might be argued that an anamorph name is not strictly necessary, the fungus is most commonly seen as the anamorph in the laboratory. We believe that having a name for this state will be useful. This is especially true because many apparently new species of *Fusicoccum* are currently being discovered and only the anamorph is known for them.

In addition to *B. ribis*, we have obtained isolates of *B. obtusa* (*Diplodia* anamorph) from *Ribes* sp. in New York. Grossenbacher and Duggar (1911) encountered a dark-spored sphaeropsis-like fungus on *Ribes*, which they did not study. It is, however, possible that these researchers inadvertently isolated this fungus as an endophyte, because not all their cultures were from spores or conidia. *Botryosphaeria* spp. are known to occur commonly as endophytes in many woody plants (Fisher et al 1993, Smith et al 1996). It is thus possible that some of the variation in cultural morphology described in the experiments of Grossenbacher and Duggar (1911) could be due to the presence of a second species of *Botryosphaeria*, viz. *B. obtusa*.

Sequence data for the three gene regions used in this study show clearly that isolates of *B. ribis* and *B. parva*, respectively, reside in two clades. In contrast, data from the ITS, mt-SSU-rDNA gene sequence, and RAPD data (Smith and Stanosz 2001, Zhou and Stanosz 2001a), did not distinguish *B. parva* from *B. ribis*, and it was suggested that these species may be synonyms. Yet again, a study using Inter Simple Sequence Repeat (ISSR) markers separated these two species (Zhou et al 2001). Our data, based on a multiple gene genealogy, strongly support the view that *B. ribis* and *B. parva* are distinct and probably recently derived.

Some of the unique polymorphisms detected in the sequence data of this study, and that distinguish *B. ribis* from *B. parva*, are repetitive elements in the intron regions. Such elements can be highly polymorphic within species (Carbone et al 1999, Fisher et al 2000). Since the three isolates representing *B. ribis* were all collected from the same site and host (*Ribes* from New York), the variation that we observed at these sites might reflect the presence of a founder population. This is especially true given that preliminary data suggest that these fungi are non-outcrossing (H van Geuns, B Slippers, and S Denman unpubl.), and that *Ribes* spp. have been introduced into the New York area. Furthermore, there is also sequence variation among isolates in the *B. parva* clade.

These results call for a study of a wider collection of isolates, using co-dominant markers to determine possible gene flow and boundaries between groups of isolates that represent these species.

The morphological description of *B. parva* (Pennycook and Samuels 1985) is indistinguishable from that of *B. ribis* by Grossenbacher and Duggar (1911) and Punithalingam and Holliday (1973), except that the conidia and ascospores in the former species are reportedly wider and slightly shorter. These differences were not evident in the present study, and the characteristics overlapped between the species. Pennycook and Samuels (1985), however, did not consider the synonymy of these species, as they treated *B. ribis* as a synonym of *B. dothidea sensu* von Arx and Müller (1954). These authors and Punithalingam and Holliday (1973) refer to septation in older spores. Our observations show that the pattern of septation and discoloration in older, discharged conidia formed in culture is consistent with the separation of *B. ribis* and *B. parva* based on sequence data. Aging conidia of both species become one to two septate and light brown after being discharged from the pycnidium. Conidia of *B. ribis* are, however, commonly one-septate and dark walls are evenly spread. In contrast, conidia of *B. parva* are more regularly two septate, with conspicuously darker brown middle cells. However, care must be taken in making these observations because not all conidia darken and become septate, even after discharge and where cultures are left to age for two months.

Isolates residing in the *B. ribis*/*B. parva* clade reportedly form microconidia or spermatia (Pennycook and Samuels 1985, Rayachhetry et al 1996). These structures are not common and were not observed in the present study. Phillips et al (2002) also recently reported spermatia of similar dimensions to those described for *B. dothidea*, formed by some isolates of *B. lutea.* Spermatia have not been reported from *B. dothidea.* In the present study, microconidia were, however, formed in anamorph fruiting structures of *B. dothidea* from *Ostrya* sp. This characteristic appears to be insufficiently consistent to be useful in distinguishing between the *B. ribis, B. parva*, and *B. dothidea.*

The *Botryosphaeria* spp. considered in this study resided in two major and well resolved clades, based on the combined sequence datasets of the ITS rDNA, β-tubulin, and EF1-α. These clades correspond to the anamorph genera with hyaline conidia in *Fusicoccum* and those with dematiaceous conidia in *Diplodia sensu lato*. These two groups have also been identified in previous studies (Denman et al 2000, Zhou and

Stanosz 2001a). Despite a report to the contrary (Zhou and Stanosz 2001b), the fusicoccum- and diplodia-like anamorph conidium phenotype, therefore, seem to be consistent with major evolutionary events in *Botryosphaeria*.

Based on the combined sequence data sets used in this study, *B. rhodina* (anamorph = *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.) grouped with other species having *Diplodia* anamorphs. These combined and separately analyzed data sets also showed that this species groups separately within the larger *Diplodia* clade, in which *B. obtusa*, *B. stevensii*, and *B. quercuum* group closely. The conidial morphology of *B. rhodina* is similar to that of other *Diplodia* spp., but conidia are also unique in having conspicuous longitudinal striations. Our data thus suggest that conidial striations are definitive at the species level, and should not be used to distinguish genera within *Botryosphaeria* or its anamorphs.

Botryosphaeria dothidea, *B. ribis*, and *B. parva* can clearly be distinguished based on morphological and DNA sequence data. However, when considering morphology, care should be taken to examine a sufficiently large number of samples to compensate for the fact that some characteristics overlap significantly. For *in vitro* studies, sporulating cultures should be allowed to age for at least three weeks and preferably longer, because septation of discharged, aged spores is useful in separating some species. Recently collected isolates should preferably be used because cultures can lose their useful characteristics and ability to sporulate after repeated sub-culturing. We thus rely strongly on sequence data linked to morphologically defined groups to confirm their identity. For phylogenetic studies of closely related species such as *B. ribis* and *B. parva*, more than one gene region should be used. However, there is a preponderance of ITS rDNA sequence data for *Botryosphaeria* spp. in public databases and this appears to be sufficient to identify the major clades.

KEY TO *B. DOTHIDEA, B. RIBIS, B. PARVA* AND *B. LUTEA*

Botryosphaeria lutea is included in the key, as it is closely related and commonly encountered in comparisons with the other species studied here (Jacobs and Rehner 1998, Zhou and Stanosz 2001a, Smith and Stanosz 2001, Phillips et al 2002). Data for *B. lutea* in this key are derived from Pennycook and Samuels (1985) and Phillips et al (2002) and were confirmed in this study.

1. Conidia in culture averaging <18 µm long, l/w ±3, colony on MEA or PDA thick felt of grey aerial mycelium 2

1. Conidia in culture averaging ≥20 µm long, l/w >3, colony on MEA or PDA appressed with occasional tufts of grey to buff aerial mycelium.....3

2. Conidia 15--20 x 5--7 µm, becoming light brown and septate after discharge.....***B. ribis***

2. Conidia 12--23 x 4--6 µm, frequently becoming light brown and one to two septate with a darker brown middle cell after discharge.....***B. parva***

3. Conidia fusiform to irregularly rod-shaped, 15--30 x 5--8 µm (average 22 x 6 µm), l/w 3--4, colony on MEA or PDA producing distinct yellow pigment after three days, becoming dull brown to buff with age.....***B. lutea***

3. Conidia narrowly fusiform, 19--30 x 4--6 µm (average 25 x 5 µm), l/w3.5--6, colonies on MEA or PDA not producing yellow pigment and becoming grey to black with age.....***B. dothidea***

LITERATURE CITED

Arx JA von. 1987. Plant pathogenic fungi. Beheifte zur Nova Hedwigia 87. Berlin, Germany: J. Cramer. 288 p.

---, Müller E. 1954. Die Gattungen der amerosporen Pyrenomyceten. Beitr Kryptogamenfl Schweiz 11(1):1--434.

---, ---. 1975. A re-evaluation of the bitunicate Ascomycetes with keys to families and genera. Stud Mycol 9:1--159.

Barr ME. 1972. Preliminary studies on the Dothideales in temperate North America. Contr Univ Mich Herb 9:523--638.

Brown EA, Britton KO. 1986. *Botryosphaeria* diseases of apple and peach in the Southeastern United States. Plant Dis 70:480--484.

Carbone I, Anderson JB, Kohn LM. 1999. A method for designing primer sets for the speciation studies in filamentous ascomycetes. Mycologia 91:553--556.

Cesati V, De Notaris G. 1863. Schema di classificazione degle sferiacei italici aschigeri piu' o meno appartenenti al genere *Sphaeria* nell'antico significato attribuitoglide Persoon. Comment Soc Crittog Ital 1, 4:177--240.

Crous PW, Palm ME. 1999. Reassessment of the anamorph genera *Botryodiplodia*, *Dothiorella* and *Fusicoccum*. Sydowia 52:167--175.

Denman S, Crous PW, Wingfield MJ. 1999. A taxonomic reassessment of *Phyllachora proteae*, a leaf pathogen of *Proteaceae*. Mycologia 91:510--516.

---, ---, Taylor JE, Kang JC, Pascoe I, Wingfield MJ. 2000. An overview of the taxonomic history of *Botryosphaeria* and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Stud Mycol 45:129--140.

De Notaris G. 1863. Sferiacei Italici. Genova: Tipi del R.I. de' Sordo-Muti. pp. 82--84.

English H, Davis JR, DeVay JE. 1975. Relationship of *Botryosphaeria dothidea* and *Hendersonula toruloidea* to a canker disease of almond. Phytopathology 65:114--122.

Eriksson T. 1998. Autodecay ver. 4.0 (program distributed by the author). Department of Botany, Stockholm University, Stockholm.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Testing significance of incongruence. Cladistics 10:315--319.

Felsenstein J. 1985. Confidence intervals on phylogenetics: an approach using bootstrap. Evolution 39:783--791.

Fisher MC, Koenig G, White TJ, Taylor JW. 2000. A test for concordance between the multilocus genealogies of genes and microsatelites in the pathogenic fungus *Coccidioides immitis*. Mol Biol Evol 17:1164--1174.

Fisher PJ, Petrini O, Sutton BC. 1993. A comparative study of fungal endophytes in leaves, xylem and bark of *Eucalyptus nitens* in Australia and England. Sydowia 45:1--14.

Fries E. 1823. Systema Mycolgicum. 2(2):423--424.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61:1323--1330.

Greuter W, McNeill J, Barrie FR, Burdet HM, Démoulin V, Filgueiras TS, Nicolson DH, Silva PC, Skog JE, Trehane P, Turland NJ, Hawksworth DL. 2000. International Code of Botanical Nomenclature (Saint Louis Code). XVI International Botanical Congress, St. Louis, Missouri, July-August 2000. Königstein, Germany: Koeltz Scientific Books. 389 p.

Grossenbacher JG, Duggar BM. 1911. A contribution to the life-history, parasitism, and biology of *Botryosphaeria ribis*. NY Agric Exp Station, Tech Bull 18:113--190.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J Hered 83:189--195.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. TREE 11:152--158.

Jacobs KA, Rehner SA. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90:601--610.

Johnson GI. 1992. Biology and control of stem end rot pathogens of mango [PhD thesis]. Queensland, Australia: University of Queensland. 265 p.

Maas JL, Uecker FA. 1984. *Botryosphaeria dothidea* cane canker of thornless blackberry. Plant Dis 68:720--726.

Morgan-Jones G, White JF Jr. 1987. Notes on Coelomycetes. II. Concerning the *Fusicoccum* anamorph of *Botryosphaeria ribis*. Mycotaxon 30:117--125.

O'Donnell K, Cigelnik L. 1997. Two divergent intragenomic rDNA ITS2 types within the monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7:103--116.

Ogata T, Sano T, Harada Y. 2000. *Botryosphaeria* spp. isolated from apple and several deciduous fruit trees are divided into three groups based on the production of warts on twigs, size of conidia, and nucleotide sequences of nuclear ribosomal DNA ITS regions. Mycoscience 41:331--337.

Pennycook SR, Samuels GJ. 1985. *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. Mycotaxon 24:445--458.

Phillips AJL, Fonseca F, Povoa V, Castilho R, Nolasco G. 2002. A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp. nov. Sydowia 54:59--77.

Punithalingam E, Holliday P. 1973. *Botryosphaeria ribis*. CMI descriptions of pathogenic fungi and bacteria, No. 395. Kew, Surrey, England: Commonwealth Mycological Institute. 2 p.

Putterill VA. 1919. A new apple tree canker. S Afr J Sci 16:258--271.

Raeder U, Broda P. 1985. Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17--20.

Rayachhetry MB, Blakeslee GM, Webb RS, Kimbrough JW. 1996. Characteristics of the *Fusicoccum* anamorph of *Botryosphaeria ribis*, a potential biological control agent for *Melaleuca quinquenervia* in South Florida. Mycologia 88:239--248.

Rayner RW. 1970. A mycological colour chart. Kew, Surrey, UK: CMI and British Mycological Society. 34 p.

Saccardo PA. 1877. Fungi Veneti novi vel critici. Michelia 1:1--72.

---. 1882. Sylloge fungorum omnium hucusque cognitorum 1:456--466.

Sassa T, Ishizaki A, Nukina M, Ikeda M, Sugiyama T. 1998. Isolation and identification of new antifungal macrophorins E, F and G as malonyl meroterpenes from *Botryosphaeria berengeriana*. Biosci Biotech Bioch 62:2260--2262.

Shoemaker RA. 1964. Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Can J Bot 42:1297--1301.

Smith H, Kemp GHJ, Wingfield MJ. 1994. Canker and die-back of *Eucalyptus* in South Africa caused by *Botryosphaeria dothidea*. Plant Pathol 43:1031--1034.

---, Wingfield MJ, Crous PW, Coutinho TA. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. S Afr J Bot 62:86--88.

---, Crous PW, Wingfield MJ, Coutinho TA, Wingfield BD. 2001. *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. Mycologia 93:277--284.

Smith DR, Stanosz GR. 2001. Molecular and morphological differentiation of *Botryosphaeria dothidea* (anamorph *Fusicoccum aesculi*) from some other fungi with *Fusicoccum* anamorphs. Mycologia 93:505--515.

Spiers AG. 1977. *Botryosphaeria dothidea* infection of *Salix* species in New Zealand. Plant Dis Rep 61:664--667.

Stevens NE, Jenkins AE. 1924. Occurrence of the current cane blight fungus on other hosts. J Agric Res 27:837--844.

Sutton BC. 1980. The Coelomycetes, Fungi Inperfecti with Pycnidia, Acervuli and Stromata. Kew, Surrey, England: Commonwealth Mycological Institute. 696 p.

Swofford DL. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21--32.

Trotter A. 1928. Sylloge fungorum omnium hucusque cognitorum 24(2):810--815.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Snisky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315--322.

Winter G. 1886. Ascomyceten: Gymnoasceen und Pyrenomyceten. Rabenh Krypt-Fl II (band 1) abth2:800--803.

Witcher W, Clayton CN. 1963. Blueberry stem blight caused by *Botryosphaeria dothidea* (*B. ribis*). Phytopathology 42:521--525.

Zhou S, Smith DR, Stanosz GR. 2001. Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. Mycol Res 105:919--926.

---, Stanosz GR. 2001a. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93:516--527.

---, ---. 2001b. Primers for amplification of mt SSU rDNA, and a phylogenetic study of *Botryosphaeria* and associated anamorphic fungi. Mycol Res 105:1033--1044.

TABLE I. Isolates of *Botryosphaeria* and *Guignardia* species considered in the phylogenetic study.

Culture no.[1,2]	Other no.[1]	Identity[3]	Host	Location	Collector	GenBank[4]		
						ITS	β tubulin	EF-1α
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236935	AY236906	AY236877
CMW7773		*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236936	AY236907	AY236878
CMW7054	CBS121	*B. ribis*	*R. rubrum*	New York, USA	N.E. Stevens	*AF241177*	AY236908	AY236879
CMW994	ATCC58189	*B. parva*	*Malus sylvestris*	New Zealand	G.J. Samuels	*AF243395*	AY236912	AY236883
CMW9077	ICMP7924	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236939	AY236913	AY236884
CMW9078	ICMP7925	*B. parva*	*A. deliciosa*	New Zealand	S.R. Pennycook	AY236940	AY236914	AY236885
CMW9079	ICMP7933	*B. parva*	*A. deliciosa*	New Zealand	S.R. Pennycook	AY236941	AY236915	AY236886
CMW9080	ICMP8002	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236942	AY236916	AY236887
CMW9081	ICMP8003	*B. parva*	*P. nigra*	New Zealand	G.J. Samuels	AY236943	AY236917	AY236888
CMW10122	BOT21	*B. parva*	*Eucalyptus grandis*	Mpumalanga, S. Africa	H. Smith	*AF283681*	AY236911	AY236882
CMW1130		*B. parva*	*Sequoia gigantea*	Hogsback, S. Africa	W. Swart	AY236945	AY236919	AY236890
CMW10123	BOT19	*B. parva*	*E. smithii*	Mpumalanga, S. Africa	H. Smith	*AF283683*	AY236910	AY236881
CMW10124	BOT681	*B. parva*	*Heteropyxis natalensis*	Kwazulu-Natal, S. Africa	H. Smith	*AF283676*		
CMW4049		*B. parva*	*E. grandis*	Sumatra, Indonesia	M.J. Wingfield	AY236937		
CMW9071		*B. parva*	*Ribes* sp.	Australia	M.J. Wingfield	AY236938	AY236909	AY236880
CMW7885		*B. parva*	*Eucalyptus* sp.	Hawaii	M.J. Wingfield	AY236944	AY236918	AY236889
	KJ94.09	*B. ribis / B. parva*	*Melaleuca quinquenervia*	Florida, USA	M.B. Rayachhetry	*AF027743*		
	KJ93.03	*B. ribis / B. parva*	*Cercis canadensis*	District of Columbia, USA	K.A. Jacobs	*AF027742*		
CMW10125	BOT24	*B. eucalyptorum*	*E. grandis*	Mpumalanga, S. Africa	H. Smith	*AF283686*	AY236920	AY236891
CMW10126	BOT16	*B. eucalyptorum*	*E. grandis*	Mpumalanga, S. Africa	H. Smith	*AF283687*	AY236921	AY236892
	KJ93.12	*B. dothidea*	*Prunus* sp.	District of Columbia, USA	K.A. Jacobs	*AF027746*		
	KJ94.26	*B. dothidea*	*P. persica*	Japan	P.L. Pusey	*AF027749*		
	KJ93.23	*B. dothidea*	*Syringa vulgaris*	Maryland, USA	K.A. Jacobs	*AF027751*		
CMW991	ATCC58188	*B. dothidea*	*P. nigra*	New Zealand	G.J. Samuels	*AF241175*	AY236924	AY236895

TABLE I. Continued.

Culture no.[1,2]	Other no.[1]	Identity[3]	Host	Location	Collector	GenBank[4]		
						ITS	β tubulin	EF-1α
CMW9075	ICMP8019	*B. dothidea*	*P. nigra*	New Zealand	G.J. Samuels	AY236950	AY236928	AY236899
CMW7780		*B. dothidea*	*Fraxinus excelsior*	Molinizza, Switzerland	B. Slippers	AY236947	AY236925	AY236896
CMW7999		*B. dothidea*	*Ostrya* sp.	Crocifisso, Switzerland	B. Slippers	AY236948	AY236926	AY236897
CMW8000		*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236927	AY236898
	ATCC58194	*B. lutea*	*Malus x domestica*	New Zealand	G.J. Samuels	*AF243396*		
CMW992/3	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels	*AF027745*	AY236923	AY236894
CMW9076	ICMP7818	*B. lutea*	*Malus x domestica*	New Zealand	S.R. Pennycook	AY236946	AY236922	AY236893
	ZS97-59	*B. mamane*	*Sophora chrysophylla*	Hawaii	D. Gardner	*AF246930*		
	ATCC22929	*B. corticis*	*Vaccinium* sp.	North Carolina, USA	R.D. Milholland	*AF243397*		
	KJ93.29	*B. quercuum*	*Quercus* sp.	California, USA	E. Hecht-Poinar	*AF027753*		
CMW7062	CBS177.89	*B. quercuum*	*Q. cerris*	Italy	A. Vannini	*AF243399*		
CMW7060	CBS 431	*B. stevensii*	*F. excelsior*	Netherlands	H.A. van der Aa	AY236955	AY236933	AY236904
	ATCC60259	*B. stevensii*	*M. pumila*	Unknown	H.J. Boesewinkel	*AF243406*		
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236953	AY236931	AY236902
CMW7775		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236954	AY236932	AY236903
	KJ93.56	*B. obtusa*	Hardwood shrub	New York, USA	G.J. Samuels	*AF027759*		
	KJ93.41	*B. rhodina*	*Pistacia* sp.	California, USA	T.J. Michailides	*AF027762*		
CMW10130	BOT977	*B. rhodina*	*Vitex donniana*	Uganda	J. Roux	AY236951	AY236929	AY236900
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236930	AY236901
CMW7063	CBS447	*Guignardia philoprina*	*Taxus baccata*	Netherlands	H.A. van der Aa	AY236956	AY236934	AY236905

[1] Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection, Fairfax, VA, USA; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; ZS = Zhou and Stanosz (2001a).

[2] Isolates in bold are ex-type (CMW7772; CMW8000; CMW9081) or from samples that have been linked morphologically to type material of the species.

[3] Identities as determined in this study.

[4] ITS sequences represented by the 22 numbers in italics were obtained from GenBank. The remaining 75 sequences were determined in the present study.

TABLE II. A comparison of the epitype specimens with holotype material and descriptions of *Sphaeria dothidea*, *Botryosphaeria dothidea* and *B. berengeriana*.

	Fries 1823[1]	Cesati and De Notaris 1863[2]	De Notaris 1863	Pennycook and Samuels 1985[3]	Epitype
Ascostromata					
Position in Substratum	Erumpent	Erumpent	Erumpent	Erumpent	Erumpent
Shape or Appearance	Oblong	Cushion- or disk like or elongate	Disk-like	Caespitose clusters (2--5 mm)	Botryose clusters or cushion-like when young
Color	'Bleek' dark-brown	Lavender blue	Dark	Black	Black
Perithecia					
Position	Initially imbedded becoming sub-emerged	Top always free	Erumpent	¼ emergent	¼ to ½ emerged, but sometimes imbedded with only ostiole visible
Number	N/a	N/a	N/a	5--50(--100) per cluster	Clusters up to 100 or solitary (less common)
Colour	White contents	N/a	Black with lighter base and white contents	Black with white contents	Black with white contents
Shape	Round when immature becoming globose	N/a	Ovate-sphaerical	Globose, smooth, non-collapsing when dry	Globose, rarely irregular, non-collapsing, except in old material
Opening	N/a	Small open ostiole	Papillate with small open ostiole	Non-papillate or short conical papilla	Small open ostiole or short conical papilla
Size	N/a			150--250 µm	100--250 µm

TABLE II. Continued.

	Fries 1823[1]	Cesati and De Notaris 1863[2]	De Notaris 1863	Pennycook and Samuels 1985[3]	Epitype
Asci					
Description	N/a	8-spored	8-spored	Bitunicate, 8-spored	Bitunicate, 8-spored
Shape	N/a	Slender, clavate	Clavate, obtuse	Clavate	Clavate
Size	N/a	N/a	N/a	(65--)75--112(--140) µm	63--125 µm
Paraphyses	N/a	N/a	N/a	N/a	Interspersed between asci
Ascospores					
Description	N/a	Unicellular, 4 locules	Granular nuclues	Unicellular, smooth	Unicellular, smooth, granular contents
Colour	N/a	Hyaline	Pale	Hyaline	Hyaline
Shape	N/a	Ovoid to oblong	Subclavate	Ellipsoid to fusoid	Fusoid (rarely ovoid) to spindle shaped
Size	N/a	25.5 x 7 µm	<30 µm in length	(13--)19--27(--35) x (6--)8--11(--14) µm	(17.2--)19--24(--26.4) x (6.2--)7--8(--9.4) µm [21.9 x 7.8 µm; l/w 2.8]
Host	Fallen branches of *Fraxinus*	*Fraxinus* sp., *Rosa* sp. and *Robinia* sp.	*Rhamnus frangula*	*Populus nigra*, *Actinidia deliciosa*	*Fraxinus* sp., *Ostrya* sp., *Prunus* sp.
Area	Unknown	Northern Italy	Italy	New Zealand	Italy, Switzerland

[1] The type material in the Fries herbarium is from *Rosa* sp., while that from the description is from fallen branches of *Fraxinus*. This information is based on the description of *S. dothidea* (Fries 1823).
[2] Ascospore size from one spore found by Johnson (1992) on the samples from Cesati and De Notaris.
[3] The description of *B. dothidea* given by Pennycook and Samuels (1985) is not based on type specimens, but this description is included here as isolates from this study are used in phylogenetic analyses in this study.

TABLE III. A comparison of the holotype material and descriptions of *Botryosphaeria ribis* (Grossenbacher and Duggar 1911) and *B. parva* (Pennycook and Samuels 1985)[1].

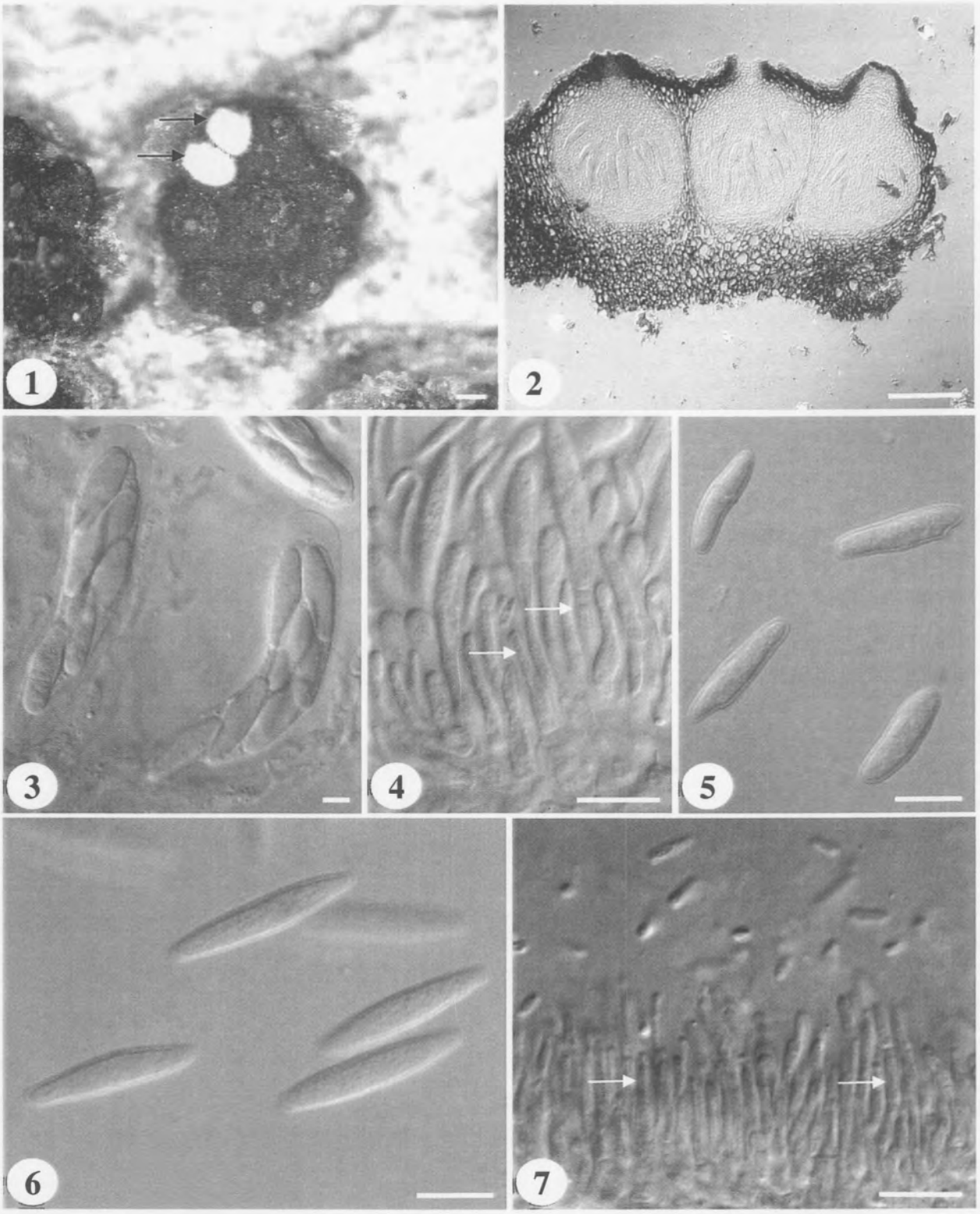
	B. ribis	*B. parva*
Ascostromata		
Position in substrate	Erumpent	Erumpent
Shape or appearance	Botryose clusters (1--4 mm)	Caespitose clusters (2--5 mm)
Ascomata		
Position	1/3 emergent, but sometimes submerged or wholly emerged	¼ emergent
Number	N/a	5--50(--100) per cluster
Colour	Black with white contents	Black with white contents
Shape	Round when immature, becoming globose	Globose, smooth, non-collapsing when dry
Opening	Papillate ostiole	Non-papillate or short conical papilla
Size	175--250 µm	150--250 µm
Asci		
Description	N/a	Bitunicate, 8-spored
Shape	Clavate	Clavate
Size	80--120 x 17--20 µm	75--143(--210) µm
Paraphyses	Filiform, interspersed between asci	
Ascospores		
Description	Unicellular	Unicellular, smooth
Color	Hyaline	Hyaline

TABLE III. Continued.

	B. ribis	*B. parva*
Shape	Fusoid	Broadly ellipsoid to fusoid
Size	(14--)18--22(--27) x 6--8(--10) µm [20.5 x 7.1 µm, l/w 2.9]	(14--)18--23(--26) x (7--)8--10(--11) µm [20.8 x 9.2 µm, l/w 2.2]
Anamorph		
General	Same stroma as teleomorph	Same stroma as teleomorph
Pycnidia (on material)	Same as ascomata or depressed globular and imbedded	Same as given for ascomata
Pycnidia (in culture)	N/a	Globose, non-papillate, single or aggregate (up to 0.5 mm diam)
Conidia	Fusoid to ellipsoid, obtuse apex and flat base, unicellular, hyaline, rarely becoming light brown with 1--2 septa	Ellipsoid with obtuse apex and flat base, unicellular, hyaline, becoming light brown with 1--2 septa with age, middle section often darker brown
Conidial size (*in vivo*)	(16--)19--22(--24) x 5--6(--7) µm [20.8 x 5.5 µm, l/w 3.8]	N/a
Conidial size (*in vitro*)	(15--)16--19(--20) x 5--6(--7) µm [17.2 x 5.5 µm, l/w 3.1]	(12--)15--19(--24) x 4--6 µm [16.9 x 5.4 µm, l/w 3.1]
Host	*Ribes* spp.	*Populus nigra*, *Malus x domestica*, *Actinidia deliciosa*, *Sequoia* sp., *Eucalyptus* spp., *Ribes* sp.
Geographic region	New York, USA	Australia, New Zealand, Hawaii, South Africa, Sumatra

[1] All information is as given in the original description and was confirmed during the current study and left unchanged, except ascospore and conidial measurements, host and area, which are given as determined in this study.

FIGS. 1-7. *Botryosphaeria dothidea*, dissecting microscope and DIC compound-microscope micrographs. 1. Botryose ascomata, from which the tops of two have been removed to show the typical white centrum contents (arrows). 2. Median, longitudinal section through a mature ascoma. Bars = 100 µm. 3. Asci and ascospores. 4. Conidiogenous cells (arrows). 5. Conidia from nature. 6. Conidia produced in culture on WA and pine needles. 7. Spermatiophores (arrows) and spermatia. Bars = 10 µm.



FIGS. 8-14. *Botryosphaeria ribis*, dissecting microscope and DIC compound-microscope micrographs. 8, 9. Ascomata. Bars = 100 µm. 10. Asci and ascospores. 11. Conidiogenous cells (arrows). 12. Conidia from nature. 13, 14. Conidia produced in culture on WA and pine needles; older conidia septate (arrows). Bars = 10 µm.

FIG. 15. *B. parva*, DIC compound-microscope micrographs. Conidia produced in culture on WA and pine needles; older conidia septate (arrows). Bar = 10 µm.

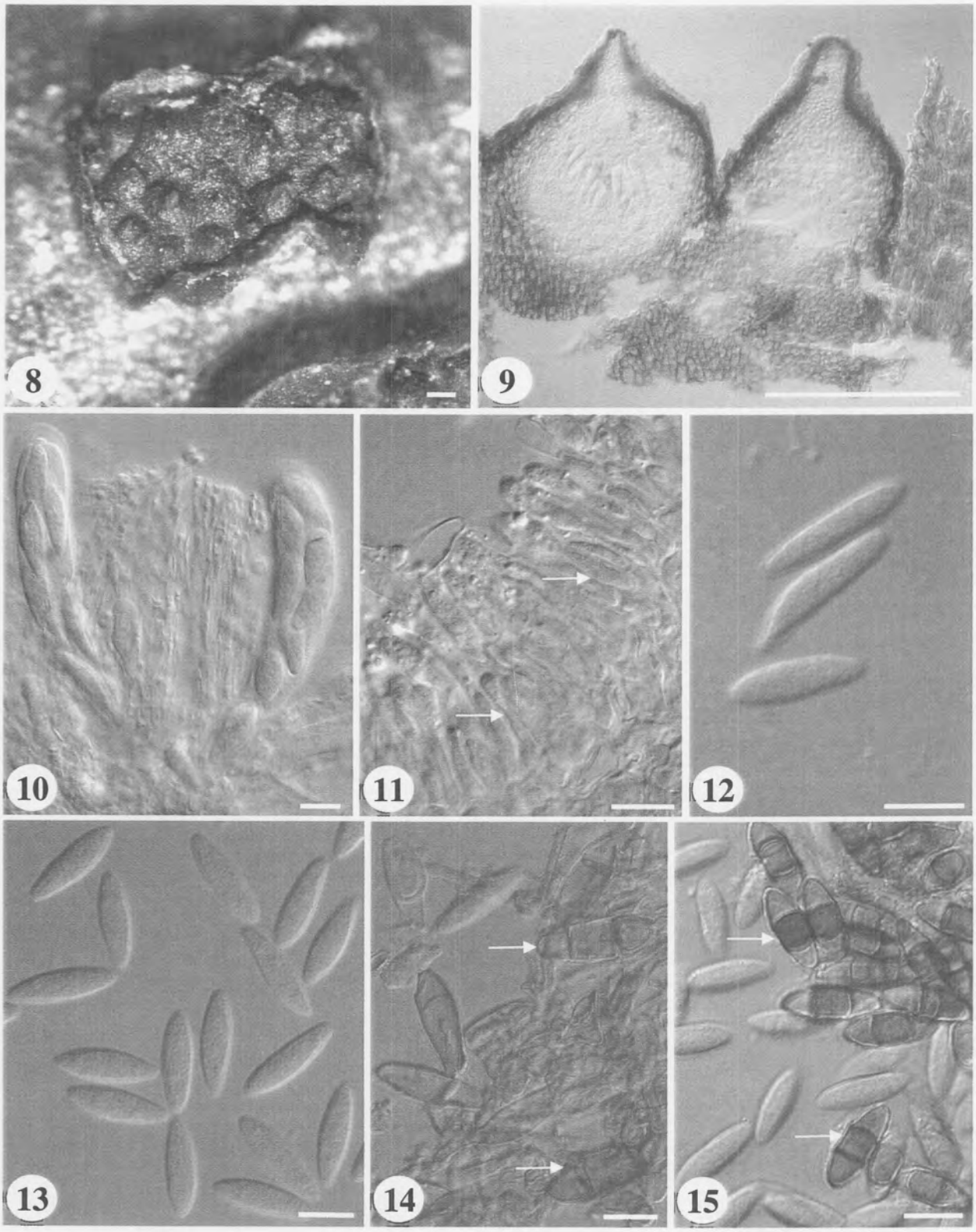


FIG. 16. Most parsimonious tree of 325 steps obtained from ITS1, 5.8S and ITS2 rDNA sequence data. Branch supports are indicated by decay indices above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to the outgroup, *Guignardia philoprina*. Clades are shaded individually and their identities are as used in this study. Host and origin (Aust = Australia, Haw = Hawaii, Ital = Italy, Jp = Japan, Neth = Netherlands, NY = New York, NZ = New Zealand, Mex = Mexico, SA = South Africa, Sum = Sumatra, Swit = Switzerland, Ug = Uganda), of each isolate are also indicated. ▒ = *Botryosphaeria* spp. with *Fusicoccum* anamorphs and ▤ = *Botryosphaeria* spp. with *Diplodia* anamorphs.

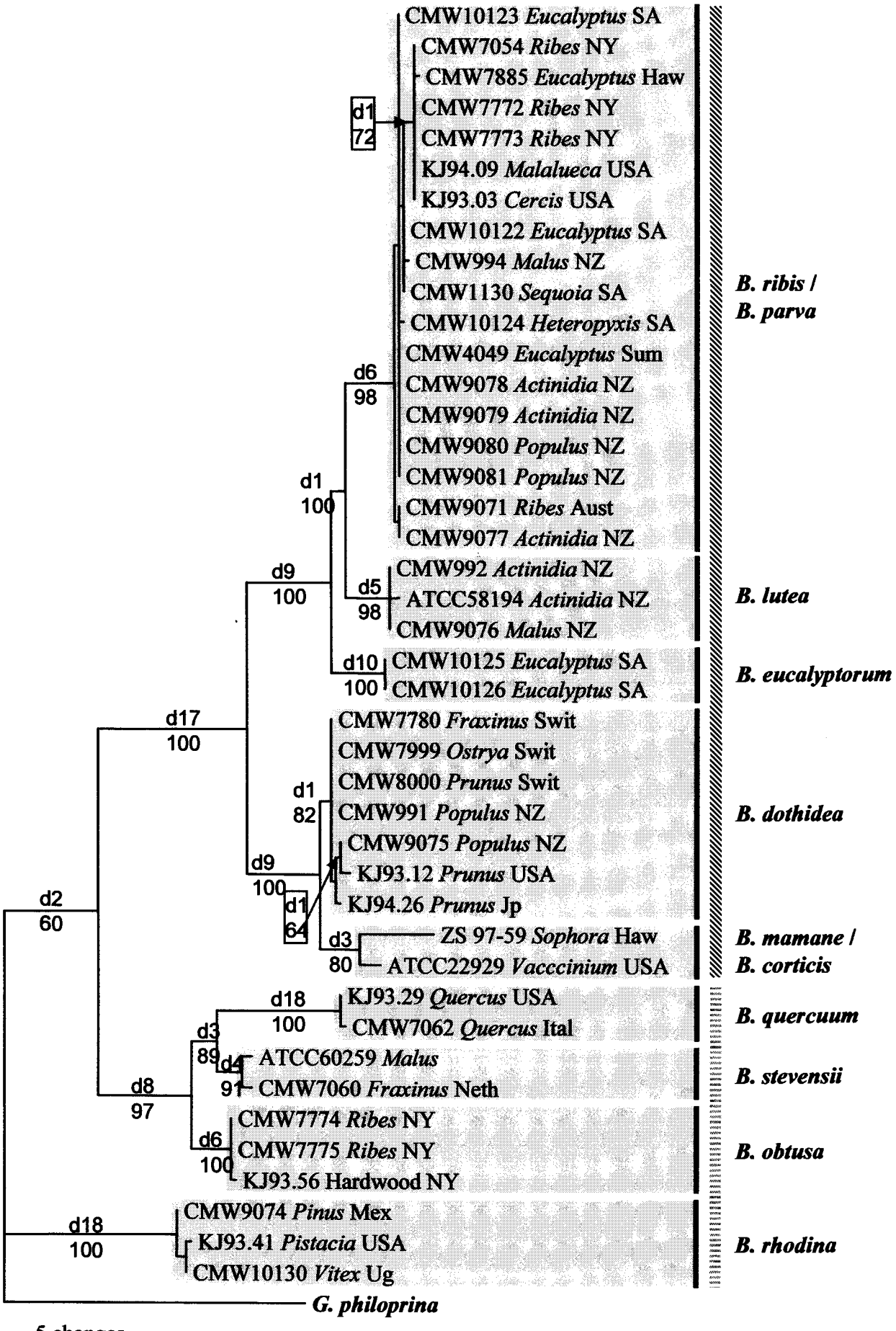
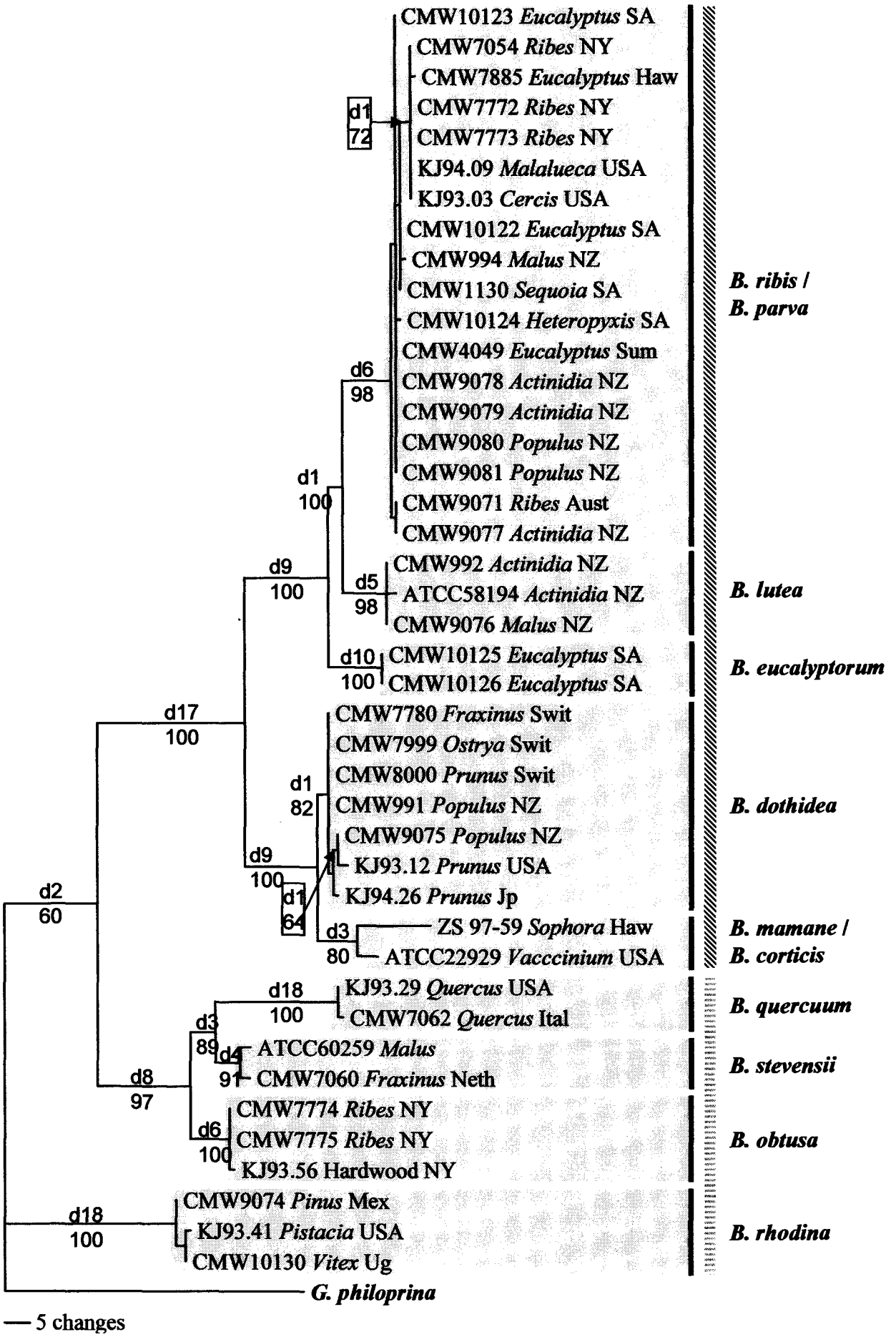


FIG. 17. Most parsimonious tree of 858 steps obtained from 5.8S and ITS2 rDNA and partial β-tubulin and EF1-α gene sequence data. Support for the branching points are given in decay values above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to the outgroup *Guignardia philoprina*. Clades are shaded individually and their identities are as used in this study. Isolates' identities are given in the order, number, host and origin (Aust = Australia, Haw = Hawaii, Neth = Netherlands, NY = New York, USA, NZ = New Zealand, Mex = Mexico, SA = South Africa, Swit = Switzerland, Ug = Uganda). ▒ = *Botryosphaeria* spp. with *Fusicoccum* anamorphs and ▤ = *Botryosphaeria* spp. with *Diplodia* anamorphs.



2

Phylogenetic and morphological re-evaluation of the *Botryosphaeria* anamorphs causing diseases of *Mangifera indica* in Australia

Abstract: Species of *Botryosphaeria* are among the most serious pathogens that affect mango trees and fruit. Several species occur on mangoes and these are identified mainly based on the morphology of the anamorphs. Common taxa include *Dothiorella dominicana*, *D. mangiferae* (= *Natrassia mangiferae*), *D. aromatica* and an unidentified species, *Dothiorella* 'long'. The genus name *Dothiorella* is, however, acknowledged as a synonym of *Diplodia*. The aim of this study was to characterise and name the *Botryosphaeria* spp. associated with disease symptoms on mangoes. To achieve this, isolates representing all four *Dothiorella* spp. mentioned above were compared with the anamorphs of known *Botryosphaeria* spp., based on conidial morphology and DNA sequence data. Two genomic regions were sequenced and analyzed, namely the ITS rDNA and β-tubulin regions. The morphological and molecular results confirmed that the fungi previously identified from mango as species of *Dothiorella*, all belong to *Fusicoccum*. *Dothiorella dominicana* isolates were identical to isolates of *F. parvum* (teleomorph = *B. parva*). A new epithet, namely *F. mangiferum*, is proposed for isolates previously treated as *D. mangiferae* or *N. mangiferae*. Isolates of *D. aromatica* were identified as *F. aesculi* (teleomorph = *B. dothidea*). A fourth *Fusicoccum* sp. was also identified as those isolates previously known as *Dothiorella* 'long'. A key is provided to distinguish these species based on anamorph morphology in culture. This study provides a basis for the identification of *Botryosphaeria* species from mango, which is important for disease control and to uphold quarantine regulations.

INTRODUCTION

Stem end rot of mango (*Mangifera indica* L.) fruit is one of the most serious diseases affecting this industry worldwide (Prakash and Srivastava 1987, Cappellini et al 1988, Prusky 1991, Mitra and Baldwin 1997). This disease is caused by a complex of fungal pathogens, of which various *Botryosphaeria* spp. are among the most dominant (Darvas 1991, Johnson et al 1991a, b, 1992, Sangchote 1991). Apart from fruit diseases, *Botryosphaeria* spp. also cause tip- and branch die-back and cankers on mango trees (Stevens 1926, Ramos et al 1991). These fungi live endophytically in healthy tissue, and mostly cause disease after stress to the trees or fruit after harvest (Johnson et al 1991a, 1992, Sangchote 1991).

Botryosphaeriaceous fungi considered as pathogens of mango trees and fruit are best known by their anamorph states. These include species of *Natrassia* B. Sutton & Dyko, *Dothiorella* Sacc., *Fusicoccum* Corda, *Diplodia* Fr. and *Lasiodiplodia* Ellis & Everh. (Johnson 1992). Some of the most commonly used names, and those considered in this study are *Dothiorella dominicana* Petr. & Cif., *D. mangiferae* Syd. & P. Syd., *D. aromatica* (Sacc.) Petr. & Syd. and an unnamed species, *Dothiorella* 'long' (Johnson 1992). These names are, however, in need of revision. *Dothiorella mangiferae* has been reduced to synonymy under *Nattrassia mangiferae* (Syd. & P. Syd.) Sutton & Dyko (Sutton and Dyko 1989). This synonymy has been recognised by some researchers (Londsdale 1992, Roux 1993), but disputed by others (Johnson 1991a, b, 1992). In addition the type species of *Dothiorella* was recently synonymised under *Diplodia*, raising questions about the correct generic affinities of all species presently placed in *Dothiorella* (Crous and Palm 1999).

Not all the *Dothiorella* spp. are of equal importance as pathogens of mango. *Dothiorella dominicana* is the most common pathogen and causes significant losses annually (Darvas 1991, Johnson et al 1991a). *Dothiorella mangiferae* is another common *Botryosphaeria* anamorph associated with mango fruit and trees world-wide, especially in Australia and Thailand (Sydow et al 1916, Johnson et al 1991a, 1992, Mitra and Baldwin 1997). *Dothiorella aromatica* (Sacc.) Petr. & Syd., and an unnamed species, *Dothiorella* 'long', have occasionally been recorded from mango in Thailand and Australia, but are of less importance (Johnson et al 1991a, Johnson 1992). *Dothiorella aromatica* has been reported from mango, but is better known as a pathogen of avocado (Johnson 1992, Johnson et al 1992, Hartill 1991).

It has been suggested that the *Dothiorella* spp. occurring on mango should all be accommodated in the genus *Fusicoccum* (Johnson 1992). In that study it is suggested that *D. dominicana* is a synonym of *F. aesculi* Corda (*B. dothidea* (Fr.: Moug.) Ces. & De Not.), and that *D. aromatica* should be renamed as *F.* "*aromaticum*", having *F. luteum* Pennycook & Samuels as synonym. He also suggested that *D. mangiferae* should be renamed as *F. 'mangiferam'*, which he considered to be the anamorph of *B. parva* Pennycook & Samuels, and that *Dothiorella* 'long' is a synonym of *F. cajani* (Syd., P. Syd. & E.J. Butler) Samuels & Singh (teleomorph *B. xanthocephala* (Syd., P. Syd. & E.J. Butler) Theissen). These synomymies were, however, never formally proposed.

Other researchers have reported a species of *Fusicoccum* from mango and avocado. Hartill (1991) examined Botryosphaeriaceous fungi from avocado in New Zealand, which have previously been described as *Dothiorella* species. He concluded that these fungi should reside in the genus *Fusicoccum* and identified *F. aesculi*, *F. parvum* Pennycook & Samuels and *F. luteum* from New Zealand collections. In California, Ramos et al (1991) reported on the presence of *Fusicoccum* anamorph of *B. ribis* Grossenb. & Duggar from mango plants.

Anamorph morphology is commonly used to identify species of *Botryosphaeria* (Shoemaker 1964, Pennycook and Samuels 1985, Jacobs and Rehner 1998, Slippers et al 2003). The morphological distinctions between some of the closely related anamorph species are, however, not always clear. Recent studies, using DNA sequence data have highlighted taxonomic groups and relationships in *Botryosphaeria* (Jacobs and Rehner 1998, Denman et al 2000, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001, Slippers et al 2003). These data combined with morphological characteristics could clarify the current taxonomic confusion. There is a clear need to use the same approach to clarify the relationships and identities of the stem end rot pathogens of mango.

The aim of this study was to re-evaluate the status of the anamorph names of *Botryosphaeria* species from mango in Australia, and determine their relatedness to other *Botryosphaeria* spp. DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and the β-tubulin gene were used, in combination with morphological characteristics to characterise and name the different '*Dothiorella*' spp. The taxonomy of *B. rhodina* (Berk. & Curt.)

Arx (anamorph = *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.), another *Botryosphaeria* sp. that commonly occurs on mango in Australia, is not considered in this study.

MATERIALS AND METHODS

Isolates and morphological characterization.--A total of 14 single spore isolates from stem-end rot lesions on mango fruit or from necrotic twigs, were used in this study (TABLE 1). These isolates had previously been characterized based on morphology by Johnson (1992). In the current study, the isolates were induced to sporulate on water agar amended with pine needles as substrate, and exposed to near UV light for a 12 h cycle at 20--25 C for up to one month. Fruiting structures and spores were mounted in lactophenol. Observations and measurements of conidial characteristics were made with a light microscope and an Axiocam digital camera (Carl Zeiss, Germany). At least 50 conidia were measured for each isolate.

Molecular characterization.--A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA, as described in Raeder and Broda (1985) and Smith et al (2001). Partial sequences from two housekeeping gene regions were used for phylogenetic comparisons between isolates. Firstly, the region spanning the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene, was amplified using the primers ITS1 (5' TCCGTAGGTGAAC CTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al 1990). Secondly, a part of the β-tubulin gene was amplified using the primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGA CCCTTGGC 3') (Glass and Donaldson 1995). PCR reaction mixtures, PCR conditions and visualization of amplicons were as described in Slippers et al (2003). ITS and β-tubulin PCR amplicons were purified and sequenced as described in Slippers et al (2003).

To compare the sequence data determined in this study with those of known taxa, 15 ITS rDNA sequences and 15 β-tubulin sequences obtained from GenBank were included in the analyses (TABLE 1). These sequence data included those of *B.*

dothidea, *B. ribis* and *B. parva* from a study of type material and ex-type cultures (Slippers et al 2003), as well as other sequence data of related *Botryosphaeria* spp. (Jacobs and Rehner 1998, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001). BLAST searches were done to identify any other related sequence data to the fungi studied here. *Guignardia philoprina* (Berk. & M.A. Curtis) Aa, which is closely related to *Botryosphaeria*, was included as an outgroup taxon in the analyses. Despite the close relationship between the outgroup and the ingroup taxa, unambiguous alignment of intron regions of the outgroup sequence with the ingroup was not always possible, due to the high degree of sequence variation within these regions. Analysis with and without these regions did not affect the relationships of isolates of the ingroup taxa and were thus left as is.

Sequence data determined in this study were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA). These data were manually aligned with each other and with the data obtained from GenBank, by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Partition homogeneity tests (Farris et al 1995, Huelsenbeck et al 1996) were run in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), to determine whether the ITS rDNA and β-tubulin sequence data sets were congruent and, therefore, combinable. These data were then analyzed together to determine possible phylogenetic relationships between the taxa using parsimony in PAUP. To construct maximum parsimonious trees from the data, heuristic searches were done using informative characters and stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were determined. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985) and decay analysis of the branch nodes using Autodecay (Eriksson 1998).

RESULTS

Isolates and morphological characterization.--The isolates used in this study had been maintained in culture for an extended period of time and sub-cultured extensively. Many of the cultures grew poorly and isolates did not sporulate as readily on pine needles as observed previously with freshly isolated strains (Slippers et al 2003). Nevertheless, all species sporulated on the needles after one to four weeks. Pycnidia were spherical (150--400 µm), with an apical pore, with or without a conical neck (50--200 µm), semi-immersed to superficial on the needle surfaces and mostly occurred singly (FIG. 1). The apical pore was often inconspicuous due to dense growth of grey mycelium covering the pycnidia.

The four taxa represented by the isolates, were distinguishable based on conidial size and shape (TABLE II) (KEY) (FIGS. 2-7). Isolates previously identified as *D. dominicana* had fusiform to ellipsoid, hyaline conidia (average of 56 conidia = 19 x 5.2 µm). These conidia were infrequently observed to become 1--2 eu-septate, often with a darker brown middle cell. Such septate, versicolored spores were usually observed after discharge from the pycnidia or on material that had been left to dry. These conidia are similar to those reported to be *F. parvum* (TABLE II). Conidia of isolates previously identified as *D. mangiferae*, were similar in shape, septation and color to those of *D. dominicana*, but were smaller (average of 54 conidia = 13.6 x 5.4 µm). Dark brown mycelial, toruloid cells were infrequently observed on pine needles or in culture. Isolates identified as *D. aromatica* produced long, fusiform conidia (average of 59 conidia = 23 x 5.1 µm). These isolates were similar to those reported as *F. aesculi*, the anamorph of *B. dothidea* (TABLE II). Isolates previously identified as *D.* 'long' also produced long conidia, but differed from the last named taxon by their broader, rod-shaped conidia (average of 59 conidia = 26.6 x 6 µm).

Molecular characterization.--Amplicons of about 550 bp were obtained using the primers ITS1 and ITS4, and approximately 450 bp using the primers Bt2a and Bt2b. Approximately 25 bp of the terminal end sequence data were excluded in each case in the final alignments. The total aligned sequence data set had 1016 characters. Only the 260 parsimony informative characters were included in the analysis.

A partition homogeneity test showed that the ITS rDNA and β-tubulin datasets were congruent (P value = 0.2). Evaluation of random trees showed that the combined datasets contained significant phylogenetic signal ($P < 0.01$; g1 = -0.72) (Hillis and Huelsenbeck 1992). Heuristic searches found two equal, most parsimonious trees (Tree length = 544 steps; CI = 0.746; RI = 0.885) (FIG. 8). The nine clades in these trees were identified as follows: clade I = *B. ribis*, clade II = *B. parva*, clade III = *F. mangiferum*, clade IV = *B. eucalyptorum* Crous, H. Smith & M.J. Wingf., clade V = *B. lutea* A.J.L. Phillips, clade VI = *Fusicoccum* sp., clade VII = *B. dothidea*, clade VIII = *B. obtusa* (Schwein.) Shoemaker and *B. stevensii* Shoemaker, and clade IX = *B. rhodina*. All these clades were supported by high bootstrap values (>99%).

Clades I-VII all represent *Botryosphaeria* spp. with *Fusicoccum* anamorphs, and formed a monophyletic group supported by a 100% bootstrap value. Within this group, clades I-II (*B. ribis*, *B. parva*) grouped together (100% bootstrap), and these two clades, were most closely related to clades III-V (*F. mangiferum*, *B. eucalyptorum* and *B. lutea*) with 85 % bootstrap support. Clade VI (undescribed *Fusicoccum* sp.) and clade VII (*B. dothidea*) grouped apart from the other groupings. There was sequence variation among isolates within each of clades VI and VII. The variation in clade VI is in the β-tubulin region in only one isolate (CMW7023). The variation within clade VII is in two bases located in a repetitive G (nine repeats) and C (10 repeats) rich area in the ITS1 region. Clade VIII and IX represent *Botryosphaeria* spp. with *Diplodia*-like conidia.

TAXONOMY

Four botryosphaeriaceous fungi were identified in this study from mango. Two species have known teleomorphs, *B. parva* and *B. dothidea*. Johnson (1992) provisionally suggested a new combination in the genus *Fusicoccum* for the fungus reported as *D. mangiferae* or *N. mangiferae* from mango and other hosts. That proposal is supported by molecular and morphological data obtained in this study and a new combination is formally proposed here. The fourth distinct species is only identified as a species of *Fusicoccum*.

Botryosphaeria parva Pennycook and Samuels, Mycotaxon 24:455. 1985.

Anamorph. ***Fusicoccum parvum*** Pennycook and Samuels, Mycotaxon 24:455. 1985. FIG. 2

Notes. The anamorph state of *B. parva* has commonly been identified from mango as *D. dominicana*. The conidia from putative *D. dominicana* isolates collected in Australia (Johnson 1992) are exactly the same as those reported from the type of *F. parvum* (Pennycook and Samuels 1985, Slippers et al 2003). These data, and that emerging from this study (TABLE II), show that this taxon can be distinguished from other Botryosphaeriaceous fungi on mango, based on conidial characteristics. The most recognizable characteristics of these conidia are that they are aseptate, hyaline, granular, broadly ellipsoid to fusoid, on ave. 17--19 x 5--6 µm (see KEY). Older, discharged conidia sometimes become 1--2 septate and light brown with darker middle cells. Septate conidia with distinctly darker middle cells in this fungus have been confused with *N. mangiferae* and *D. mangiferae* (Sutton and Dyko 1989, Roux 1993).

Although the '*D. dominicana*' isolates considered here are conspecific with *B. parva*, the true identity of the type of *D. dominicana* remains unclear. The dimensions reported in the original description of *D. dominicana* from mango leaves by Petrak and Ciferri (1930) fall within the range of *F. parvum*. Johnson (1992) re-examined and described the type material of *D. dominicana*, which he considered to be synonymous with *B. dothidea*. The conidia reported by Johnson (1992) from the *D. dominicana* type material are, however, smaller than the anamorphs of either *B. dothidea* or *B. parva*. Despite this uncertainty, it is clear that the name *D. dominicana* is not appropriate for isolates associated with stem end rot and other diseases of mango in Australia.

Fusicoccum mangiferum (Syd. & P. Syd.) Johnson, Slippers & M.J. Wingf. comb. nov. FIG. 3-5

Basionym. *Dothiorella mangiferae* Syd. & P. Syd., Ann. Mycol. 14:192. 1916.

Synonyms. *Natrassia mangiferae* (Syd. & P. Syd.) B. Sutton & Dyko, Mycol. Res. 93:484. 1989.

Hendersonula toruloidea Natrass, Trans. Br. Mycol. Soc. 18:197. 1933.

Hendersonula cypria Natrass, Cypress fungi, Nicosia: 43. 1937.

Teleomorph. ***Botryosphaeria*** sp.

Notes. Various morphological descriptions have been given for this taxon. The holotype was first described by Sydow et al (1916) from *M. indica* in India. Sutton and Dyko (1989) reviewed all previous type material connected with this taxon, including the holotype, and provided a very thorough description. Other clear descriptions are found in Natrass (1933), Punithalingam and Waterston (1970) and Johnson (1992).

Duplication of previous descriptions is avoided here, but the most distinctive features are highlighted. The conidia are distinct from other *Fusicoccum* spp. by their shorter average length (ave. ~13--14 µm) and smaller length/width ratio (2--2.5) (see KEY). The conidia often become one to two septate, light brown with distinctly darker middle cells. This feature is also shared with *F. parvum*. *Fusicoccum mangiferum* produces vegetative, toruloid cells in culture and in nature. This species also produces fluffy, evenly grey-coloured aerial mycelium, lacking the white tufts found in other similar species such as *F. parvum*.

Sydow et al (1916) described *D. mangiferae* from mango, but noted only the aseptate conidia. Re-examination of the type material, however, confirmed the presence of 1--2 septate, pigmented conidia (Sutton and Dyko 1989). It is possible that the spores on the type material aged and became septate after the description by Sydow. Natrass (1933) studied the taxon from pome and stone fruit trees and first noticed the pigmented conidia, which led him to describe it as *Hendersonula toruloidea* Natrass. He also studied the fungus in culture and noted the characteristic brown one or two celled, toruloid, vegetative cells. Sutton and Dyko (1989) revised the genus *Hendersonula* and synonymized both *D. mangiferae* and *H. toruloidea*, amongst other genera and species, with the newly described *N. mangiferae*.

Natrass (1933) and Sutton and Dyko (1989) reported fragmented mycelial cells or toruloid cells in culture and in nature. In the last named study, this form was described as the synanamorph *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko. Johnson (1992) reported no toruloid state, but referred only to these cells as fragmented mycelia, and he did not use the last named epithet. Such cells as described in Natrass (1933) and Sutton and Dyko (1989) were rarely observed in this study and when seen, resembled fragmented, thick-walled hyphae.

Sutton and Dyko (1989) reduced *Fusicoccum eucalypti* Sousa da Câmara and *H. agathi* to synonymy with *F. mangiferum* (as *N. mangiferae*). These synonymies are not accepted here, as the taxa both have conidia that differ from those of *F. mangiferum* in length and in length/width ratio (Young 1948, Sutton and Davison 1983, Sutton and Dyko 1989). The conidial sizes reported in the last named studies for *F. eucalypti* and *H. agathi* were more similar to those of *F. parvum*.

The teleomorph of *F. mangiferum* is a *Botryosphaeria* sp. The DNA sequence data presented here group this species with the type species, *B. dothidea*, and other *Botryosphaeria* sp. Johnson (1992) also reported *Botryosphaeria* ascomata and ascospores forming in cultures of *F. mangiferum* (Johnson 1992). Sufficient material was, however, not available to formally describe a specific name for the teleomorph.

Botryosphaeria dothidea (Moug.: Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital. 1:212. 1863.

Anamorph. ***Fusicoccum aesculi*** Corda in Sturm, Deutschl. Fl., Abth. 3, 2:111. 1829. FIG. 6

Notes. Previous reports of this fungus from mango and avocado listed it as *D. aromatica*. Conidial morphology of this species is similar to that described for the anamorph of *B. dothidea* (Pennycook and Samuels 1985, Slippers et al 2003). The most distinctive feature of this taxon is its conidia, which are aseptate, hyaline, fusiform to narrowly fusiform and on average 23-25 x 4-5 µm (see KEY).

The type specimen of *D. aromatica* has not been compared with that of *F. aesculi* in this or any other study. Johnson (1992) reports that this material is not available and we have been unable to locate it. The synonymy of *D. aromatica* and *F. aesculi* is, therefore, not considered. The fungus occurring as a pathogen of mango and avocado and generally identified as *D. aromatica* is, however, *F. aesculi.*

***Fusicoccum* sp.** FIG. 7

Notes. Johnson et al (1991a) and Johnson (1992) identified an unknown *Dothiorella* or *Fusicoccum* sp. from mango in Australia and Thailand, which was referred to only as *Dothiorella* 'long'. Our DNA sequence and morphological data confirm that this is a *Fusicoccum* sp., and that it probably represents an undescribed species.

Johnson (1992) considered this *Fusicoccum* sp. as possibly synonymous with *F. cajani* (teleomorph = *B. xanthocephala*). Samuels and Singh (1986) described *F. cajani* from *Cajanus* spp. (pigeon pea) from India, Fiji and the USA. Conidial measurements of *F. cajani* [(17-) 21.6--27.8 (-32) x (5-) 6.5--8 (-9) µm] and the *Fusicoccum* sp. [average = 26.6 x 6 µm, see TABLE II] considered here, overlap. This alone is, however, not sufficient evidence for synonymy. For example, the measurements and the shape of the conidia of *F. cajani* overlap with the anamorphs of a number of other *Botryosphaeria* spp., such as *B. lutea* (Pennycook and Samuels 1985, Phillips et al 2002), *B. eucalyptorum* (Smith et al 2001), *B. protearum* Denman and Crous (Denman et al 2003), and others. Furthermore, the host and geographical differences make this view impossible to confirm without further evidence. No isolates of *B. xanthocephala* could be located to further test this hypothesis, using molecular or cultural characters.

KEY TO *BOTRYOSPHAERIA* SPP. AND THEIR ANAMORPHS FROM MANGO IN AUSTRALIA

Conidial characters are used to separate the Botryosphaeriaceous fungi treated here. The anamorph state is most frequently encountered in nature and is also readily induced *in vitro* on nutrient poor medium (e.g. water agar) supplemented with sterilized pine needles. Differences among the species are more pronounced in anamorph than teleomorph features. Teleomorphs have not been described or even observed for all the species treated here, but these names are used preferentially where they are known. The unnamed species of *Fusicoccum* refers to the fungus previously known as *Dothiorella* 'long'.

1. Conidia in culture on average <18 µm in length, l/w 2--3.5, unicellular, but occasionally becoming light brown and 1--2 septate with a darker brown middle cell after discharge, colony on MEA or PDA thick felt of grey aerial mycelium.....2

1. Conidia in culture on average >18 µm in length, l/w >4, unicellular, colony on MEA or PDA appressed with only occasional tufts of grey to buff aerial mycelium...3

2. No toruloid cells, conidia 12--23 x 4--6 µm (average 19 x 5.2 µm), l/w 3--3.5 .....***B. parva***

2. Toruloid cells, conidia 12--14 x 4--6 µm (average 13.6 x 5.4 µm), l/w 2--3..... .....***F. mangiferum***

3. Conidia hyaline, unicellular, rod-shaped, 20--32 x 5--7 µm (average 26.6 x 6 µm), l/w 3.5--4.5.....***Fusicoccum* sp.**

3. Conidia hyaline, unicellular, narrowly fusiform, 19--30 x 4--6 µm (average 23 x 5.1 µm), l/w 4--5.....***B. dothidea***

DISCUSSION

Four *Fusicoccum* spp. were identified as endophytes and pathogens of Australian mango fruit and trees in the present study. Identification of these species is based on a combination of morphological and molecular phylogenetic analyses. These species are *F. parvum*, *F. mangiferum, F. aesculi*, and an undescribed *Fusicoccum* sp. They were all previously known as species of *Dothiorella* or *Natrassia*. The data emerging from this study show that all these taxa should be seen as species of *Fusicoccum*, and that their teleomorphs, although some are presently still unknown, should all reside in *Botryosphaeria*.

The description of *Dothiorella* spp. from mango as *Fusicoccum* spp. is in accordance with recent proposals for the correct use of these two generic names (Crous and Palm 1999, Denman et al 2000). *Fusicoccum* and *Dothiorella* have often been confused, because both have commonly been used to describe anamorphs of *Botryosphaeria* (Saccardo 1882, Petrak 1922, Von Arx and Müller 1954). The common use of the name *Dothiorella* for *Botryosphaeria* anamorphs from mango, follows the preference of this name for botryosphaeriaceous fungi from this host by Sydow et al (1916) and Petrak (1922). Recently, *D. pyrenophora* Sacc., the type species of *Dothiorella*, was re-described as *Diplodia pyrenophora* (Sacc.) Crous & M.E. Palm (Crous and Palm 1999). These authors suggested that all *Botryosphaeria* anamorphs that are placed in *Dothiorella* should be re-examined. Denman et al (2000) argued that all hyaline, thin walled fusiform conidial *Botryosphaeria* anamorphs should reside in *Fusicoccum*.

Results of this study and those of Johnson (1992) show clearly that *B. parva* (reported as *D. dominicana*) is one of the most common pathogens that cause stem

end rot, die-back and blossom blight on mango. The species was first described by Pennycook and Samuels (1985) from *Populus*, *Malus* and *Actinidia* species in New Zealand. Subsequently, it was shown that this species occurs world-wide on a number of hardwood species, including native Australian flora, such as *Eucalyptus* spp. (Slippers et al 2003). *Botryosphaeria parva* has often been misidentified as *B. ribis* and *B. dothidea*, due to overlapping host ranges, morphological similarities and taxonomic confusion over the use of the names (Slippers et al 2003). It is thus also likely that the fungus described as *B. ribis* from mango in Florida (Ramos et al 1991) is *B. parva*. These identifications from Florida were done based on conidial dimensions, which overlap between *B. ribis* and *B. parva* (Slippers et al 2003).

The name *F. mangiferum* has been proposed in this study for the mango pathogen that was previously identified as *D. mangiferae* and *N. mangiferae*. Johnson (1992) first suggested that *D. mangiferae* and *N. mangiferae* should be described in the genus *Fusicoccum*. This proposal is supported in the present study by the phylogenetic monophyly of this taxon with the type species, *F. aesculi*, and other *Fusicoccum* spp. Isolates used for sequence analyses were not ex-type cultures. The conidia of these isolates were, however, similar to those from the type specimens of *D. mangiferae* and *N. mangiferae*, which are unique in morphology (Sutton and Dyko 1989, Sydow et al 1916).

There are obvious similarities in the septation and pigmentation of conidia of *F. mangiferum* and *F. parvum*. This has led to confusion between these taxa in the past. These species can, however, be separated based on conidial size, as the conidia of the former species are smaller in average length and width. Moreover, in culture *F. parvum* has more fluffy aerial mycelium than the appressed grey aerial mycelium of *F. mangiferum*.

Botryosphaeria dothidea is of little importance as a pathogen of mango in Australia or other parts of the world. It is less common on mango than *B. parva* and *F. mangiferum* and is often omitted from lists of important pathogens of this host (Johnson et al 1991a, b, 1992, Johnson 1992). This name is, however, one of the most commonly used for *Botryosphaeria* pathogens on a wide variety of other hosts (McGlohon 1982, Pennycook and Samuels 1985, Brown and Britton 1986, Hartill 1991, Jacobs and Rehner 1998, Smith et al 2001). Some of these identifications, however, need to be viewed with care, as many species have incorrectly been relegated to the name *B. dothidea*. This followed the extensive synonymy of many

species with *B. dothidea* by Von Arx and Müller (1954). Due to this synonymy, *B. ribis* was treated as a synonym of *B. dothidea*. *Botryosphaeria parva* was often not distinguished from *B. ribis*, and was consequently also treated under *B. dothidea* (Slippers et al 2003).

Reports of *B. dothidea* from Australasia and other southern hemisphere countries are from exotic hosts (Pennycook and Samuels 1985, Hartill 1991, Slippers et al 2003). Studies of pathogens of native hosts in Australasia have not reported this pathogen (Denman et al 2003). This species is, however, common on both cultivated and indigenous hosts in the northern hemisphere (Zhou and Stanosz 2001, Slippers et al 2003). This suggests a northern hemisphere origin for this fungus and implies that it was introduced into the southern hemisphere with planting material of agricultural and ornamental crops.

The taxon previously known from mango as *Dothiorella* 'long' is identified in this study as an undescribed species of *Fusicoccum*. This fungus was identified from mango from Australia and Thailand by Johnson et al (1991a) and Johnson (1992). This species was rarely found in extensive surveys during these studies and is not considered important in causing pre- or post-harvest diseases of mango. This species of *Fusicoccum* is also not known from any other hosts. The suggested synonymy of this taxon with *F. cajani* (Johnson 1992) is rejected based on the distinctive hosts of each species and the lack of any further evidence to their conspecificity.

Johnson (1992) both suggested that *F. luteum* (teleomorph *B. lutea*) occurs on mango in Australasia. Based on DNA sequence data produced in this study, none of the *Fusicoccum* spp. from mango in Australia group with this taxon. This finding is surprising as *F. luteum* seems to be common in the Australasian region. *Fusicoccum luteum* was initially described from *Actinidia*, *Malus* and *Pyrus* in New Zealand (Pennycook and Samuels 1985) and subsequently also from avocado (Hartill 1991).

Sequence variation was observed among isolates of clade VI (*Fusicoccum* sp.) and clade VII (*B. dothidea*) that was not phylogenetically informative. Among the three isolates identified in clade VI one isolate had sequence variation only in the β-tubulin region. In clade VII the three isolates from mango grouped together based on two variable bases in the ITS1 region in a highly repetitive region. In both cases these variable characters were thus found only in one of the two sequenced regions.

Additional data and a larger number of isolates are required to determine the extent of variation and its phylogenetic relevance to populations of the above clades.

The many misidentifications of Botryosphaeriaceous fungi from mango in the past illustrate aptly how confusing morphological characterisation of these fungi has been. This problem results from the fact that sizes of structures for these species overlap. There is also some variation between morphological features in nature and in culture, which has added to the confusion (Slippers et al 2003, Johnson 1992). Furthermore, conidial septation and colour, which has been used to characterize species, is not always consistent. Spores tend to age only after discharge from the pycnidia and their color and septation changes with age.

This study provides a basis on which future identifications of *Botryosphaeria* and its anamorphs from mango can be made. The combination of molecular data and average conidial size and shape, as well as cultural characteristics, has successfully been used here to identify these fungi from mango. Correct identifications of these pathogens are becoming more crucial due to increased quarantine requirements. These data will also facilitate studies to better understand the epidemiology of the different fungal species.

LITERATURE CITED

Arx JA von, Müller E. 1954. Die Gattungen der amerosporen Pyrenomyceten. Beitr Kryptogamenfl Scheiz 11, 2:1--434.

Brown EA, Britton KO. 1986. *Botryosphaeria* diseases of apple and peach in the Southern United States. Plant Dis 70:480--484.

Cappellini RA, Ceponis MJ, Lightner GW. 1988. Disorders in Avocado, Mango, and Pineapple Shipments to the New York Market, 1972-1985. Plant Dis 72:270--273.

Crous PW, Palm ME. 1999. Reassessment of the anamorph genera *Botryodiplodia*, *Dothiorella* and *Fusicoccum*. Sydowia 52:167--175.

Darvas JM. 1991. *Dothiorella dominicana*, a new mango pathogen in South Africa. Phytophylactica 23:295--298.

Denman S, Crous PW, Taylor JE, Kang JC, Pascoe I, Wingfield MJ. 2000. An overview of the taxonomic history of *Botryosphaeria* and a re-evaluation of its

anamorphs based on morphology and ITS rDNA phylogeny. Stud Mycol 45:129--140.

---, ---, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. Mycologia 95:294--307.

Eriksson T. 1998. Autodecay ver. 4.0 (program distributed by the author). Department of Botany, Stockholm University, Stockholm.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Testing significance of incongruence. Cladistics 10:315--319.

Felsenstein J. 1985. Confidence intervals on phylogenetics: an approach using bootstrap. Evolution 39:783--791.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61:1323--1330.

Hartill WFT. 1991. Postharvest diseases of avocado fruit in New Zealand. NZ J Crop Hort Sci 19:297--304.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J Hered 83:189--195.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. TREE 11:152--158.

Jacobs KA, Rehner SA. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90:601--610.

Johnson GI. 1992. Biology and control of stem end rot pathogens of mango. [PhD thesis]. Queensland, Australia: University of Queensland. 265 p.

---, Cooke AW, Mead AJ, Wells IA. 1991a. Stem end rot of mango in Australia: cause and control. Acta Hort 291:288--295.

---, Mead AJ, Cooke AW, Dean JR. 1991b. Mango stem end rot pathogens – Infection levels between flowering and harvest. Ann Appl Biol 119:465--473.

---, ---, ---, ---. 1992. Mango stem en rot pathogens – Fruit infection by endophytic colonisation of the inflorescence and pedicel. Ann Appl Biol 120:225--234.

Lonsdale JH. 1992. Etiology and control of some mango blossom diseases in Transvaal. [M.Sc. thesis]. Pretoria, South Africa: University of Pretoria. 103 p.

McGlohon NE. 1982. *Botryosphaeria dothidea* – Where will it stop? Plant Dis 66:1202--1203.

Mitra SK, Baldwin EA. 1997. Mango. In: Mitra MA ed. Postharvest physiology and storage of tropical and subtropical fruits. Oxon, UK: CAB International. p. 85--122.

Natrass RM. 1933. A new species of *Hendersonula* (*H. toruloidea*) on deciduous trees in Egypt. Trans Br Mycol Soc 18:189--198.

Pennycook SR, Samuels GJ. 1985. *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. Mycotaxon 24:445--458.

Petrak F. 1922. Beiträge zur Kentnis der Pilzflora der südlichen Alpenländer und Norditaliens. Ann Mycol 20:126--159.

Phillips AJL, Fonseca F, Povoa V, Castilho R, Nolasco G. 2002. A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp. nov. Sydowia 54:59--77.

Prakash OM, Srivastava KC. 1987. Mango diseases and their management. A world review. Today and Tomorrow's Printers and Publishers, New Delhi, India. 180p.

Prusky D. 1991. Mango diseases: An overview. Acta Hort 291:279--287.

Punithalingam E, Waterston JM. 1970. *Hendersonula toruloidea*. CMI descriptions of pathogenic fungi and bacteria, No. 274. Kew, Surrey, England: Commonwealth Mycological Institute. 2 p.

Ramos LJ, Lara SP, McMillan RT Jr, Narayanan KR. 1991. Tip dieback of mango (*Mangifera indica*) caused by *Botryosphaeria ribis*. Plant Dis 75:315--318.

Raeder U, Broda P. 1985. Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17--20.

Roux C. 1993. *Nattrassia mangiferae*, a fungus causing several diseases of mango. SA Mango Growers' Yearbook 13:93--96.

Saccardo PA. 1882. Sylloge fungorum omnium hucusque cognitorum 1:1--768.

Samuels GJ, Singh B. 1986. *Botryosphaeria xanthocephala*, cause of stem canker in pigeon pea. Trans Br Mycol Soc 86:295--299.

Sangchote S. 1991. *Botryodiplodia* stem end rot of mango and its control. Acta Hort 291:296--303.

Shoemaker RA. 1964. Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Can J Bot 42:1297--1301.

Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ. 2003. Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia (in press).

Smith H, Crous PW, Wingfield MJ, Coutinho TA, Wingfield BD. 2001. *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. Mycologia 93:277--284.

Smith DR, Stanosz GR. 2001. Molecular and morphological differentiation of *Botryosphaeria dothidea* (anamorph *Fusicoccum aesculi*) from some other fungi with *Fusicoccum* anamorphs. Mycologia 93:505--515.

Stevens NE. 1926. Occurrence of the currant cane blight fungus on numerous hosts in the southern states. Mycologia 18:278--282.

Sutton BC, Davison EM. 1983. Three stromatic Coelomycetes from Western Australia. Trans Br Mycol Soc 81:291--301.

Sutton BC, Dyko BJ. 1989. Revision of *Hendersonula*. Mycol Res 93:466--488.

Swofford DL. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Sydow H, Sydow P, Butler EJ. 1916. Fungi Indiae Orientalis. V. Ann Mycol 14:177--220.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Snisky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p. 315--322.

Young HE. 1948. *Hendersonula agathi* n. sp., the cause of leaf cast of Kauri pine in Queensland. Queensland J Agric Sci 5:9-12.

Zhou S, Stanosz GR. 2001. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93:516--527.

TABLE I. Isolates from mango fruit and trees considered in the phylogenetic study.

Culture no.[1]	Other no.[1]	Identity[2]	Host	Location	Collector	GenBank[3]	
						ITS	β-tubulin
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236935	AY236906
CMW7054	CBS121	*B. ribis*	*Ribes* sp.	New York, USA	N.E. Stevens	AF241177	AY236908
CMW9078	ICMP7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236940	AY236914
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236917
CMW7798	BRIP23348	*B. parva*	*Mangifera indica*	Australia	G.I. Johnson		
CMW7796	BRIP23349	*B. parva*	*M. indica*	Australia	G.I. Johnson		
CMW7799	BRIP23300	*B. parva*	*Persea americana*	Australia	G.I .Johnson		
CMW7026	BRIP19684	*B. parva*	*M. indica*	Australia	G.I. Johnson		
CMW7025	BRIP24083	*B. parva*	*M. indica*	Australia	G.I. Johnson		
CMW78701	BRIP23396	*Fusicoccum mangiferum*	*M. indica*	Australia	G.I. Johnson		
CMW7024	BRIP24101	*F. mangiferum*	*M. indica*	Australia	G.I. Johnson		
CMW7797	BRIP23350	*F. mangiferum*	*M. indica*	Australia	G.I. Johnson		
CMW7802	BRIP23491	*Fusicoccum* sp.	*M. indica*	Australia	G.I. Johnson		
CMW7023	BRIP19560	*Fusicoccum* sp.	*M. indica*	Australia	G.I. Johnson		
CMW7022	BRIP19782	*Fusicoccum* sp.	*M. indica*	Australia	G.I. Johnson		
CMW7803	BRIP23750	*B. dothidea*	*M. indica*	Australia	G.I. Johnson		
CMW7020	BRIP24286	*B. dothidea*	*M. indica*	Australia	G.I. Johnson		
CMW7027	BRIP24172	*B. dothidea*	*M. indica*	Australia	G.I. Johnson		
CMW7780		*B. dothidea*	*Fraxinus excelsior*	Switzerland	B. Slippers	AY236947	AY236925
CMW8000		*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	AY236949	AY236927
CMW9076	ICMP7818	*B. lutea*	*Malus* x *domestica*	New Zealand	S.R. Pennycook	AY236946	AY236922
CMW992	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels	AF027745	AY236923
CMW10125	BOT24	*B. eucalyptorum*	*Eucaluptus grandis*	South Africa	H. Smith	AF283686	AY236920
CMW10126	BOT16	*B. eucalyptorum*	*E. grandis*	South Africa	H. Smith	AF283687	AY236921
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236953	AY236931
CMW7060	CBS431	*B. stevensii*	*F. excelsior*	Netherlands	H.A. van der Aa	AY236955	AY236933
CMW10130	BOT977	*B. rhodina*	*Vitex donniana*	Uganda	J. Roux	AY236951	AY236929
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236930
CMW7063		*Guignardia philoprina*	*Taxus baccata*	Netherlands	H.A. van der Aa	AY236956	AY236934

[1] Abbreviations for culture collections and isolates: BRIP = Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; BOT = *Botryosphaeria* sub-collection of CMW; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998).

[2] Identities are given as determined in this study. Isolates from *M. indica* were previously known by the anamorph names *D. dominicana* (*B. parva*), *D. mangiferae* (*F. mangiferum*), *D.* 'long' (*Fusicoccum* sp.) and *D. aromatica* (*B. dothidea*).

[3] Sequences obtained from GenBank are from Jacobs and Rehner (1998), Smith et al (2001) and Slippers et al (2003).

TABLE II. Conidial measurements for *Botryosphaeria* spp. and their *Fusicoccum* anamorphs associated with mango.

Identity[1]	**Previously used name**[2]	**Conidial size *in vitro* (µm)**	**L/W**	**Source of data**[3]
B. parva / *F. parvum*	*D. dominicana*	(12-)15-19(-24) x 4-6 [Ave. 16.9 x 5.4]	3.1	Pennycook & Samuels 1985
		14.5-18.7(-17.1) x 4.6-7 [Ave. 17.1 x 4.7]	3.6	Johnson 1992
		(14.7-)17-21(-25.5) x 4.5-6(-7) [Ave. 19 x 5.2]	3.7	This study
B. dothidea / *F. aesculi*	*D. aromatica*	(20-)23-27(-30) x 4-5(-6) [Ave. 24.7 x 4.9]	5	Slippers et al 2003
		19.9-26.2 x 3.9-5.5 [Ave. 22.8 x 4.6]	4.9	Johnson 1992
		(18.8-)21-24(-30.4) x 4.5-6(-7) [Ave. 23 x 5.1]	4.5	This study
F. mangiferum	*D. mangiferum* or *N. mangiferum*	9-13 x 3.5-5		Sydow et al 1916
		10-16 x 3.5-6.5	N/a	Sutton & Dyko 1989
		11-14 x 4.5-5.5 [Ave. 12.8 x 5]	2.6	Johnson 1992
		(11-)12-15(-17.3) x 5-6.6 [Ave. 13.6 x 5.4]	2.5	This study
Fusicoccum sp.	*D.* 'long'	18.6-37.2 x 4.6-7 [Ave. 22.4 x 5]	4.5	Johnson 1992
		(20.2-)23-30(-35.5) x (4.7-)5.5-7 [Ave. 26.6 x 6]	4.4	This study

[1] Teleomorph (where known) and anamorph names as used in this study.
[2] Common anamorph name used for this taxon from mango in earlier literature.
[3] The isolates studied in this study and by Johnson (1992) are the same, but measurements from both studies are given because of the difference in the technique used to induce sporulation.

FIGS. 1-7. Anamorphs of various *Botryosphaeria* spp. formed in culture. 1. Pycnidium forming on a pine needle. Bar = 100 μm. 2. *Fusicoccum parvum* conidia. 3, 4. Young, aseptate conidia of *F. mangiferum*, which become septate and discolored after discharge. 5. Toruloid cells of *F. mangiferum*. 6. Fusiform conidia of *F. aesculi*. 7. Rod-shaped conidia of a *Fusicoccum* sp. Bars = 10 μm.

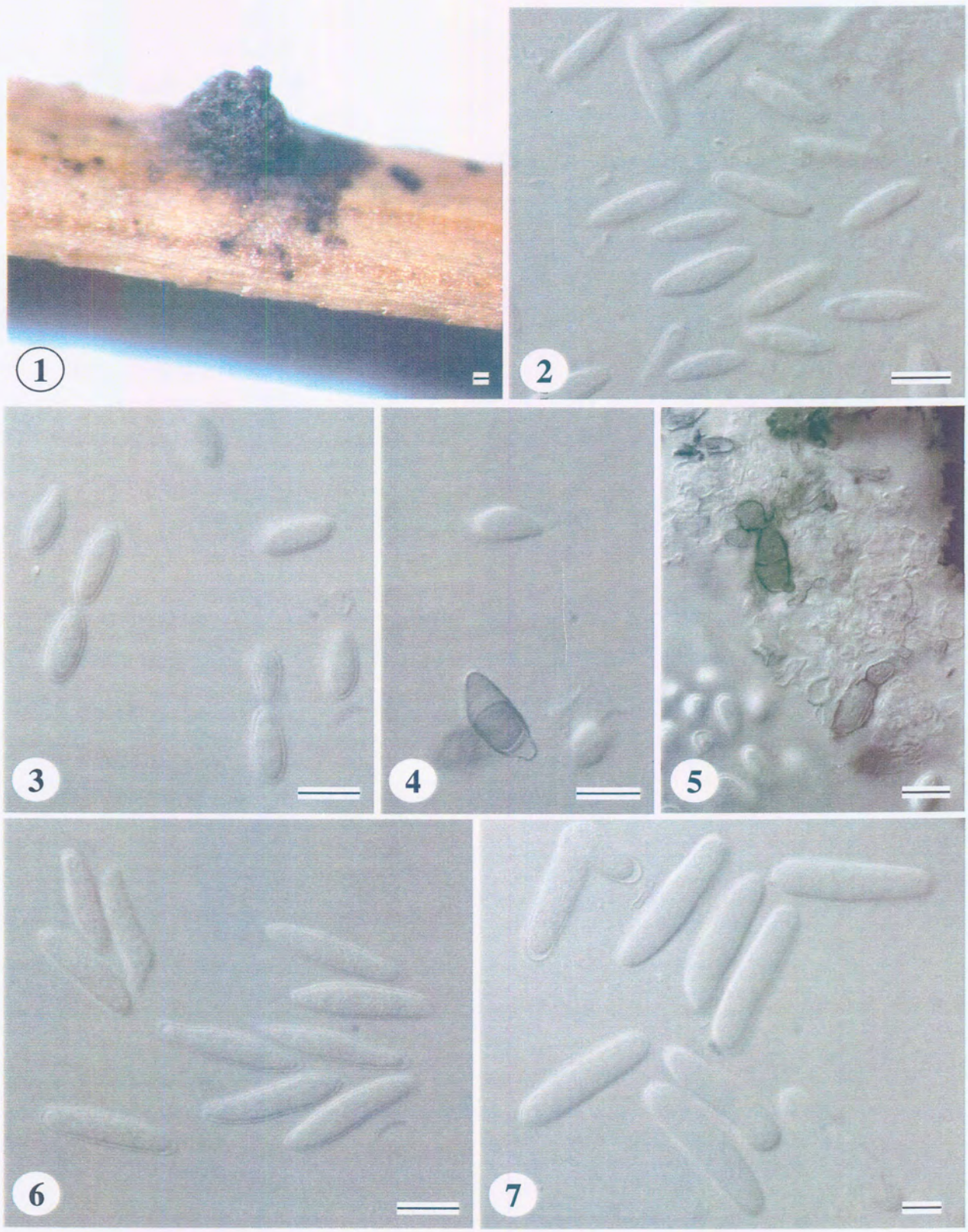


FIG. 8. One of the two equal, most parsimonious trees obtained by heuristic searches of the ITS rDNA and β-tubulin sequence datasets in PAUP. Branch supports are indicated by decay indices below and bootstrap values above the branches. Nine clades or taxa are identified. Clade VI represents an unknown *Fusicoccum* sp. (previously identified as *Dothiorella* 'long').