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

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Abstract: *Botryosphaeria dothidea* is one of the most commonly reported species in a genus of important pathogens of woody plants. This taxon generally is accepted to represent a species complex, and hence its identity remains unclear. Previous studies either have 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 datasets 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.

Key words: *Botryosphaeria*, epitypification, *Fusicoccum*, key, phylogeny, systematics

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 four 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 proposals 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 species originally 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

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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. The name *B. berengeriana* is not commonly used currently, 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 often are 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 also is 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 should be included in *Fusicoccum* and those with conidia that are dark and opaque when mature should 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 or 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, 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* remains in taxonomic disarray. The objective of our 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 Oct 2000. This is the same time of year, area and included the same hosts upon which Cesati and De Notaris based the original descriptions.

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

Culture No.[1,2]	Other No.[1]	Identity[3]	Host	Location	Isolator	GenBank[4]		
						ITS	ß tubulin	EF-1α
CMW7772	107	*Botryosphaeria ribis*	*Ribes sp.*	New York	B. Slippers/G. Hudler	AY236935	AY236906	AY236877
CMW7773	108	*B. ribis*	*Ribes sp.*	New York	B. Slippers/G. Hudler	AY236936	AY236907	AY236878
CMW7054	CBS121	*B. ribis* (chromagena)	*R. rubrum*	New York	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	80	*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	94	*B. parva*	*E. grandis*	Sumatra, Indonesia	M.J. Wingfield	AY236937		
CMW9071	160	*B. parva*	*Ribes sp.*	Australia	M.J. Wingfield	AY236938	AY236909	AY236880
CMW7885	X34	*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
CMW9075	ICMP8019	*B. dothidea*	*P. nigra*	New Zealand	G.J. Samuels	AY236950	AY236928	AY236899
CMW7780	115	*B. dothidea*	*Fraxinus excelsior*	Molinizza, Switzerland	B. Slippers	AY236947	AY236925	AY236896
CMW7999	119	*B. dothidea*	*Ostrya sp.*	Crocifisso, Switzerland	B. Slippers	AY236948	AY236926	AY236897

TABLE I. Continued

Culture No.[1,2]	Other No.[1]	Identity[3]	Host	Location	Isolator	GenBank[4]		
						ITS	ß tubulin	EF-1α
CMW8000	118	*B. dothidea*	*Prunus sp.*	Crocifisso, Switzerland	B. Slippers	AY236949	AY236927	AY236898
	ATCC58194	*B. lutea*	*Malus 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 × 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	CBS431	*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*	*Pistachia*	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 Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection; CBS = Centraal Bureau voor Scimmelcultures, 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.

Isolations were made from ascomata or pycnidia on dead or dying twigs of various hardwood species (TABLE I). Isolations similarly were made from twigs of *Ribes* spp. showing symptoms of cane dieback from Ithaca, New York. 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, 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 only in molecular comparisons.

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 speciments, 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′ GGTAACCAAATCGGTGCTGCTTTC 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′ TACTTGAAGGAACCCTTACC) (Carbone et al 1999). PCR reaction mixtures contained final concentrations of: 2.5 units *Taq* DNA polymerase (Roche Molecular Biochemicals, Almeda, California), 1× buffer and $MgCl_2$ 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 first was 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) 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 100 bp 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). 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) as indicated by the manufacturer and run on an ABI PRISM 377 autosequencer (Perkin-Elmer Applied BioSystems, Foster City, California).

To compare *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) 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) also were determined in PAUP. Decay analyses of the branch nodes were determined using Autodecay (Eriksson 1998). Phylogenetic hypotheses also were 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 datasets 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 datasets subsequently were 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 *Rosa* sp. This sample, thus, cannot 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, however, is not definitive of the species because the specimens are immature and contain no spores.

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 our 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 neotype 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 dieback, which were collected on our behalf from the same geographical area as the original type material (New York, U.S.A.), 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, clearly were 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 dataset consisted of 563 characters after alignment; 418 uninformative characters were excluded, and 145 parsimony-

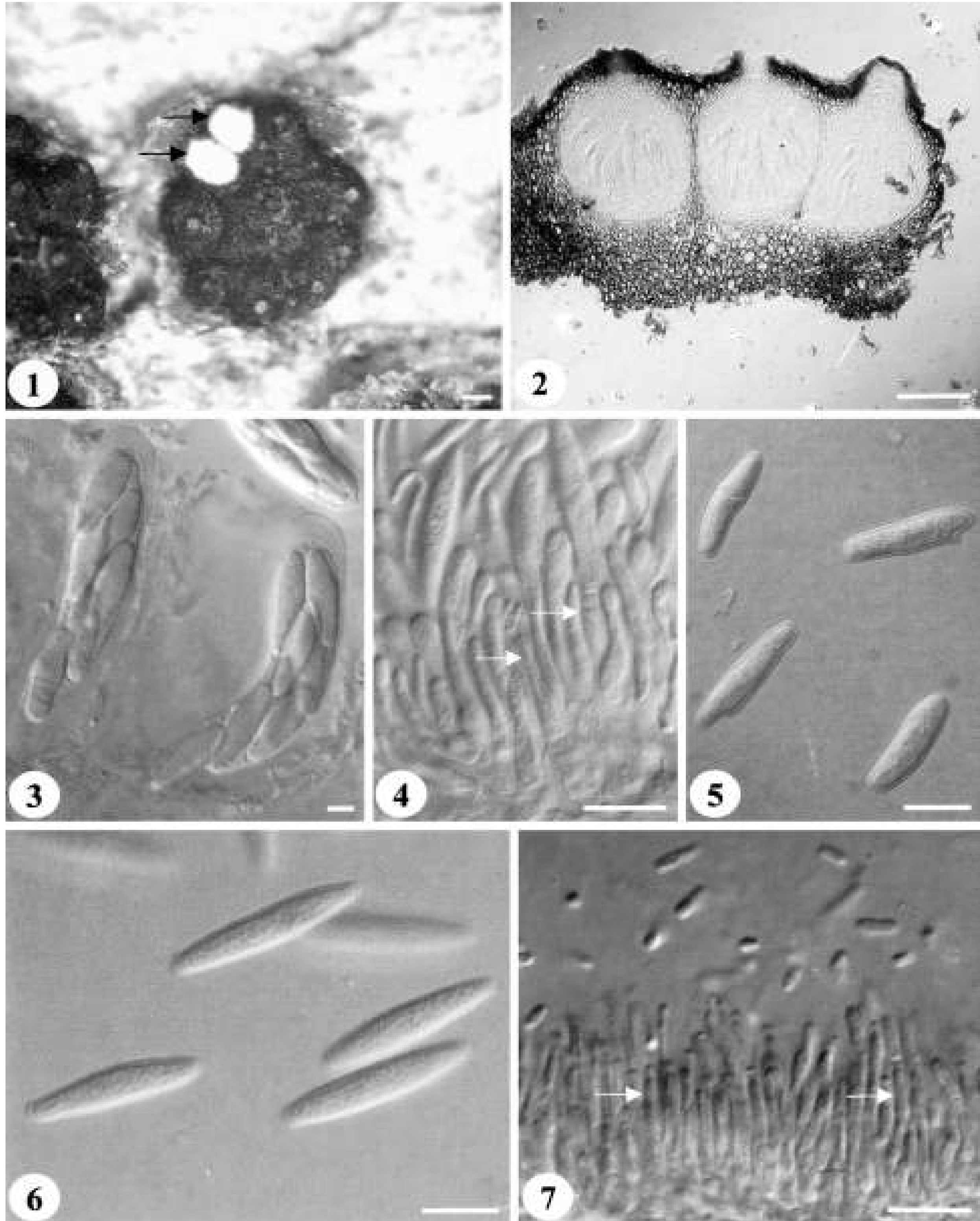
TABLE II. A comparison of the epitype specimens with holotype material and descriptions of *Sphaeria dothidea*, *Botryosphaeria dothidea* and *B. berengeriana*

Ascostromata	Fries 1823[1]	Cesati and De Notaris 1863[2]	De Notaris 1863	Pennycook and Samuels 1985[3]	Epitype
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)
Color	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
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
Color	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 × 7 µm	<30 µm in length	(13–)19–27(–35) × (6–)8–11(–14)µm	(17.2–)19–24(–32) × (6.2–)7–8(–10) [21.9 × 7.8] [1/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

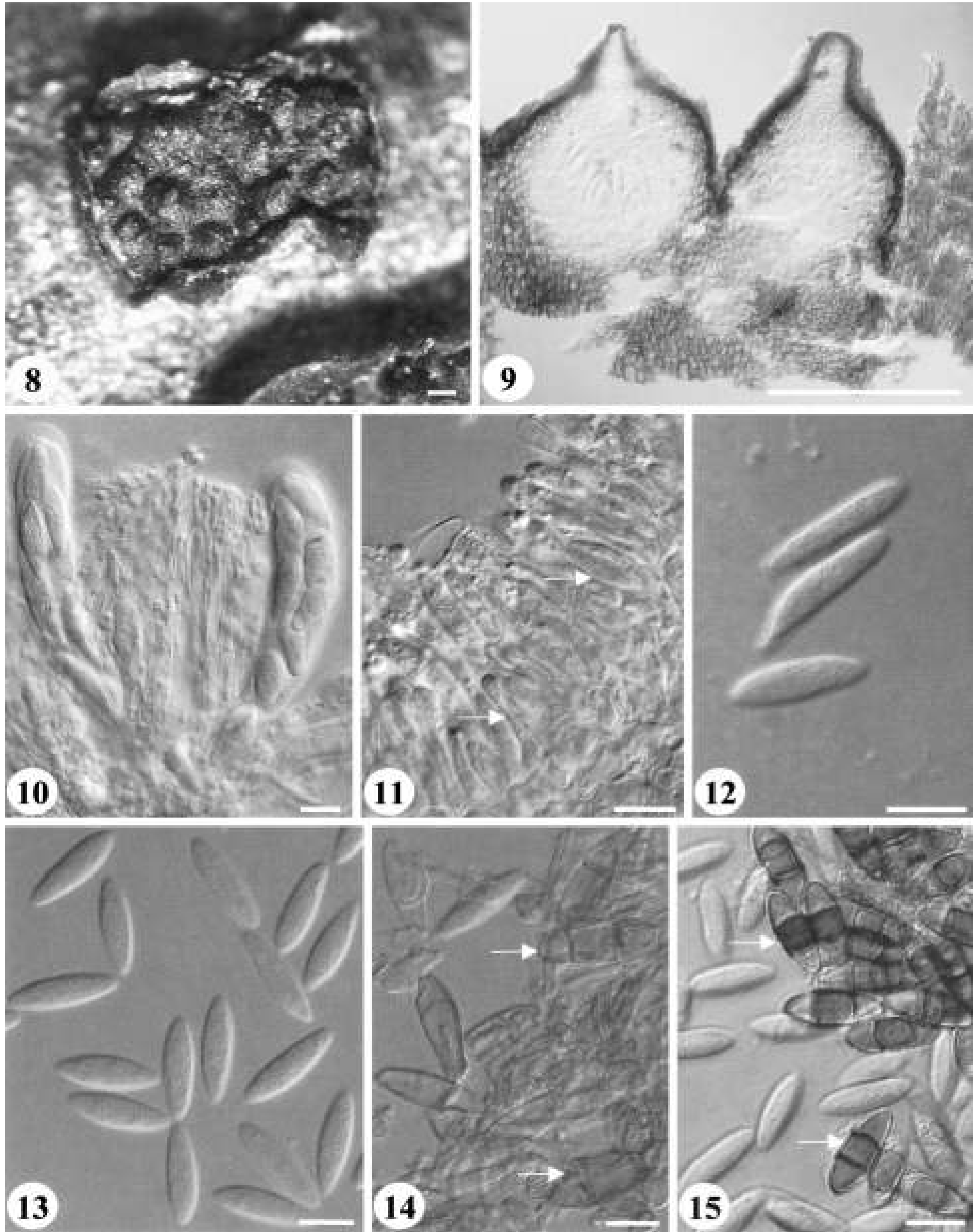
[1] The type material in the Fries herbarium is from *Rosa* sp., while that from the description is from fallen branches of *Fraxinus*. This information is based on the description of *S. dothidea* (Fries 1823).

[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.



FIGS. 1–7. *Botryosphaeria dothidea*, dissecting microscope and DIC light-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 light-microscope micrographs. 8, 9. Ascomata. Bars = 100 μm. 10. Asci and ascospores. 11. Conidiogenous cells. 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 light-microscope micrograph. Conidia produced in culture on WA and pine needles; older conidia septate (arrows). Bar = 10 μm.

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
Color	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 × 17–20 µm	75–143(–210) µm
Paraphyses	Filiform interspersed between asci	
Ascospores		
Description	Unicellular	Unicellular, smooth
Color	Hyaline	Hyaline
Shape	Fusoid	Broadly ellipsoid to fusoid
Size	(14–)18–22(–27) × 6–8(–10)[20.5 × 7.1] µm [1/w 2.9]	(14–)18–23(–26) × (7–)8–10(–11) [20.8 × 9.2 µm] [1/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) × 5–6(–7) [20.8 × 5.5] µm [1/w 3.8]	N/a
Conidial size (in vitro)	(15–)16–19(–20) × 5–6(–7) [17.2 × 5.5] µm [1/w 3.1]	(12–)15–19(–24) × 4–6 [16.9 × 5.4] µm [1/w = 3.1]
Host	*Ribes* spp.	*Populus nigra, Malus* × *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.

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 dataset, combining ITS-rDNA, β-tubulin and EF1-α, indicated that the datasets could be combined (P value = 0.32). The combined dataset consisted of 1344 characters after alignment (TreeBASE S861, M1396). A total of 968 characters were excluded, including 954

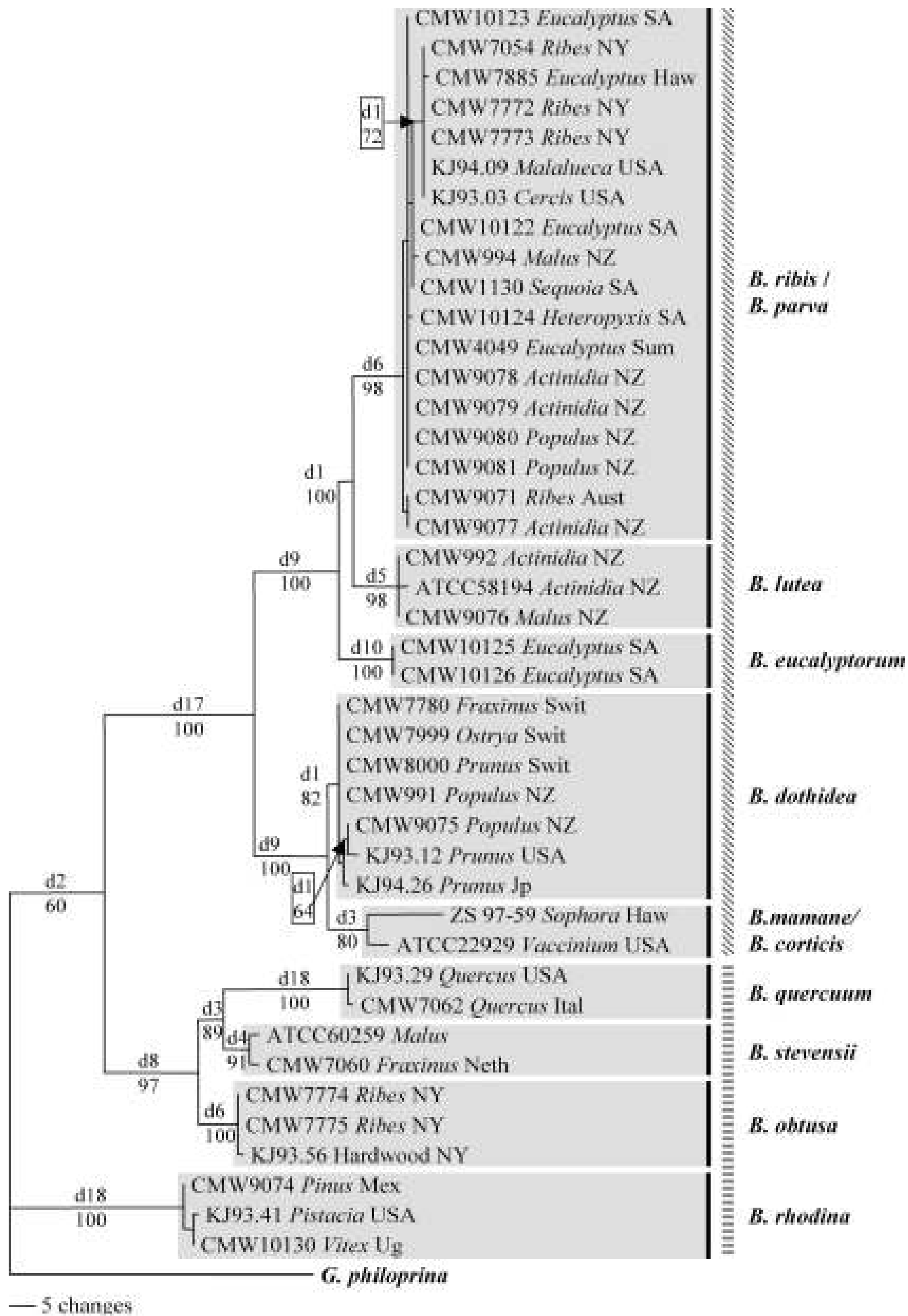


FIG. 16. Most-parsimonious tree of 325 steps obtained from ITS1, 5.8S and ITS2 rDNA sequence data. Branch supports are indicated by decay indices above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to the outgroup *Guignardia philoprina*. Clades are shaded individually 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 also are indicated. ▧ = *Botryosphaeria* spp. with *Fusicoccum* anamorphs and ▤ = *Botryosphaeria* spp. with *Diplodia* anamorphs.

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 datasets, 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* 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 related more closely 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 supported strongly (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 among 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 also is considered in this study, is not repeated here because 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, papilate 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 × 16–20 μm, 8-spored, between numerous filiform, septate, rarely branched toward the tip, pseudoparaphyses, 2–4 μm wide. *Ascospores* fusoid to ovoid, sometimes with tapered ends giving a spindle shaped appearance, (17–)19–24(–32) × (6–)7–8(–10) μm (average of 102 ascospores 22.7 × 7.8 μm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* pycnidial in nature (anamorph structures were present only on the sample from *Ostrya* sp.), morphologically indistinguishable from the ascomata. *Conidia* narrowly fusiform, or irregularly fusiform, base subtruncate to bluntly rounded, (17–)18–20(–22) × 4–5 μm (average of 35 conidia 19.6 × 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 × 2–5 μm, proliferating percurrently with 1–2 proliferations and periclinical thickening. *Spermatia* unicellular, hyaline, allantoid to rod-shaped, 3–6 × 1.5–2 μm. *Spermatophores* hyaline, cylindrical to subcylindrical, 4–10 × 1–2 μm.

Cultural characteristics. Colonies olivaceous buff (21′″d), becoming olivaceous gray (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

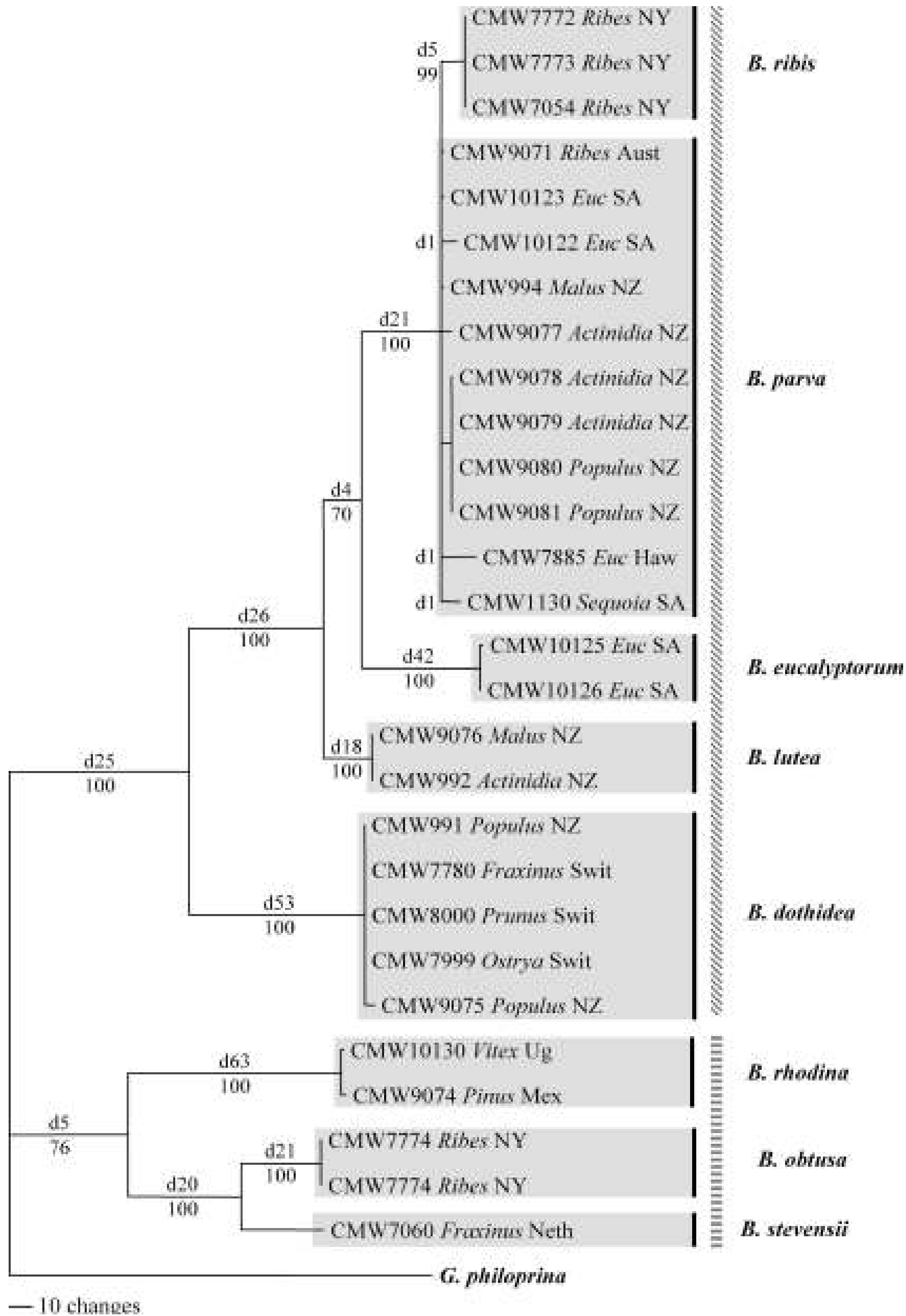


FIG. 17. Most-parsimonious tree of 858 steps obtained from 5.8S and ITS2 rDNA and partial β-tubulin and EF1-α gene sequence data. Support for the branching points are given in decay values above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to outgroup *Guignardia philoprina*. Clades are shaded individually and their identities are as used in this study. Isolate identities are given in this 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.

reaching 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) × 4–5(6) μm (average of 102 conidia 24.7 × 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., Oct 2000, *B. Slippers* (EPITYPE designated here, PREM57372, culture CMW8000); *Ostrya* sp., Oct 2000, *B. Slippers* (PREM57373, culture CMW7999); Molinizza, *Fraxinus* sp., Oct 2000, *B. Slippers* (PREM57374, culture CMW7780). ITALY. Pusiano, *Populus* sp., 31 Oct 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) × 5–6(–7) μm. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 6–22 × 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 × 5–6(–7) μm.

Ascostroma erumpent through the bark, pulvinate, 100–400 μm in diam. *Ascomata* pseudothecial, forming botryose aggregate of 5–50, globose with central ostiole, papilate 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 × 17–20 μm, 8-spored, between numerous filiform, septate, rarely branched and then toward the tip, pseudoparaphyses, 2–4 μm wide. *Ascospores* fusoid to ellipsoid, often round at the ends then broadly ellipsoidal, (14–)18–23(27) × 6–8(–10) μm (average of 80 ascospores 20.5 × 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) × 5–6(–7) μm (average of 90 conidia 20.8 × 5.5 μm, l/w 3.8), hyaline, unicellular, rarely septate with age, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 6–22 × 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 gray (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 × 5–6(–7) μm (average of 85 conidia 17.2 × 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 neotype and syntype ma-

terial of *B. dothidea*, the type species of *Botryosphaeria*. Ex-type cultures from designated type specimens and other representative specimens have 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 because the only material under this name from the Fries herbarium (which has been viewed as the type before; annotated by A.J.L. Phillips and J.A. von Arx with the sample) contains only material from what appears to be a *Rosa* sp. The holotype material from *Fraxinus* also could 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, however, is 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 also are 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 because 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* most likely was due to the variation in host and some variation in ascocarp morphology. However, we currently 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 generally is 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* thus was 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, thus is accepted.

The type specimen of *B. ribis* is well preserved, providing ample material to define this taxon. However, no cultures are 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 thus could not be designated as epitype of the teleomorph (Greuter et al 2000; ICBN article 59.2). The structures on this material, however, morphologically were identical to anamorph structures and conidia on the lectotype specimen. The isolates from this freshly collected material thus are 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 achroma-

gena 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 thus they all would be classified as achromagena variants. This characteristic initially was believed to relate to pathogenicity (Grossenbacher and Duggar 1911, Stevens and Jenkins 1924), but this notion later was rejected (Witcher and Clayton 1963). These designations no longer are 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 also were 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, however, is designated here as holotype for the anamorph name, to 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 most commonly is 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* currently are 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 possible, however, 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 thus is 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*, namely *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 might 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* all were 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 codominant 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 our study, and the characteristics overlapped between the species. Penny-

cook and Samuels (1985), however, did not consider the synonymy of these species because 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*, however, are 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 in cases where cultures are left to age 2 mo.

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 our study. Phillips et al (2002) also recently reported spermatia of similar dimensions to those described for *B. ribis/B. parva*, formed by some isolates of *B. lutea*. Spermatia have not been reported from *B. dothidea*. In our study, however, microconidia were 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 also have 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 datasets used in this study, *B. rhodina* (anamorph = *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.) grouped with other species having *Diplodia* anamorphs. These combined and separately analyzed datasets 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 also are 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* clearly can 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 at least 3 wk and preferably longer because septation of discharged, aged spores is useful in separating some species. Recently collected isolates should be used because cultures can lose their useful characteristics and ability to sporulate after repeated subculturing. 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 because it is closely related and commonly encountered in comparison 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 gray aerial mycelium 2
1. Conidia in culture averaging ≥20 μm long, l/w >3, colony on MEA or PDA appressed with only occasional tufts of gray to buff aerial mycelium 3
   2. Conidia 15–20 × 5–7 μm, becoming light brown and septate after discharge . . . . *B. ribis*
   2. Conidia 12–23 × 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 × 5–8 μm (average 22 × 6), l/w 3–4, colony on MEA or PDA producing distinct yellow pigment after 3 d, becoming dull brown to buff with age . . . .*B. lutea*
3. Conidia narrowly fusiform, 19–30 × 4–6 μm (average 25 × 5), l/w 3.5–6, colonies on MEA or PDA not producing yellow pigment and becoming gray to black with age . . . . *B. dothidea*

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