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Speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees in Australia and South Africa

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Abstract: *Botryosphaeria* spp. are important canker and die-back pathogens that affect *Eucalyptus* spp. They also occur endophytically in *Eucalyptus* leaves and stems. For the purpose of this study, *Botryosphaeria* strains were isolated from diseased and symptomless *Eucalyptus* material from Australia and South Africa. These isolates were induced to sporulate in culture, and compared with known species of *Botryosphaeria*. Selected isolates were also compared with authentic isolates of known *Botryosphaeria* spp. based on nuclear DNA sequence data of the ITS rDNA, β-tubulin and elongation factor 1-α regions. Five *Botryosphaeria* spp. were identified from *Eucalyptus* plants. The ITS rDNA sequence data were then used to develop a PCR RFLP technique that could distinguish these species. *Botryosphaeria eucalyptorum* and a new species, *B. eucalypticola*, were the most common species on *Eucalyptus* in eastern Australia. These species also occur on *Eucalyptus* in South Africa, where they have most likely been introduced. *Botryosphaeria parva* was common on *Eucalyptus* in exotic environments, but rare on this host in Australia. Although *B. dothidea* was previously thought to be common on eucalypts, only one isolate of each of *B. dothidea* and *B. australis* were found in all the areas surveyed. No isolates of *B. ribis*, which was also commonly reported from *Eucalyptus*, were identified during this survey from *Eucalyptus*. Data from the present study provide the first holistic overview of the species of *Botryosphaeria* associated with *Eucalyptus* in both native and exotic environments.

Taxonomic novelties: *Botryosphaeria eucalypticola* Slippers, Crous & M.J. Wingf. sp. nov. (anamorph *Fusicoccum eucalypticola* Slippers, Crous & M.J. Wingf. sp. nov.).
Key words: *Botryosphaeria*, *Eucalyptus*, *Fusicoccum*, *Idiocercus*, Multigene phylogeny, PCR-RFLP, Sympatric speciation.

INTRODUCTION

Botryosphaeria spp. are common and widely distributed ascomycetes that cause canker and die-back diseases on many woody plant hosts (Von Arx 1987). A part of the life-cycle of these fungi is, however, spent as endophytes within healthy plant tissue (Smith *et al.* 1996). For this reason, their introduction into new environments on germ plasm could go unnoticed, e.g. *Diplodia pinea* (Desm.) J. Kickx is thought to have been introduced to various regions of the world in this way (Burgess *et al.* 2004). In a new environment, a *Botryosphaeria* sp. has the potential of infecting different hosts or to contribute to the genetic diversity and fitness of an existing population (Wingfield *et al.* 2001a, Burgess & Wingfield 2002a). Identification and knowledge of the *Botryosphaeria* spp. that occur on plants that are moved across the world for commercial purposes is, therefore, crucially important (Palm 1999, Wingfield *et al.* 2001b).

Most *Eucalyptus* species are native to Australia, but are planted worldwide as an important source of fibre, especially in the Southern Hemisphere and the tropics and subtropical regions. *Botryosphaeria* spp. are endophytes of *Eucalyptus*, but also cause severe canker and die-back diseases in exotic plantations of these plants (Figs 1−6) (Wingfield *et al.* 1991, Smith *et al.* 1994, 1996). Species of *Botryosphaeria* are, therefore, considered to be a significant threat to the production and sustainability of *Eucalyptus* plantations.

In the past, *Botryosphaeria* spp. have been reported from native *Eucalyptus* in Australia (Davison & Tay 1983, Shearer *et al.* 1987, Old *et al.* 1990). The possible influence of these pathogens on tree health is currently of interest, because *Eucalyptus* plantations in Australia are increasing in extent and economic importance (Burgess & Wingfield 2002b, National Forest Inventory 2003). The risk of diseases is high in these plantations due to the increased genetic uniformity of the plants. Furthermore, planted trees are often on marginal sites and can subsequently be subjected to environmental and other stresses. Native stands adjacent to plantations might also be adversely affected by

increased inoculum pressure of pathogens (Strauss 2001).

A number of *Botryosphaeria* spp. have been reported from exotic *Eucalyptus* (Sankaran *et al.* 1995). *Botryosphaeria ribis* Grossenb. & Duggar has been associated with seed capsule abortion, as well as leaf and stem diseases of *Eucalyptus* worldwide (Webb 1983, Shearer *et al.* 1987, Crous *et al.* 1989, Old *et al.* 1990). *Botryosphaeria dothidea* (Moug. : Fr.) Ces. & De Not. has been commonly reported from areas around the world with temperate climates, as the cause of cankers and die-back of *Eucalyptus* (Barnard *et al.* 1987, Fisher *et al.* 1993, Smith *et al.* 1994). In tropical environments, *B. rhodina* (Berk. & M.A. Curtis) Arx, however, appears to be the dominant taxon causing these diseases (Roux *et al.* 2001). Recently, Smith *et al.* (2001) identified a new species, *B. eucalyptorum* Crous, H. Smith & M.J. Wingf., causing cankers on *Eucalyptus* spp. in South Africa.

Previous identifications of *Botryosphaeria* spp. on *Eucalyptus* should in many cases be viewed with circumspection due to the confused taxonomy of the species involved. *Botryosphaeria dothidea* and *B. ribis*, for example, have been treated as synonyms (Von Arx & Müller 1954), but this view has not been accepted by all researchers working with *Eucalyptus* pathogens. Recent studies have shown that *B. dothidea* and *B. ribis* are distinct species (Zhou & Stanosz 2001, Slippers *et al.* 2004a). Furthermore, Slippers *et al.* (2004a) showed that isolates identified as *B. dothidea* from South Africa, represent *B. parva* Pennycook & Samuels. This taxon is morphologically similar to *B. ribis* and some reports mentioned above could also have inadvertently been referring to this species.

Anamorph morphology and DNA sequence data have been used with substantial success to distinguish species of *Botryosphaeria*. Anamorph structures and conidia of these fungi are more commonly encountered in nature than their respective teleomorphs (Pennycook & Samuels 1985). Cultures can also readily be induced to produce the anamorph, and conidial morphology is more characteristic than that of the ascospores (Pennycook & Samuels 1985, Slippers *et al.* 2004a). Sequence data of the ribosomal DNA region have been most widely used to distinguish *Botryosphaeria* spp., often in combination with morphological characters (Jacobs & Rehner 1998, Denman *et al.* 2000, Zhou & Stanosz 2001, Phillips *et al.* 2002). In some cases, a combination of different gene regions, together with morphological characters, was necessary to delimit and describe some closely related or cryptic species (De Wet *et al.* 2003, Slippers *et al.* 2004a, b).

In this study the *Botryosphaeria* spp. that infect *Eucalyptus* spp. in native forests and plantations in eastern Australia are compared with those found in exotic plantations of these trees in South Africa. Characterisation of species is based on sequence data of the internal transcribed spacer (ITS) of the ribosomal RNA operon, β-tubulin and elongation factor 1-α gene regions. Species were also characterized based on morphology. Furthermore, a reliable PCR RFLP identification tool was developed to distinguish the *Botryosphaeria* spp. that occur on *Eucalyptus*.

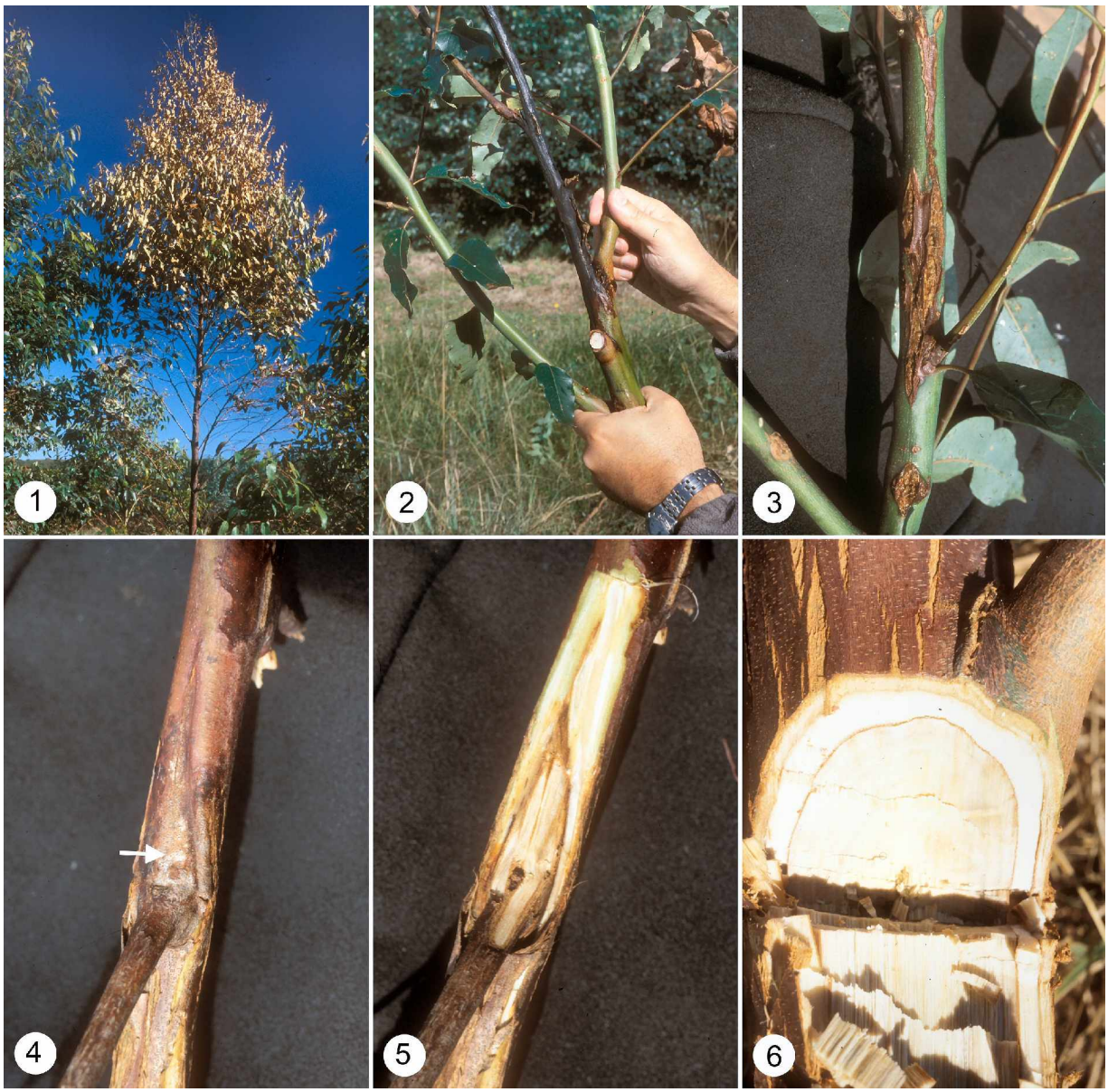
MATERIALS AND METHODS

Fungal isolates and DNA isolation

A total of 86 isolates were used in this study. Of these, 55 isolates were collected from the Mpumalanga and KwaZulu-Natal Provinces of South Africa between 1990 and 2001, and 27 isolates from Eastern Australia between July and December 2001. Five isolates collected in 2001 from *Tibouchina* in Eastern Australia were also included, as pathogens of this host are known to also occur on *Eucalyptus*. The latter isolates have also been included in a study of Heath (2003), and are used here for comparative purposes. Isolates were grown on malt and yeast extract agar (MYA) (2 % malt extract, 0.2 % yeast extract and 2 % agar; Biolab, Johannesburg, South Africa) at 25 °C in the dark or under near-UV light. Cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Reference strains have also been deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands.

Prior to the study of Smith *et al.* (1996), two other *Botryosphaeria*-like species were reported from *Eucalyptus* in South Africa, namely *B. ribis* (Crous *et al.* 1989), and *Idiocercus australis* (Cooke) H.J. Swart (Crous *et al.* 1990), which was noted to resemble *Botryosphaeria* in morphology (Swart 1988). Because part of the aim of the present study was to resolve South African records from *Eucalyptus*, the original specimens on which these records were based, were re-examined.

A modified phenol and chloroform extraction method described by Reader & Broda (1985) was used to extract DNA from all isolates. The basic procedure is similar to that described by Slippers *et al.* (2004a). Extracted DNA was precipitated by adding 0.1 vol. 3 M NaAc (pH 5–5.5) and 2 vol. absolute EtOH at 4 °C. The precipitated DNA was washed (70% EtOH), dried and resuspended to approximately 80–100 ng/μL in sterile water. DNA concentration was estimated using λ-marker standard (λ-DNA digested with *Hae*III and *Eco*RI) after electrophoresis on a 1 % Ethidium Bromide-stained agarose gel and visualized under UV light.



Figs 1–6. Disease symptoms associated with infection by *Botryosphaeria* spp. on *Eucalyptus* trees. 1. Die-back of tree tops often after damage by late frost or hot winds. 2. Stem canker and die-back taken from the growing tip of a tree. The young bark associated with these cankers is typically blackened and kino is also commonly exuded. 3. Localised cankers on stem following wounding. 4, 5. Canker commencing from infection of a side branch and *Botryosphaeria* pseudothecia on the dead bark (arrow). Internally the wood is killed and has a brown to blackish-brown colour. 6. Canker of the main stem of a tree causing cracking of the bark and brown discoloured, dead xylem.

DNA extraction and sequencing

The extracted DNA was used as template in the amplification reactions. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) operon was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTATTGATATGC) (White *et al.* 1990). A part of the β-tubulin gene of selected isolates was amplified using Bt2a (5' GGTAACCAAATCGGTGCTGCTTC) and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC) (Glass & Donaldson 1995). Part of the elongation factor 1-α (EF 1-α) was amplified using the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTG AAGGAACCCTTACC) (Carbone *et al.* 1999). The same amplification protocol was used to amplify the ITS and β-tubulin regions (using *Taq* polymerase; Roche Molecular Biochemicals, Alameda, CA) and the EF-1-α region (using Expand *Taq* Polymerase; Roche Molecular Biochemicals) as described in Slippers *et al.* (2004a). All PCR products were visualized under UV light on 1 % agarose gels stained with Ethidium Bromide. Sizes of fragments were estimated against a standard 100 bp marker (Roche Molecular Biochemicals).

A selected number of the *Botryosphaeria* isolates were sequenced (Table 1). The PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals).

Table 1. Isolates considered in the phylogenetic study.

Culture no.[1]	Other no.[1]	Identity	Host	Location	Collector	ITS[2]	β-tubulin[2]	EF 1-α[2]
CMW 7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, U.S.A.	B. Slippers/G. Hudler	AY236925	AY236906	AY236877
CMW 7054	CBS 121.26	*B. ribis*	*R. rubrum*	New York, U.S.A.	N.E. Stevens	AF241177	AY236908	AY236879
CMW 6235		*B. parva*	*Tibouchina lepidota*	Melbourne, Victoria, Australia	M.J. Wingfield	AY615136	AY615120	AY615128
CMW 6237		*B. parva*	*T. urvilleana*	Melbourne, Victoria, Australia	M.J. Wingfield	AY615137	AY615121	AY615129
CMW 9071		*B. parva*	*Ribes* sp.	Australia	M.J. Wingfield	AY236938	AY236909	AY236880
CMW 9078	ICMP 7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236940	AY236914	AY236885
CMW 9081	ICMP 8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236917	AY236888
CMW 10122	BOT 21	*B. parva*	*Eucalyptus grandis*	Mpumalanga, R.S.A	H. Smith	AF283681	AY236911	AY236882
CMW 10123	BOT 19	*B. parva*	*E. smithii*	Mpumalanga, R.S.A	H. Smith	AF283683	AY236910	AY236881
CMW 6233	CBS 15768	*B. eucalyptorum*	*E. nitens*	Canberra, NSW, Australia	M.J. Wingfield	AY615138	AY615122	AY615130
CMW 6804		*B. eucalyptorum*	*E. dunnii*	Toowoomba, Queensland, Australia	M.J. Wingfield	AY615139	AY615123	AY61531
CMW 10125	CBS 115791	*B. eucalyptorum*	*E. grandis*	Mpumalanga, R.S.A	H. Smith	AF283686	AY236920	AY236891
CMW 10126		*B. eucalyptorum*	*E. grandis*	Mpumalanga, R.S.A	H. Smith	AF283687	AY236921	AY236892
CMW 6217	CBS 115766	*B. eucalypticola*	*E. rossii*	Tidbinbilla, NSW, Australia	M.J. Wingfield	AY615143	AY615127	AY615135
CMW 6229	CBS 115767	*B. eucalypticola*	*E. grandis*	Orbost, Victoria, Australia	M.J. Wingfield	AY615142	AY615126	AY615134
CMW 6539	CBS 115679	*B. eucalypticola*	*E. grandis*	Orbost, Victoria, Australia	M.J. Wingfield	AY615141	AY615125	AY615133
CMW 6543	CBS 115770	*B. eucalypticola*	*Eucalyptus* sp.	Orbost, Victoria, Australia	M.J. Wingfield	AY615140	AY615124	AY615132
CMW 992/3	KJ 93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels	AF027745	AY236923	AY236894
CMW 10309	CAP 002	*B. lutea*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY339258	AY339250	AY339266
CMW 9073		*B. australis*	*Acacia* sp.	Melbourne, Victoria, Australia	J. Roux/ D. Guest	AY339261	AY339253	AY339269
CMW 6837		*B. australis*	*Acacia* sp.	Batemans Bay, NSW, Australia	M.J. Wingfield	AY339262	AY339254	AY339270
CMW 9075		*B. dothidea*	*Populus* sp.	New Zealand	G.J. Samuels	AY236950	AY236928	AY236899
CMW 8000		*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236927	AY236898
CMW 7060	CBS 431.82	*B. stevensii*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa	AY236955	AY236933	AY236904
CMW 7774		*B. obtusa*	*Ribes* sp.	New York, U.S.A.	B. Slippers/G. Hudler	AY236953	AY236931	AY236902
CMW 10130	BOT 977	*B. rhodina*	*Vitex* sp.	Uganda	J. Roux	AY236951	AY236929	AY236900

[1]Designation of isolates and culture collections: C.A.P. = Culture collection of A.J.L. Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; C.M.W. = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; K.J. = Jacobs and Rehner (1998). [2]GenBank accession numbers.

Each PCR product was sequenced in both directions with the same primers as used for PCR. The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) was used to perform the sequencing reactions and the sequences were run on an ABI PRISM 377/3100 Autosequencer (Perkin-Elmer Applied Biosystems).

DNA sequence analyses

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems) and sequences were manually aligned. In order to determine the phylogenetic relationship and the identities of the *Botryosphaeria* spp. used in this study, sequences of known *Botryosphaeria* spp. were obtained from GenBank, and included in the alignment (Table 1). The *Botryosphaeria* spp. with *Fusicoccum* anamorphs from *Eucalyptus* trees were the focus of this study. The trees were thus rooted to the GenBank sequences of the sister group of *Botryosphaeria* spp. (*B. stevensii* Shoemaker, *B. obtusa* (Schwein.) Shoemaker and *B. rhodina*) with *Diplodia* or *Lasiodiplodia* anamorphs (Jacobs & Rehner 1998, Slippers *et al.* 2004a).

Phylogenetic relationships were determined from these aligned sequences in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford 1999). Nucleotides were treated as unordered, unweighted characters, and gaps were treated as a fifth character. A partition homogeneity test was done to determine the congruence of the three datasets (Farris *et al.* 1995, Huelsenbeck *et al.* 1996). After a positive outcome, the datasets were analyzed together. Heuristic searches, using random stepwise addition and tree bisection and reconstruction (TBR) as branch swapping algorithm, were used to find the most parsimonious trees. The phylogenetic signal from the dataset was evaluated against random trees as described by Hillis & Huelsenbeck (1992). One thousand bootstrap replicates (Felsenstein 1985) were done to determine the support for branches. Decay indices for the branches were determined using the program Autodecay (Eriksson 1998) in combination with PAUP. To confirm phylogenetic species hypotheses inferred from parsimony, the data were also analyzed by distance analyses with the neighbour-joining algorithm, using both an uncorrected p-factor and HKY85 parameters alternatively in PAUP. To test the consistency of branches in the combined dataset, the three partial gene sequence datasets were also analyzed separately, but in the same way as described above.

PCR-RFLP

Sequence data of the ITS region of sequenced isolates were analyzed in Webcutter 2.0 (www.firstmarket.com/cutter.cut2) to identify polymorphisms of restriction enzyme sites in different *Botryosphaeria* spp., which could potentially discriminate between these species. Restriction fragment maps were constructed and the restriction enzymes (RE) *Cfo*I, *Ksp*I and *Sty*I (Roche Diagnostics, Indianapolis, U.S.A.) were selected to identify the remaining isolates that were not identified using sequence data.

Each RFLP reaction consisted of 20 µL PCR reaction with ITS DNA template, 0.3 µL restriction enzyme, 2.2 µL matching enzyme buffer and 2.5 µL sterile Sabax water. The reaction mixture was incubated at 37 °C for 3 h. Restriction fragments were separated on 1.5 or 2 % agarose gels that were stained with Ethidium Bromide and visualized under UV light. Fragments sizes were estimated against a standard 100 bp marker.

Morphological characterisation

All samples from Australia of the various *Botryosphaeria* spp. that were identified in this study, were characterized by light microscopy. Teleomorph structures and spores were studied from field-collected samples from Australia. Anamorph characters were studied from these samples and from structures produced *in vitro*. Cultures were induced to sporulate by plating on water agar (WA) (2 % agar; Biolab, Johannesburg, South Africa), amended with sterilized pine needles as substrate and incubating these at 25 °C under near-UV light. Sections of sporocarps were made with an American Optical Freezing Microtome or by hand and mounted in clear lactophenol. Measurements and photographs were taken with an Axiocam digital camera (Carl Zeiss, Germany). Growth rate was determined at 5 °C intervals between 10 and 30 °C, and colony morphology and colour (Rayner 1970) was assessed for cultures grown at 25 °C, in the dark and on potato-dextrose agar (PDA) (0.4 % potato extract, 2 % dextrose, 1.5 % agar; Biolab).

RESULTS

DNA-based characterisation

DNA fragments of approximately 600 bp (ITS-rDNA), 450 bp (β-tubulin) and 300 bp (EF1-α) were amplified in PCR reactions. A partition homogeneity test showed that the sequence data sets from these gene regions were congruent (P value = 0.84). After alignment, the combined dataset consisted of 1321 characters, of which 331 were parsimony-informative. Fourteen variable sites in the EF1-α were made up of two identical repeats of seven base pairs in isolates of *B. ribis*. These sites were coded as two evolutionary events by excluding twelve of the fourteen base pairs of the repeat. The combined data set contained significant phylogenetic signal compared to random sampling ($P < 0.01$; $g1 = -0.82$) (Hillis & Huelsenbeck 1992).

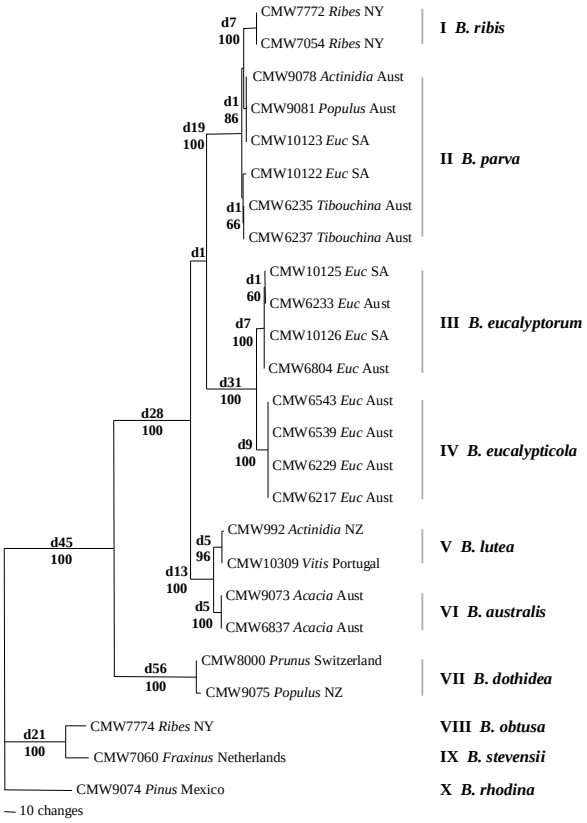


Fig. 7. One of two most parsimonious trees retained after heuristic searches of the combined dataset of ITS rDNA, β-tubulin and EF1-α sequence data. Branch supports are given as decay values above and bootstrap values (1000 replicates) below the branches. The trees are rooted to *B. obtusa*, *B. rhodina* and *B. stevensii*, which are all in the subsection characterized by *Diplodia*-like conidia, unlike the ingroup taxa that all have *Fusicoccum*-like conidia. Isolates numbers, host and origin (Aust = Australia, NY = New York, USA, NZ = New Zealand, SA = South Africa), as well as the identity of the clades, are indicated.

Two most parsimonious trees of 559 steps were retained (CI = 0.839; RI = 0.929) after heuristic searches in PAUP (Fig. 7). Both trees had the same topology and varied only within the identified clades. The topology of the MP trees and conclusions drawn from them were the same as for trees generated by distance analyses.

Seven ingroup clades (I–VII) were identified and these correspond to *B. ribis*, *B. parva*, *B. eucalyptorum*, a *Botryosphaeria* sp. (described below as a new species), *B. lutea* A.J.L. Phillips, *B. australis* Slippers, Crous & M.J. Wingf. and *B. dothidea* (Fig. 7). All isolates from *Eucalyptus* and *Tibouchina* trees grouped in clades II, III, IV, VI and VII.

Sequence variation in Clade II (*B. parva*) resulted in a separate branch with high bootstrap support (86 %), although short (2 steps) and with a low decay value (d1). Analysis of polymorphisms/alleles within this group showed that there are significantly more fixed alleles that group these isolates with *B. parva*, than alleles that separate them as two species (Table 2).

Clades III and IV represent closely related, but distinct phylogenetic species. There were 19 polymorphisms among isolates in clades III and IV. Of these, 16 were fixed in both groups and in each of the three gene regions (Table 3) (Fig. 8A–C).

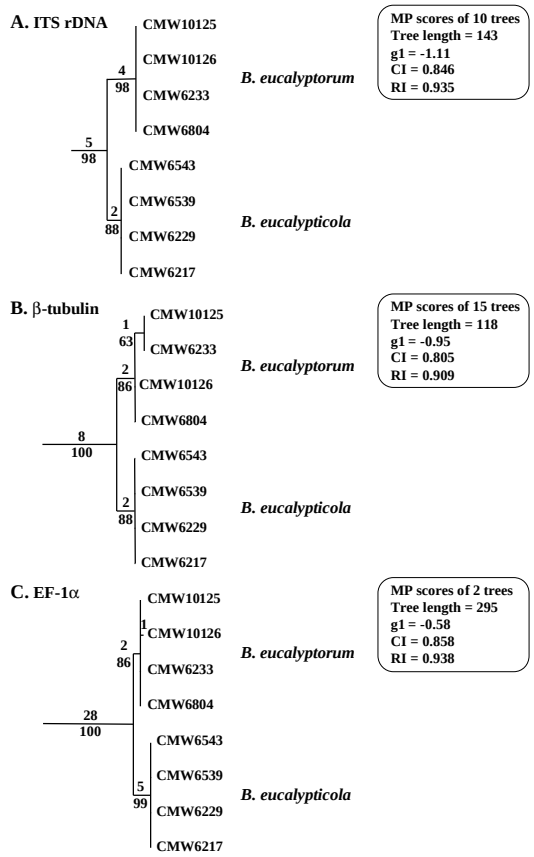


Fig. 8. Most parsimonious (MP) trees retained after analyzing the sequence data of three gene regions separately, showing the consistent separation of the *B. eucalyptorum* and *B. eucalypticola* clades. Relationships to other species considered are the same as in the tree obtained from the combined dataset (Fig. 7) and are not shown. Trees were obtained from sequence data of the following DNA regions: (A) ITS rDNA, (B) β-tubulin and (C) EF1-α. Data of the tree length, phylogenetic signal (g1), and consistency and retention indexes (CI, RI) are given directly opposite each tree.

PCR-RFLP

Restriction maps were determined for three restriction endonucleases (RE), *Cfo*I, *Ksp*I and *Sty*I, that would give distinct digestion patterns of ITS amplicons for all *Botryosphaeria* spp. identified by sequence data from *Eucalyptus* in this study (Fig. 9A–C). The enzymes were used separately and in a specific order. *Cfo*I produced distinctive fragment patterns for *B. parva* and *B. eucalyptorum* (Fig. 10A). From the remaining three species, *Ksp*I allows for the distinction of *B. australis*, and *Sty*I separated *B. dothidea* and the unknown *Botryosphaeria* sp. (Fig. 10B, C). The identities of 81 isolates could thus be determined using these three enzymes (Table 4).

Table 2. Polymorphic nucleotides[1] (or alleles) from sequence data of the ITS rDNA, β-tubulin and EF1-α, from isolates in the *B. ribis* and *B. parva* clades. *Botryosphaeria eucalyptorum* and *B. lutea* are included for outgroup comparisons.

Identity	Culture number	β-tubulin					ITS				EF1-α							
		95	128	187	418	436	512	584	863	936	1082	1083	1094	1101	1191	1252	1310	1314
B. ribis	CMW 7772	C	G	T	T	T	A	G	T	-	T	G	1	1	C	A	G	A
	CMW 7045	C	G	T	T	T	A	G	T	-	T	G	1	1	C	A	G	A
B. parva	CMW 9080	T	A	C	T	T	T	-	C	-	C	A	0	0	T	G	A	A
	CMW 9081	T	A	C	T	T	T	-	C	-	C	A	0	0	T	G	A	A
	CMW 10123	T	A	C	T	T	T	-	C	-	C	A	0	0	T	G	A	A
	CMW 6235	T	A	C	C	C	A	-	C	A	C	A	0	0	T	G	G	***C***
	CMW 6237	T	A	C	C	C	A	-	C	A	C	A	0	0	T	G	G	C
B. eucalyptorum	CMW 11705	T	A	C	C	C	T	-	C	-	-	-	0	0	T	G	G	A
B. lutea	CMW 10309	T	A	C	C	C	C	-	T	-	-	-	0	0	T	A	G	G

[1]The polymorphisms that are unique to a specific group are highlighted.

Table 3. Polymorphic nucleotides[1] (or alleles) from sequence data of the ITS rDNA, β-tubulin and EF1-α, from isolates in the *B. eucalyptorum* and *B. eucalypticola*. *Botryosphaeria lutea* and *B. dothidea* are included as outgroup sequence to illustrate derived characters.

Identity	Culture number	β-tubulin					ITS							EF1-α						
		80	98	275	331	367	566	567	570	599	654	843	969	1123	1127	1212	1228	1270	1309	1330
B. eucalyptorum	CMW 10125	t	T	A	G	C	T	-	c	T	A	T	C	G	T	T	G	C	T	C
	CMW 11705	c	T	A	G	C	T	-	c	T	A	T	C	G	T	T	G	C	T	C
	CMW 6233	t	T	A	G	C	T	c	-	T	A	T	C	G	T	T	G	C	T	C
	CMW 6804	c	T	A	G	C	T	c	-	T	A	T	C	G	T	T	G	C	T	*C*
B. eucalypticola	CMW 6543	c	C	G	A	T	C	c	-	C	C	C	T	A	C	C	A	T	C	T
	CMW 6539	c	C	G	A	T	C	c	-	C	C	C	T	A	C	C	A	T	C	T
	CMW 6229	c	C	G	A	T	C	c	-	C	C	C	T	A	C	C	A	T	C	T
	CMW 6217	c	C	G	A	T	C	c	-	C	C	C	T	A	C	C	A	T	C	T
B. lutea	CMW 10309	C	C	G	G	C	C	C	G	C	A	C	C	G	T	C	G	C	C	C
B. dothidea	CMW 8000	C	C	G	G	C	C	C	C	C	A	C	C	G	T	C	G	C	C	*C*

[1]Polymorphisms that are not fixed in both populations are in lower case. The derived (apomorphic) characters in either of *B. eucalyptorum* or *B. eucalypticola* are shaded.

Table 4. Identities of *Botryosphaeria* spp. isolated from *Eucalyptus* in different regions, identified using the PCR RFLP profiles of the ITS rDNA region.

Identity	Australia	South Africa	Total
B. dothidea	1	0	1
B. parva[1]	4	26	30
B. australis	1	0	1
B. eucalyptorum	13	11	24
B. eucalypticola	8	17	25

[1] *Botryosphaeria parva* and *B. ribis* cannot be distinguished using the PCR RFLP profiles, but no isolates could be identified as *B. ribis* in this or previous studies, based on sequence data.

Table 5. Conidial measurements for anamorphs of *Botryosphaeria* spp. isolated from *Eucalyptus* and *Tibouchina* in Australia.

Identity	Culture No.	Conidial measurements[1]	Host	Location
B. parva	CMW 6237	(15−)17.5(−20) × 5	*Tibouchina urvilleana*	Melbourne
	CMW 6235	(15−)16.3(−20) × (5−)5.5(−6.04)	*T. lepidota*	Melbourne
	CMW 6236	(15−)16.5(−20) × 5	*T. lepidota*	Melbourne
	CMW 6536	(17.5−)18.75(−20) × 5	*T. lepidota*	Melbourne
	CMW 6797	(17.5−)18.75(−22.5) × 5	*Tibouchina* sp.	Coffs Harbour
	CMW 6799	(15−)17.5(−20) × (5−)5.25(−7.5)	*Eucalyptus grandis*	Kyogle
	CMW 6802	(17.5−)17.25(−20) × 5	*E. grandis*	Kyogle
	CMW 6798	(17.5−)19.75(−20) × 5	*E. grandis*	Kyogle
	CMW 6812	(21−)24.2(−25) × (5−)5.8(−7)	*E. pilularis*	Zuills Grafton
B. eucalyptorum	CMW 6550	(22.5−)24.5(−25) × 7.5	*E. nitens*	Uriarra
	CMW 6551	(20−)23(−27.5) × (5−)7(−7.5)	*E. nitens*	Uriarra
	CMW 6804	(22.5−)24(−27.5) × (5−)7(−7.5)	*E. dunnii*	Towoomba
	CMW 6805	(25−)26.5(−27.5) × (5−)6.8(−7.5)	*E. dunnii*	Towoomba
	CMW 6810	(22.5−)24.8(−27.5) × (7.5−)7.7(−10)	*E. grandis*	Zuills Grafton
	CMW 6807	(20−)23.5(−27.5) × (5−)7.3(−10)	*E. rossii*	Canberra
	CMW 6545	(20−)25(−30) × (7.5−)8.3(−10)	*E. rossii*	Tidbinbilla
	CMW 6808	(17.5−)22(−25) × (7.5−)7.3(−7.5)	*Eucalyptus* sp.	Canberra
	CMW 6811	(22.5−)25(−30) × (7.5−)7.3(−10)	*E. pilularis*	Zuills Grafton
	CMW 6808	(17.5−)22(−25) × 7.3	*E. dunnii*	Towoomba
	CMW 6818	(27.5−)30.5(−35) × (7.5−)8(−10)	*E. nitens*	Canberra
	CMW 6815	(25−)26.3(−27.5) × (5−)6.8(−7.5)	*E. dunnii*	Towoomba
B. eucalypticola	CMW 6229	(25−)25.75(−30) × (7.5−)8.25(−10)	*E. grandis*	Orbost
	CMW 6539	(22.5−)26.75(−30) × (5−)7.25(−7.5)	*E. grandis*	Orbost
	CMW 6220	(22.5−)25.5(−27.5) × (7.5−)8.5(−10)	*Eucalyptus* sp.	Tidbinbilla
	CMW 6543	(25−)29.25(−35) × 7.5	*Eucalyptus* sp.	Orbost
	CMW 6219	(25−)29.25(−32.5) × (7.5−)9.25(−10)	*E. rossii*	Tidbinbilla
	CMW 6221	(20−)24.75(−27.5) × 7.5(−10)	*Eucalyptus* sp.	Tidbinbilla
	CMW 6545	(20−)25(−30) × (7.5−)8.25(−10)	*Eucalyptus* sp.	Orbost
	CMW 6222	(22.5−)25.25(−30) × (5−)7.75(−10)	*Eucalyptus* sp.	Tidbinbilla
	CMW 6217	(25−)25.5(−30) × (7.5−)8.5(−10)	*E. rossii*	Tidbinbilla
	CMW 6229	(25−)25.75(−30) × (7.5−)8.25(−10)	*E. grandis*	Orbost
B. australis	CMW 6230	(20−)23.4(−25) × (5−)5.5(−7.5)	*E. grandis*	Orbost
B. dothidea	CMW 6801	(25−)26(−27.5) × 5	*Eucalyptus* sp.	Kyogle

[1] Measurements in brackets are actual ranges. Values outside brackets are averages of 15 conidia.

Morphological characterisation

Ascospores were observed from a limited number of samples, but representing all species. These ascospores were hyaline, aseptate, ovoid to ellipsoidal, smooth with granular contents, 8 spores were transversely biseriate in bi-tunicate asci, produced in spherical to papillate black ascomata, single or in botryose clusters, with a central ostiole. Conidia of all isolates included in this study were hyaline, aseptate and shapes were ellipsoidal to fusiform, produced holoblastically on hyaline, subcylindrical conidiogenous cells, with percurrent proliferation producing periclinal thickening. These characteristics are typical of *Botryosphaeria* spp. with *Fusicoccum* anamorphs, and represent *B. parva*, *B. australis*, *B. eucalyptorum* and *B. dothidea* and an unknown *Botryosphaeria* sp. (described below as a new species) (Table 5; Figs 11–20). Morphological and cultural characters that distinguish the species from *Eucalyptus* treated here are presented in the key below.

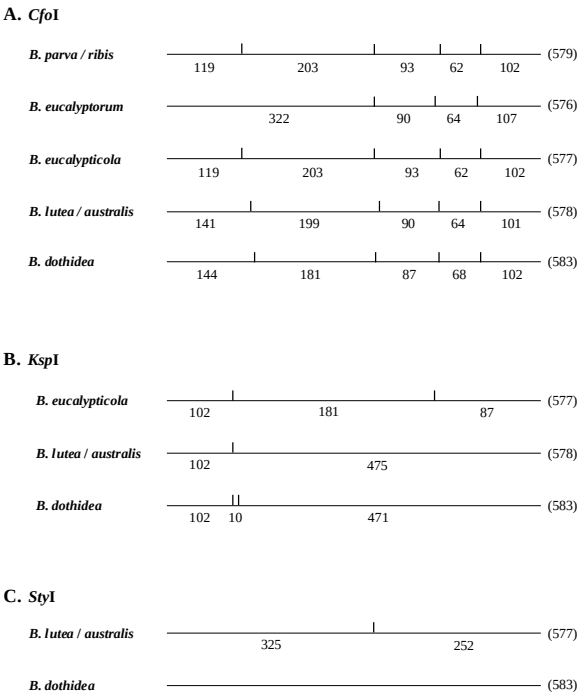


Fig. 9. Restriction fragment length polymorphism maps of ITS rDNA PCR amplicons of five *Botryosphaeria* spp. when digested with the restriction enzymes *Cfo*I (A), *Ksp*I (B) and *Sty*I (C). The enzymes are used in succession to distinguish all five species. The total length (in base pairs) of each fragment is

given in brackets, and fragment lengths are given below each line. These RFLP fragments could not distinguish *Botryosphaeria parva* from *B. ribis*, or *B. lutea* from *B. australis*.

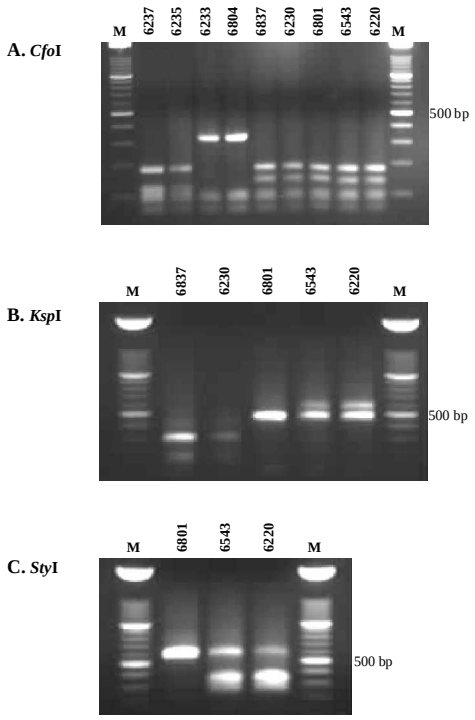


Fig. 10. Agarose gels showing fragments of ITS PCR amplicons of five *Botryosphaeria* spp., namely *B. parva* (CMW 6237, CMW 6235), *B. eucalyptorum* (CMW 6233, CMW 6804), *B. eucalypticola* (CMW 6837, CMW 6230), *B. australis* (CMW 6543, CMW 6220) and *B. dothidea* (CMW 6801), after digestion with (A) *Cfo*I, (B) *Ksp*I and (C) *Sty*I. The numbers above the lanes refer to the CMW numbers. Lane M contains a 100 bp size marker.

TAXONOMY

Based on morphology, some specimens and isolates collected from *Eucalyptus* in Australia resembled *B. eucalyptorum* (Smith *et al.* 2001) and might not have been separated from this species based solely on these characters. These isolates were, however, identified as a distinct sister species to *B. eucalyptorum* using combined sequence data for the three gene regions considered in this study. Subsequently it was shown that the conidia of these species are also distinct (see key). The fungus is thus described here as a new species as follows:

Botryosphaeria eucalypticola Slippers, Crous & M.J. Wingf., **sp. nov.** MycoBank MB500089. Figs 11–19.

Anamorph: *Fusicoccum eucalypticola* Slippers, Crous & M.J. Wingf., sp. nov.

Etymology: Referring to the only known host of this fungus.

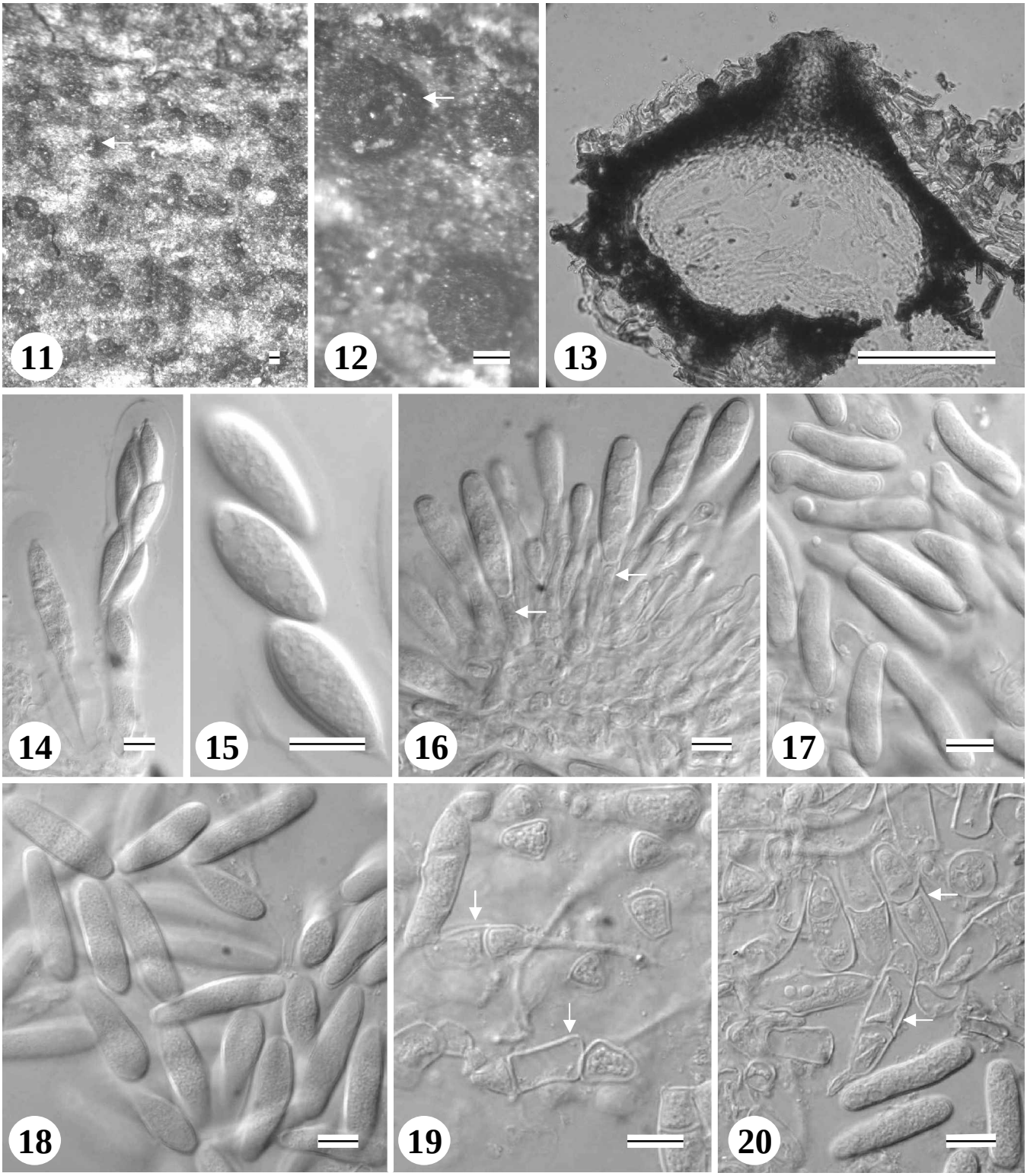
Ascostroma indistincta. Ascomata pseudothecia, plerumque solitaria, interdum 2–3 aggregatae, globosa, ostiolo centrali, papillata, 1/3 vel 2/3 parte emergente, nigra, 160–340 µm diam.; paries pseudothecii e 5–8 stratis texturae angularis, extus e cellulis atrobrunneis vel brunneis composita, intus e cellulis hyalinis revestimentum loculi facientibus. Asci bitunicati, clavati, 70–110 × 20–25 µm, octospori, paraphysibus multis filiformibus septatis raro apicem versus ramosis, 2–4 µm latis interspersi. Ascosporae fusoideae vel ovoideae, 20–22(–23.5) × 7–8 µm, unicellulares, hyalinae, laeves, contentu granulari, in asco biseriatae.

Ascostroma indistinct. *Ascomata* pseudothecia, mostly solitary, sometimes forming a botryose aggregate of 2–3 structures, globose with a central ostiole, papillate, imbedded with 1/3 to 2/3 emerging, black, 160–340 µm diam.; pseudothecial wall comprising 5–8 layers of *textura angularis*, outer region of dark or medium brown cells, inner region of hyaline cells lining the locule. *Asci* bitunicate, clavate, 70–110 × 20–25 µm, 8-spored, interspersed with numerous filiform, septate pseudoparaphyses, rarely branched towards the tip, 2–4 µm wide. *Ascospores* fusoid to ovoid, 20–22(–23.5) × 7–8 µm (av. of 50 ascospores = 21.7 × 7.6 µm, l/w 2.8), unicellular, hyaline, smooth with granular contents, biseriate in the ascus.

Fusicoccum eucalypticola Slippers, Crous & M.J. Wingf., **sp. nov.** MycoBank MB500090.

Pycnidia in agaro acquoso in acubus pinorum sterilifactis post 7–21 dies formata, superficialia, globosa, plerumque solitaria, mycelio tecta. Conidia in cultura fusiformia vel baculata, saepe flexa fel forma irregularia, basi subtruncata vel obtuse rotundata, (20–)25–27(–35) × (5–)7–9(–10) µm, hyalina, unicellularia, ante germinationem septata, laevia, contentu exigue granulari.

Pycnidia (formed on WA on sterilized pine needles within 7–21 d) superficial, globose, mostly solitary, and covered by mycelium. *Conidia* produced in culture fusiform to rod-shaped, often bent or irregularly shaped, apex obtuse, bases subtruncate to bluntly rounded, (20–)25–27(–35) × (5–)7–9(–10) µm (av. of 135 conidia 26.3 × 7.2 µm, l/w 3.6), hyaline, unicellular, sometimes forming 1–2 transverse septa before germination, smooth with fine granular contents. *Cultural characteristics:* Colonies white to buff (19’’f) or olivaceous-grey (21’’’’’i), sometimes becoming olivaceous-black (21’’’d) at the centre after 7 d, with a dense mat of aerial mycelium, edges smooth to crenulate, sometimes not reaching the edge of the plate. Optimum temperature for growth 25 °C, colonies slow-growing compared with other *Botryosphaeria* spp., reaching 34–43 mm radius on PDA after 4 d at 25 °C in the dark.



Figs 11–20. *Botryosphaeria* spp. 11-19. Dissecting microscope and DIC compound-microscope micrographs of *Botryosphaeria eucalypticola*. 11, 12. Spherical, singular ascomata that erupt through the bark (arrows). 13. Median, longitudinal section through an ascoma. Bars = 100 µm. 14. Bi-tunicate asci. 15. Mature ascospores. 16. Conidiogenous cells (arrows) and immature conidia. 17, 18. Fusiform to rod-shaped mature conidia that are often bent or irregularly shaped. 19. Septate germinating conidia (arrows). 20. *Botryosphaeria eucalyptorum* septate germinating- (arrows) and aseptate conidia. Scale bars = 10 µm.

Specimens examined: **Australia**, Victoria, Orbost, *Eucalyptus grandis*, 2001, M.J. Wingfield, **holotype** PREM 57848; culture ex-type CBS 115679; Tidbinbilla, *Eucalyptus rossii*, M.J. Wingfield, PREM 57845; *Eucalyptus* sp., M.J. Wingfield, PREM 57846; *Eucalyptus* sp., M.J. Wingfield, PREM 57847.

Botryosphaeria eucalyptorum Crous, H. Smith & M.J. Wingf., Mycologia 93: 280. 2001.
Anamorph: *Fusicoccum eucalyptorum* Crous, H. Smith & M.J. Wingf., Mycologia 93: 280. 2001. Fig. 20.

= *Phoma australis* Cooke, Grevillea 15: 17. 1886.

≡ *Idiocercus australis* (Cooke) H.J. Swart, Trans. Brit. Mycol. Soc. 90: 283. 1988.

Notes: The South African specimen of *I. australis* collected in 1988 (PREM 50452) has smaller conidia (4.5–11 ×1.5–4 µm) than the Australian type (K 121467), and is morphologically distinct. As no cultures were obtained, fresh collections would be required to resolve its taxonomy. Type material of *I. australis* from Australia, however, was morphologically indistinguishable from *F. eucalyptorum*. Conidia were hyaline, clavate to ellipsoid, sometimes irregular, smooth, granular, with obtuse apices and subtruncate bases, sometimes with basal frill, (17–)20–23(–29) × (6–)7(–8) µm (av. 21 × 7 µm) (Fig. 21). In his treatment of the genus *Idiocercus*, Nag Raj (1993) excluded *I. australis*, but did not suggest an alternative genus for it. This situation has now been resolved.

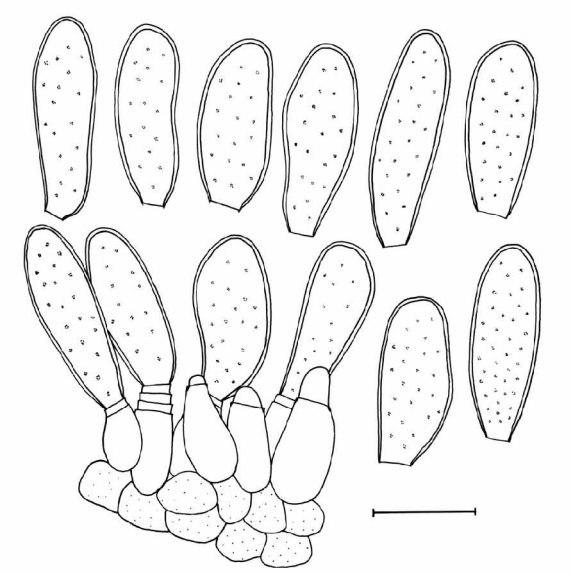


Fig. 21. Conidia and conidiogenous cells of *Idiocercus australis* (= *Fusicoccum eucalyptorum*) (Holotype, K). Scale bar = 10 µm.

Idiocercus australis should be recombined into *Fusicoccum*, and with the name being older, it should receive preference over *F. eucalyptorum*. However, *F. australis* Slippers, Crous & M.J. Wingf. has recently been introduced for a species occurring on *Acacia* in Australia (Slippers *et al.* 2004b) and the epithet is thus not available. The next valid and available name, i.e. *Fusicoccum eucalyptorum*, is, therefore, retained.

The earlier record of *B. ribis* on *Eucalyptus cladocalyx* leaves in the Western Cape Province, South Africa (Crous *et al.* 1989) is incorrect. From DNA sequence (STE-U 53–57) and morphology, it can be concluded that this record is representative of the recently described *B. australis* (Crous, unpubl. data).

Specimens and cultures examined: **Australia**, Victoria, Melbourne, leaves of *Eucalyptus* sp., H. Watts 12, 27 Apr. 1886, K(M) 121467 (**holotype** of *Idiocercus australis*). **South Africa**, Western Cape Province, Stellenbosch Farmers Winery, *E. cladocalyx*, P.W. Crous, Sept. 1988, PREM 50452 (reported as *I. australis*); Western Cape Province, Stellenbosch, *E. cladocalyx*, P.W. Crous, 1988, PREM 49298, STE-U 53−57 (reported as *B. ribis*).

DISCUSSION

Five *Botryosphaeria* spp. have been identified from *Eucalyptus* spp. growing in plantations and native environments in eastern Australia or as exotics in South Africa. Isolates represented *B. parva*, *B. dothidea*, *B. eucalyptorum*, *B. australis*, and the newly described species, *B. eucalypticola*. These species were readily distinguishable based on comparisons of ITS, β-tubulin and EF1-α sequence data. They could, however, also be distinguished from one another by using morphology (see key below) and a PCR-RFLP DNA fingerprinting technique developed as part of this study.

Botryosphaeria eucalyptorum (anamorph *Fusicoccum eucalyptorum* H. Smith, Crous & M.J. Wingf.) was the dominant species collected from *Eucalyptus* spp. in native forests and plantations in eastern Australia. This species represented almost 50% of isolates from this host genus and area. *Botryosphaeria eucalyptorum* was first described from South Africa as a pathogen of plantation eucalypts (Smith *et al.* 2001). This species appears to be restricted to *Eucalyptus* spp. and this is the first report of *B. eucalyptorum* from Australia, where *Eucalyptus* spp. are native. The abundance, wide distribution in eastern Australia, and unique host association suggest that *B. eucalyptorum* is native on *Eucalyptus* in Australia.

In this study, *B. eucalyptorum* was collected from dead *Eucalyptus* branches and twigs in Australia. The fungus could have contributed to the death of these branches, or might only have sporulated on this tissue during the saprophytic phase of its life cycle. Smith *et al.* (2001) showed that *B. eucalyptorum* is pathogenic to *Eucalyptus*, although less so than *B. parva* (reported as *B. dothidea* in that study). The role of *B. eucalyptorum* in natural Australian ecosystems deserves further investigation.

Botryosphaeria eucalyptorum was commonly isolated from exotic *Eucalyptus* in South Africa. All evidence available to us suggests that this fungus was introduced into this area with planting stock or with seed from Australia. Such introductions of *B. eucalyptorum* would not be surprising, given its common occurrence in Australia from where *Eucalyptus* plant-

ing material and seed is commonly obtained for plantation development.

Idiocercus australis has previously been reported from diseased *Eucalyptus* leaves in Australia (Swart 1988) and from South Africa (Crous *et al*. 1990). Examination of type material of this taxon confirmed that it is identical to *F. eucalyptorum*, although some of the South African material was morphologically distinct, and its taxonomy remains unresolved. Denman *et al*. (2000) recorded 18 anamorph genera previously used to accommodate *Botryosphaeria* anamorphs. This is, however, the first species of *Idiocercus* B. Sutton shown to be a *Botryosphaeria* anamorph. This plethora of genera highlights previous difficulties with identification of *Botryosphaeria* anamorphs.

To the best of our knowledge, there is no prior evidence for *Botryosphaeria* spp. having been transported between continents on *Eucalyptus* planting stock. *Botryosphaeria* species on other tree genera, e.g. *B. protearum* S. Denman & Crous that occurs on South African *Proteaceae*, have been moved with their host to different continents (Denman *et al*. 2003). *Botryosphaeria* spp. could easily be overlooked because they live as endophytes in healthy plant tissue, especially in *Eucalyptus* (Fisher *et al.* 1993, Smith *et al.* 1996). These fungi also occur in seed, and various species have been moved across the world in this way, e.g. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Diplodia pinea* (Cilliers *et al.* 1993, Burgess & Wingfield 2002a). The common introduction of pathogenic *Botryosphaeria* spp. into new environments is of concern and should receive greater attention when importing germplasm in the future.

A relatively large number of *Botryosphaeria* isolates from *Eucalyptus* spp. in Australia grouped in a sister clade to *B. eucalyptorum*. The taxon represented by this sister clade of *B. eucalyptorum* is described in this study as *B. eucalypticola*. The sequence divergence between *B. eucalyptorum* and *B. eucalypticola* was small in each of the three gene regions investigated, but consistent across them. Each clade contained at least two synapomorphic characters per gene region, seven in total for *B. eucalyptorum* and nine for the sister clade. There is no exchange or mixing of these unique alleles, despite the sympatric occurrence of these two species on the same hosts and from the areas in Australia and South Africa. This suggests a complete sexual barrier between the groups (Taylor *et al.* 2000, Steenkamp *et al.* 2002). For this reason, these clades are treated here as representing sibling species. The above observations would be uncertain when based on single-gene phylogenies and illustrate the need for multiple-gene analyses to identify species boundaries among closely related *Botryosphaeria* spp.

The distinction between *B. eucalyptorum* and *B. eucalypticola* was overlooked in initial identifications based solely on morphology. Subsequent to identification based on DNA sequence comparison, the value of culture morphology, differences in average conidial size (length × width) and ascospore width could be appreciated and weighted taxonomically. Morphological similarity is not uncommon between recently diverged sibling species, as has been discussed previously (Brasier 1997, Harrington & Rizzo 1999). Taylor *et al.* (2000) thus predict that morphological species recognition is likely to amalgamate two or more species that can be recognised by refined methods. This is confirmed here, and in other cryptic *Botryosphaeria* spp. (De Wet *et al.* 2003, Slippers *et al.* 2004a, b).

Botryosphaeria eucalypticola was the second most common species isolated from *Eucalyptus* trees in eastern Australia. The dominance of the fungus in this endemic niche is a strong indication that *B. eucalypticola* is native to Australia and *Eucalyptus* spp. Furthermore, its sibling species, *B. eucalyptorum*, also appears to be native to this environment. The common occurrence of this species in South Africa is of concern. As with *B. eucalyptorum* it shows how frequently such potential pathogens can be moved around the world with planting stock such as seed or other germplasm. It also illustrates how such incursions have proceeded without recognition, and presumably over a long period of time.

In this study, a large number of isolates produced a RFLP profile that represents the *B. parva-B. ribis* complex. None of the selected isolates from this group from *Eucalyptus* could, however, be confirmed as *B. ribis* based on sequence data. Slippers *et al.* (2004a) also found that it is *B. parva*, rather than *B. ribis* or *B. dothidea*, which is associated with diseases of *Eucalyptus* in South Africa. Most of the isolates from this RFLP group are, therefore, expected to belong to *B. parva*. Clearer identification techniques are, however, needed to confirm this assumption.

The fact that no *B. ribis* isolates and only one *B. dothidea* isolate were identified from *Eucalyptus* in the areas surveyed during this study, is contrary to many previous reports. *Botryosphaeria ribis* (anamorph *F. ribis* Slippers, Crous & M.J. Wingf.) has been reported from *Eucalyptus* spp. in Australia, South Africa and the USA (Webb 1983, Shearer *et al.* 1987, Crous *et al.* 1989, Old *et al.* 1990, Carnegie 2000) and *B. dothidea* is known from this host in Britain, Hawaii, South Africa and the USA (Hodges 1983, Barnard *et al.* 1987, Fisher *et al.* 1993, Smith *et al.* 1994, 2001). The confusion between these species stems from the fact a number of *Botryosphaeria* spp., including *B. ribis*, have been treated as synonyms of *B. dothidea* (Von Arx & Müller 1954). These synonymies were not accepted by all researchers working with *Botryosphaeria* spp. on *Eucalyptus*. Furthermore, the morphological characteristics of *B. ribis* and *B. parva* overlap considerably (Slippers *et al.* 2004a).

Botryosphaeria ribis and *B. dothidea* have, therefore, probably been misidentified in previous reports.

Isolates from the *B. parva-B. ribis* complex represented approximately 15% of isolates from *Eucalyptus* in Australia, but almost 50% of isolates from this host in South Africa. All isolates from exotic *Tibouchina* in Australia were, however, represented by *Botryosphaeria parva*. It appears that fungi from this group are important pathogens of *Eucalyptus* in exotic plantations, but less common in the native environment of this host. A similar situation exists in the distribution of "morphotypes" of *D. pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. on pines. These fungal groups differ on native and introduced pines, and one type often dominates in a specific geographical area (De Wet *et al.* 2000, Burgess *et al.* 2004). Therefore, the species of *Botryosphaeria* affecting a specific host needs to be individually identified in every different country or environment where the host occurs.

There was significant variation within the *B. parva* clade. This is reflected by the high internal bootstrap values for partitions in this clade. Analysis of the polymorphic sites showed that two isolates from Australia had four unique alleles, two of which are shared with more distantly related *Botryosphaeria* spp. The subclades in *B. parva*, however, share 11 unique alleles that differ from the sibling species, *B. ribis*. There are also no phenotypic or other distinguishing characters for isolates in the subclades and they are, thus, not considered distinct species. Further work on more representative populations of this group should determine patterns of gene flow and ecological or other differentiations. These data would reveal whether these subclades represent normal variation in the population, or might be an indication of speciation (Davis & Nixon 1992).

One *Botryosphaeria* isolate from *E. grandis* in eastern Australia was shown in this study to represent *B. australis*. Another isolate from *E. marginata* (Smith and Stanosz 2001), has previously been shown to belong to this taxon (Slippers *et al.* 2004b). *Botryosphaeria australis* is common on native plants such as *Acacia* spp. and *Banksia* spp. in Australia (identified as *B. lutea* in Smith & Stanosz 2001 and Denman *et al.* 2003, but see Slippers *et al.* 2004b). DNA sequence data for the culture on which the original record of *B. ribis* from South African *Eucalyptus* was based (Crous *et al*. 1989), concerned *B. australis*, and not *B. ribis* as reported (Crous, unpubl. data). This fungus can thus infect *Eucalyptus* spp., although it does not seem to be the dominant *Botryosphaeria* spp. in this niche.

All *Botryosphaeria* spp. from *Eucalyptus* identified in this study using RFLP profiles and DNA sequence data could also be identified based on the size and shape of their conidia. Compared to other *Botryosphaeria* anamorphs from *Eucalyptus*, conidia of *B. parva* isolates are short, narrow and fusiform to ellipsoidal. *Botryosphaeria eucalyptorum* conidia are considerably longer and wider than those of *B. parva*, and more or less clavate. These conidia are very similar to those of *B. eucalypticola*, but are smaller (as reflected by length × width ratios). *Botryosphaeria dothidea* has long, narrowly fusiform conidia, and those of *B. australis* are longer than those of *B. parva*, and their length falls between those of *B. eucalyptorum* and *B. dothidea*. *Botryosphaeria australis* is, however, easily distinguished by a yellow pigment in young cultures. These characters, however, overlap and should be used with caution and ideally in combination with other methods. This is especially true when only small numbers of isolates are available for study.

PCR-RFLP fingerprinting profiles were useful in this study to distinguish the five *Botryosphaeria* spp. identified from *Eucalyptus*. The technique is rapid and reliable, and provides an efficient means to screen larger numbers of isolates, that sporulate with difficulty and which would be costly to subject to DNA sequence comparisons. The technique could also be useful to identify *Botryosphaeria* spp. in other environments and from other hosts. Overlapping patterns between some *Botryosphaeria* spp. using the enzymes described here will occur. For example, *B. ribis* and *B. parva*, and *B. lutea* and *B. australis*, respectively, have the same profiles with all three enzymes. Additional information, including sequence data, would be needed to do a final identification of such isolates.

This study provides a basis for future work to understand the occurrence and importance of *Botryosphaeria* spp. on *Eucalyptus*. Clearly *Botryosphaeria* spp. are a threat to both native forests and plantations of exotic *Eucalyptus*. These pathogens appear to have been moved both in and out of Australia and between other countries and continents. Quarantine measures, specifically designed to restrict further introductions of new genotypes, as well as currently unknown pathogens, is of increasing importance, especially in Australia (Burgess & Wingfield 2002a, b). The current survey focused on the eastern coastal region of Australia. Almost 40% of *Eucalyptus* plantations, however, occur in the western parts of Australia (National Forest Inventory 2003). A survey of these plantations, as well as native trees in that area, should provide important additional knowledge pertaining to species of *Botryosphaeria* occurring on *Eucalyptus* in Australia. Little is known regarding the pathogenicity of these fungi on *Eucalyptus* in Australia and trials aimed at expanding such knowledge would be valuable.

Key to *Botryosphaeria* spp. that occur on *Eucalyptus* in Australia and South Africa

Information of *B. ribis* (anamorph *F. ribis*) from Slippers *et al.* 2004 is included, because it has been reported from *Eucalyptus* and could be confused with *B. parva.*

1. Conidia in culture on average ≥20 µm long, l/w >3; colonies on MEA or PDA with sparse to moderately dense grey to buff mycelium and fast growing, or thicker grey mycelium and slow growing **2**
1. Conidia in culture on average <20 µm long, l/w *ca*. 3; colonies on MEA or PDA with thick felt of grey aerial mycelium and fast growing **5**

2. Conidia fusiform to irregularly rod-shaped, 20–25 × 5–8 µm (av. 22 × 6 µm), l/w 3–4; colonies on MEA or PDA producing light yellow pigment after 3 d, becoming dull brown to buff with age ..... ***B. australis***
2. Conidia frequently >25 µm long; colonies on MEA or PDA not producing yellow pigment and becoming grey to black with age **3**

3. Conidia narrowly fusiform, 24–30 × 4–6 µm, l/w 3.5–6 ..... ***B. dothidea***
3. Conidia clavate, 20–30 × 5–10 µm, l/w 3–3.5 ..... **4**

4. Conidial length × width <200 ..... ***B. eucalyptorum***
4. Conidial length × width >200 ..... ***B. eucalypticola***

5. Conidia 12–25 × 5–7.5 µm, becoming pale brown and 1–2-septate with a darker brown middle cell after discharge ..... ***B. parva***
5. Conidia 15–20 × 5–7 µm, rarely becoming pale brown and septate after discharge ..... ***B. ribis***

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REFERENCES

Arx JA von (1987). *Plant pathogenic fungi*. Beheifte zur Nova Hedwigia 87. J. Cramer, Berlin, Germany.

Arx JA von, Müller E (1954). Die Gattungen der amerosporen Pyrenomyceten. *Beiträge zur Kryptogamenflora der Schweiz* **11(1)**: 1–434.

Barnard EL, Geary T, English JT, Gilly SP (1987). Basal cankers and coppice failure of *Eucalyptus grandis* in Florida. *Plant Disease* **71**: 358–361.

Brasier CM (1997). Fungal species in practice: identifying species units in fungi. In: *Species. The units of biodiversity* (Claridge MF, Dawah HA, Wilson MR, eds). Chapman & Hall, London, UK: 135–170.

Burgess T, Wingfield MJ (2002a) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. *International Forestry Review* **4**: 56–64.

Burgess T, Wingfield MJ (2002b). Impact of fungal pathogens in natural forest ecosystems: A focus on *Eucalyptus*. In: *Microorganisms in plant conservation and biodiversity* (Sivasithamparam K, Dixon KW, eds). Kluwer Academic Press, Dordrecht, The Netherlands: 285–306.

Burgess T, Wingfield MJ, Wingfield BD (2004). Global distribution of the pine pathogen *Sphaeropsis sapinea* revealed by SSR markers. *Australasian Plant Pathology* in press.

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

Carnegie AJ (2000). State and company overviews of eucalypt plantation estates and pest problems – New South Wales. In: *Proceedings of a workshop on managing pests of eucalypt plantations* (Elliott H, McArthur C, Floyd R, de Little D, eds). Department of Forestry, Australian National University, Canberra: 15–25.

Cilliers AJ, Swart WJ, Wingfield MJ (1993). A review of *Lasiodiplodia theobromae* with particular reference to its occurrence on coniferous seeds. *South African Forestry Journal* **166**: 47–52.

Crous PW, Knox-Davies PS, Wingfield MJ (1989). Newly-recorded foliage fungi of *Eucalyptus* spp. in South Africa. *Phytophylactica* **21**: 85–88.

Crous PW, Phillips AJL, Baxter AP (2000). *Phytopathogenic fungi from South Africa*. University of Stellenbosch, Department of Plant Pathology Press, University of Stellenbosch Printers. Stellenbosch, South Africa.

Crous PW, Wingfield MJ, Schoch SH (1990) New and interesting records of South African fungi. X. New records of *Eucalyptus* leaf fungi. *South African Journal of Botany* **56**: 583–586.

Davis JI, Nixon KC (1992). Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology* **41**: 421–435.

Davison EM, Tay CS (1983). Twig, branch and upper trunk cankers of *Eucalyptus marginata*. *Plant Disease* **67**: 1285–1287.

Denman S, Crous PW, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ (2003). Circumscription of *Botryosphaeria* species associated with *Proteaceae* on morphology and DNA sequence data. *Mycologia* **95**: 294–307.

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. *Studies in Mycology* **45**: 129–140.

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

Farris JS, Kallersjø 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 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.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Harrington TC, Rizzo DM (1999). Defining species in fungi. In *Structure and dynamics of fungal populations* (Worrall JJ, ed.). Kluwer Academic, Dordrecht, The Netherlands: 43–70.

Heath R (2003). *Studies to consider the possible origin of three canker pathogens of Eucalyptus in South Africa*. M.Sc. thesis. Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

Hillis DM, Huelsenbeck JP (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83**: 189–195.

Hodges CS (1983). Pine mortality in Hawaii associated with *B. dothidea*. *Plant Disease* **67**: 555–556.

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.

National Forest Inventory (2003). *National Plantation Inventory Annual Update* - March 2003. Bureau of Rural Sciences, Canberra, Australia.

Palm ME (1999). Mycology and world trade: a view from the front line. *Mycologia* **91**: 1–12.

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.

Old KM, Gibbs R, Craig I, Myers BJ, Yaun ZQ (1990). Effect of drought and defoliation on the susceptibility of *Eucalyptus* to cankers caused by *Endothia gyrosa* and *Botryosphaeria ribis*. *Australian Journal of Botany* **38**: 571–581.

Raeder U, Broda P (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Rayner RW (1970). *A mycological colour chart.* CMI and British Mycological Society, Surrey, UK. 34 pp.

Roux J, Coutinho TA, Mujuni Byabashaija D, Wingfield MJ (2001). Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16–18.

Sankaran KV, Sutton BC, Minter DW (1995). A checklist of fungi recorded on *Eucalyptus*. *Mycological Papers* **170**: 1–376.

Shearer BL, Tippett JT, Bartle JR (1987). *Botryosphaeria ribis* infection associated with death of *Eucalyptus radiata* in species selection trials. *Plant Disease* **71**: 140–145.

Slippers B, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ (2004b). Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. from *B. lutea*. *Mycologia* **96**: 1028–1039.

Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ (2004a). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. *Mycologia* **96**: 83–101.

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.

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

Smith H, Wingfield MJ, Crous PW, Coutinho TA (1996). *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**: 86–88.

Steenkamp ET, Wingfield BD, Desjardins AE, Marasas WFO, Wingfield MJ (2002). Cryptic speciation in *Fusarium subglutinans*. *Mycologia* **94**: 1032–1043.

Straus SY (2001) Benefits and risks of biotic exchange between *Eucalyptus* plantations and native Australian forests. *Australian Ecology* **26**: 447-457.

Swart HJ (1988). Australian leaf-inhabiting fungi. XXVI. Some noteworthy Coelomycetes on *Eucalyptus*. *Transactions of the British Mycological Society* **90**: 279–291.

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

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

Webb RS (1983). Seed capsule abortion and twig dieback of *Eucalyptus camaldulensis* in South Florida induced by *Botryosphaeria ribis*. *Plant Disease* **67**:108–109.

Wet J De, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a

new species of *Diplodia*. *Mycological Research* **107**: 557–566.

Wet J De, Wingfield MJ, Coutinho TA, Wingfield BD (2000). Characterisation of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. *Plant Disease* **84**: 151–156.

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

Wingfield MJ, Roux J, Coutinho T, Govender P, Wingfield BD (2001a). Plantation disease and pest management in the next century. *South African Forestry Journal* **190**: 67–71.

Wingfield MJ, Slippers B, Roux J, Wingfield BD (2001b). Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. *BioScience* **51**: 134–140.

Wingfield MJ, Swart WJ, Kemp GHJ (1991). Pathology considerations in clonal propagation of *Eucalyptus* with special reference to the South African situation. In *Intensive Forestry – the role of Eucalyptus. Proceedings of the 1991 IUFRO Symposium*: 811–830.

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.