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Preliminary studies on *Botryosphaeria* species from *Wollemia nobilis* and related southern hemisphere conifers in Australasia and South Africa

Abstract: *Wollemia nobilis* is an ancient coniferous tree species that was recently discovered in Eastern Australia. This plant species is highly threatened due to its limited distribution. Not only are only 40 adult plants known from the wild, but there is also no genetic variation within this small population. A recent study revealed a species of *Botryosphaeria* to be highly pathogenic to *W. nobilis*. The aim of this study was to identify this fungus to species level. Furthermore, *Botryosphaeria* isolates from other southern hemisphere coniferous hosts, namely *Araucaria* from New Zealand and *Widdringtonia* from South Africa, were also included. To facilitate their identification, sequence data for the ITS rDNA, as well as the β-tubulin and elongation factor 1-α genes were combined to determine the phylogenetic relationship of these isolates with known *Botryosphaeria* spp. Isolates from *W. nobilis* included two *Botryosphaeria* spp. The first is closely related to *B. ribis*, but also shares some unique sequence polymorphisms with *B. parva*. One isolate grouped with *B. australis*, but also varied slightly from this taxon in the gene regions analysed. Additional isolates will be needed to determine whether these sequence variations represent speciation events or merely variation within populations of *B. ribis* and *B. australis*. As part of this study, *B. parva* was identified from *Araucaria* in New Zealand, and *B. australis* was found on *Widdringtonia* trees in South Africa. All three reports of these fungi are new records for their various hosts and could represent important pathogens of these trees in the future.

INTRODUCTION

The Araucariaceae is an ancient conifer family that occurs mainly in the southern hemisphere, and includes the genera *Araucaria* de Jussieu, *Agathis* Salisbury and *Wollemia* Jones, Hill & Allen (Gilmore and Hill 1997). Some genera and species from this group are widely grown as ornamentals. Others, however, occur only in small populations and areas, and are threatened by habitat loss, loss of genetic diversity, environmental changes and diseases.

Wollemia nobilis Jones, Hill & Allen is a unique member of the family Araucariaceae that was discovered for the first time in Australia less than ten years ago (Jones et al 1995). This monotypic genus is an ancient plant relic that is linked to fossils dating to the Mesozoic era (Gilmore and Hill 1997, Hill 1997). The total population of adult plants of *W. nobilis* is approximately 40 individuals which occur as two groups that are 2 km apart in a gorge in the Wollemi National Park, north-west of Sydney (Offord et al 1999). Population studies using allozymes and AFLP markers could not detect any genetic variation among the adult individuals (Hogbin et al 2000).

The small population size, close proximity of the trees to each other and lack of genetic variation makes this genus vulnerable to diseases (Offord 1996, Bullock et al 2000, Hogbin et al 2000). Surveys to determine the level of fungal diversity associated with *W. nobilis* have identified more than 50 fungal taxa from these plants, including potential pathogens such as a *Botryosphaeria* sp. and a *Fusarium* sp. (Summerell, www.rbgsyd.gov.au/wollemi/research). A subsequent pathogenicity study revealed that *Phytophthora cinnamomi* (which was not found on the site) and a *Botryosphaeria* sp. were highly pathogenic to *W. nobilis*, killing plants in the glasshouse within four weeks (Bullock et al 2000). *Phytophthora cinnamomi* is a serious pathogen of exotic and indigenous plants in Australia, while *Botryosphaeria* spp. are endophytes and stress related pathogens of various woody hosts in this region (Chapters 4, 6, Shearer and Smith 2000).

Seedlings and cuttings have been produced in an effort to conserve *W. nobilis* (Offord 1996, Hogbin et al 2000). This material is used for two purposes. Firstly, it is being distributed to discourage poaching. Secondly, the *ex situ* population are kept in different localities to reduce the risk of diseases. The genetic diversity of this planting stock is obviously as small as that of the wild population. Diseases in the nursery,

such as those already experienced due to *Botryosphaeria* die-back, thus threaten this planting stock (Hogbin et al 2000). A concern about the distribution of *W. nobilis* plants is, however, that unique pathogens that have evolved on *W. nobilis* could be spread in this way. This in turn could influence other endangered Araucariaceae.

Apart from the Auraucariaceae, other unique conifers are found in the southern hemisphere. These include the African cypresses (*Widdringtonia* Endlicher). The four species in this genus have a limited distribution in southern Africa, and are all considered rare to highly endangered (Pauw and Linder 1997). Similar to *W. nobilis*, efforts are made to produce sufficient numbers of these plants in nurseries, both to discourage poaching and to replenish natural populations (Mustart and Bond 1995). The limited stocks of nursery and field plants put these plants at risk of pathogens. In a preliminary study no significant diseases were, however, found in natural stands and plantations of adult *W. cedarbergensis* Marsh plants (Wingfield et al 1988).

Botryosphaeria spp. are notoriously difficult to identify and the taxonomy of this group of fungi has been confused for many years (Denman et al 2000). In a suite of recent studies, species have been successfully defined using a combination of morphological (mostly of anamorph) and molecular data (Smith et al 2001, Phillips et al 2002, Denman et al 2003). In some cases combined gene genealogies using sequences of ITS rDNA, β-tubulin and EF-1α regions have, however, been necessary to distinguish closely related species (Slippers et al 2003, Chapter 4, 6). The aim of this study was thus to identify Botryosphaeriaceous fungi that have been isolated from *W. nobilis* as well as some other conifers in the southern hemisphere.

MATERIALS AND METHODS

Isolates and morphological characterization.--Eight *Botryosphaeria* isolates from southern hemisphere coniferous hosts were used in this study (TABLE I). Three isolates were from die-back symptoms on *W. nobilis* plants in a nursery in Sydney. Two isolates were obtained from *Araucaria hetrophylla* (Salisb.) Franco. (Norfolk Island Pine) in Auckland, New Zealand and one isolate was from *A. cunninghamii* Aiton ex D. Don (Hoop Pine) in Sydney, Australia. A further two isolates were obtained from diseased *Widdringtonia nodiflora* (L.) Powrie (Mountain Cypress) plants from the Cape Province, South Africa.

Isolates were maintained 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. Isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Molecular phylogenetic characterization.--The eight isolates from southern hemisphere coniferous hosts that formed the basis of this study were compared to other *Botryosphaeria* spp. that are known from the area or from similar hosts in other parts of the world. These comparisons were made based on phylogenetic reconstruction using sequence data 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 region of the β-tubulin gene and a part of the elongation factor 1-α gene.

A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA, as described in Raeder and Broda (1985) and Smith et al (2001). PCR reaction mixtures, PCR conditions and visualization of amplicons were as described in a previous study by Slippers et al (2003). The amplicons of all three DNA regions were also cleaned and sequenced as described in Slippers et al (2003). Sequences for fungi other than those produced in this study were obtained from GenBank or from Slippers et al (2003) (TABLE I).

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), using heuristic searches with 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.

After partition homogeneity tests (Farris et al 1995, Huelsenbeck et al 1996), the sequence data sets of all three gene regions were combined and treated as one. Retention and consistency indices, a g1-value (Hillis and Huelsenbeck 1992) and bootstrap support (1000 replicates) (Felsenstein 1985) for branches were also determined in PAUP. Decay analysis of the branch nodes was determined using

Autodecay (Eriksson 1998). Phylogenetic hypotheses were also tested using distance analyses with the Neighbor-Joining algorithm and an uncorrected p-factor and the HKY85 parameter in PAUP.

Morphological characterization.--Isolates from *W. nobilis* were induced to sporulate by transferring them onto sterilized pine needles placed on the surface of 2% water agar (WA) (Biolab) in Petri dishes and incubating these at 25°C under near UV light. Fruiting structures were sectioned by hand and mounted in clear lactophenol. Morphological observations and photographs were made using an Axiocam digital camera (Carl Zeiss, Germany).

RESULTS

Phylogenetic relationships.--PCR amplicons of approximately 600, 450 and 300 base pairs were amplified respectively for the ITS rDNA, β-tubulin and EF1-α regions. A partition homogeneity test showed that the sequences of these three regions were significantly concordant (P value = 0.36) and could, thus, be treated as a single larger data set. The total data set contained 1324 characters after alignment. Twelve characters that were repetitive in isolates of *B. ribis* were excluded from the EF1-α region. The 327 parsimony informative characters contained significant phylogenetic signal compared to random sampling ($P < 0.01$; $g1 = -1.09$) (Hillis and Huelsenbeck 1992). Three most parsimonious trees were retained after heuristic searches (543 steps, CI = 0.838, RI = 0.918) (FIG. 1).

Two isolates from *W. nobilis* (CMW3389, CMW9070) and one isolate from *A. cunninghamii* (CMW3388) grouped most closely to *B. ribis* Grossenb. & Dugg., but formed a strongly supported separate branch (Clade II, d2/93% bootstrap) (FIG. 1). Analysis of the polymorphic sites that separate the closely related clades I-III (*B. ribis*, *Botryosphaeria* sp. and *B. parva*) revealed that Clade II had four unique polymorphisms, 4 shared polymorphisms with *B. parva* and eight shared polymorphisms with *B. ribis* (TABLE II).

Two isolates from *Araucaria* from New Zealand (CMW10120, CMW10121) grouped with isolates of *B. parva* Pennycook & Samuels (FIG. 1, Clade III). Two isolates from *Widdringtonia* (CMW1110, CMW1112) grouped with *B. australis* Slippers, Crous & M.J. Wingf. (FIG. 1, Clade V). One isolate from *W. nobilis*

grouped most closely with *B. australis*, but also distinctly separate from them (FIG. 1). Given that only one isolate was available in this case, further phylogenetic analysis could not be performed.

Morphological characterization.--Isolates from *W. nobilis* sporulated on pine needles and *Populus* sticks after two to three weeks of incubation. Pycnidia were globose, mostly solitary, with apical pores, occasionally with small conical necks, superficial on the needles and 100-300 µm in diameter. Conidia of isolates residing in Clade II were hyaline, fusiform to ellipsoid and (17--)18--19(--20) x 5--6 µm (FIG. 2). Conidia of isolate CMW3386 from *W. nobilis* were similar in shape to those in Clade II, but were slightly longer, (19--)21--22(--25) x 5--6 µm (FIG. 3).

DISCUSSION

At least four *Botryosphaeria* spp. were identified from the small collection of isolates associated with the southern hemisphere coniferous hosts, *Wollemia*, *Araucaria* and *Widdringtonia*. Two species were identified as *B. parva* and *B. australis*. The identities of the other two other taxa remain uncertain, but they are most closely related to *B. ribis* and *B. australis* respectively. All these species have *Fusicoccum* anamorphs and all are new records for the hosts concerned.

Three isolates (Clade II) from southern hemisphere coniferous hosts were most closely related to *B. ribis*, based on multiple gene sequence analysis. This is the first molecular evidence to show that isolates more similar to *B. ribis* than to *B. parva* occur in Australia. *Botryosphaeria ribis* has previously been reported from Australia on *Eucalyptus* and this record was based on morphological characters (Davison and Tay 1983, Shearer et al 1987, Old et al 1990). It is, however, likely that these reports refer to the morphologically similar *B. parva* (Chapter 5). *Botryosphaeria ribis* and *B. parva* can currently only be separated with certainty by using DNA sequence data (Slippers et al 2003). Based on these data, *B. ribis* has thus far been identified only from *Ribes* sp. in the USA. In contrast, *B. parva* has a wide host range and has been reported from native and exotic hosts in Australia and elsewhere in the southern hemisphere (Slippers et al 2003, Chapter 5).

The DNA sequences for Clade II isolates were not identical to those of *B. ribis*. Isolates residing in this clade share eight polymorphisms with *B. ribis* and four

with *B. parva*. They also have four alleles that are different to those of *B ribis* and *B. parva*. All four of these unique alleles are also present in the outgroup taxa with *Diplodia* anamorphs, and are, thus, either plesiomorphic characters or homoplasies. These results suggest that Clade II isolates represent an older lineage that share a common ancestor with both *B. ribis* and *B. parva*, but have begun to accumulate unique alleles due to geographic or host separation. Alternatively, these isolates from Clade II might represent remnants of past genetic exchange between populations of *B. ribis* and *B. parva,* before these lineages were reproductively separated (Davis and Nixon 1992). The limited sample size and absence of other distinguishing characters for Clade II isolates, however, makes it impossible to test whether these isolates represent a distinct species.

Isolates that group in Clade II, and that remain unidentified, originated from nursery plants of both *W. nobilis* and an *Araucaria* sp. This is of concern for the conservation of rare species in these host genera and their origin might be explained by various hypotheses. The pathogen could have evolved on *Wollemia* and have been introduced into nurseries on cuttings, seeds and other plant material of this host. This is quite possible given that *Botryosphaeria* spp. are often endophytic and are apparently commonly moved around the world in this way (Smith et al 1996, Burgess and Wingfield 2002, Chapter 5). It would also imply that a new pathogen of *Wollemia* has appeared and that it could threaten *Araucaria* in the nurseries or gardens where *Wollemia* are grown. Alternatively, the pathogen could have evolved on both *Wollemia* and *Araucaria*, occurring naturally on these hosts. In this case there would not be a significant threat to either group, except in situations where the plants are under stress. Another possibility is that that the pathogen is not native to the wild population of *Wollemia* and efforts should be made to prevent its introduction or spread into the area where *W. nobilis* grows naturally.

Botryosphaeria parva was identified from an *Araucaria* sp. from New Zealand. This fungus is well-known from this region were it was initially described by Pennycook and Samuels (1985) from the exotic hosts, such as *Malus domestica*, *Populus nigra* and *Actinidia deliciosa*. It has subsequently been shown that this species has a wide distribution and host range in the southern hemisphere (Slippers et al 2003, Chapter 2-5). *Botryosphaeria parva* is known to be a virulent pathogen when plants are under stress (reported as *B. dothidea* in Smith et al 2001).

Conidial morphology of *B. parva* and isolates of the species represented by Clade II from *Araucaria* and *W. nobilis* resemble *Hendersonula agathi* Young both in size and in shape. Young (1948) described *H. agathi* as the pathogen responsible for a leaf and twig disease of *Agathis* spp. in nurseries in Queensland, Australia. Sutton and Dyko (1989) considered *H. toruloidea* and *H. agathi* to be synonymous with a new taxon, *Natrassia mangiferae* Sutton & Dyko. In Chapter 2 this last named taxon is described as *F. mangiferum* Slippers, Johnson & M.J. Wingf. based on molecular and morphological similarities with other *Fusicoccum* species. The synonymy of *H. agathi* and *F. mangiferum* was, however, rejected in this study, because the conidia of the former species are more similar to those of *B. ribis* and *B. parva* in size and appearance (Chapter 2). It is thus possible that the *B. parva* and Clade II isolates, reported here from coniferous hosts that are also related to *Agathis*, are conspecific with *H. agathi*. A more extensive sample, including isolates from *Agathis*, is required to test this hypothesis.

Botryosphaeria australis was identified from *Widdringtonia nodiflora* from South Africa in this study. This fungus was first described from *Acacia* and *Sequoiadendron* trees in Australia, but also occurs on other native and exotic hosts in Australia (*Banksia* and *Eucalyptus*) and South Africa (*Prunus*, *Malus* and *Protea*) (Chapter 3-5). It is not clear whether the current finding of this fungus represents that of a native pathogen on a South African native host, or whether *B. australis* has been introduced into South Africa. In both cases, this fungus poses a risk to this rare and threatened genus of plants, especially where these plants are produced in nurseries to replenish depleted natural populations (Mustart and Bond 1995). As discussed earlier, *Botryosphaeria* spp. are known to cause nursery diseases on other coniferous hosts in the southern hemisphere (Young 1948, Bullock et al 2000). Wingfield et al (1988) did not find *Botryosphaeria* spp. associated with *Widdringtonia* in an earlier survey, but warned of the potential dangers of introducing pathogens into natural populations on plants produced in nurseries.

One isolate from *W. nobilis* grouped sister to the *B. australis* clade. Conidia of this isolate were morphologically similar to those of *B. australis*, although they were slightly shorter on average than those of the latter taxon (Chapter 3). *Botryosphaeria australis* conidia are on average 24--25 µm long, unlike the isolate from *Wollemia,* which had conidia that were 21--22 µm long. It is, however, not possible to judge the value of these measurements based on a single isolate. This isolate might represent a

unique species or simply a variant of *B. australis*, but more isolates will need to be studied to test these hypotheses. Nevertheless, this second species of *Botryosphaeria* on *Wollemia* could be important when considering conservation strategies.

The number of samples of *Botryosphaeria* spp. from *Wollemia*, *Araucaria* and *Widdringtonia* in this study was relatively limited. Results should thus be seen as providing a preliminary reflection of the identity and variation of *Botryosphaeria* spp. that could potentially affect southern hemisphere coniferous hosts. Although *Botryosphaeria* spp. are mostly stress related pathogens, the potential impact of these pathogens is well illustrated by the pathogenicity trials of Bullock et al (2000). We hope that this investigation will stimulate further collections of isolates and studies to determine the extent of variation among and within these *Botryosphaeria* spp. The accurate identification of these fungi, their origin and distribution, as well as their pathogenicity to the coniferous and other hosts in the southern hemisphere, will clearly be important in managing their potential impact.

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TABLE I. Isolates compared in the phylogenetic study.

Culture no.[1]	Other no. [1]	Identity[2]	Host	Location	Collector
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler
CMW7773		*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler
CMW7054	CBS121	*B. ribis*	*Ribes* sp.	New York, USA	N.E. Stevens
CMW3386		*Botryosphaeria* sp.	*Wollemia nobilis*	Oueensland, Australia	M. Ivory
CMW3388		*Botryosphaeria* sp.	*Araucaria cunninghamii*	Oueensland, Australia	M. Ivory
CMW3389		*Botryosphaeria* sp.	*W. nobilis*	Queensland, Australia	M. Ivory
CMW9070		*Botryosphaeria* sp.	*W. nobilis*	Sydney, Australia	B. Summerell
CMW9078	ICMP7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels
CMW10120		*B. parva*	*A. hetrophylla*	Auckland, New Zealand	M.J. Wingfield
CMW10121		*B. parva*	*A. hetrophylla*	Auckland, New Zealand	M.J. Wingfield
CMW9072		*B. australis*	*Acacia* sp.	Australia	D. Guest/J. Roux
CMW1110		*B. australis*	*Widdringtonia nodiflora*	Cape province, S Africa	W.J. Swart
CMW1112		*B. australis*	*W. nodiflora*	Cape province, S Africa	W.J. Swart
CMW9075	ICMP8019	*B. dothidea*	*P. nigra*	New Zealand	G.J. Samuels
CMW8000		*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers
CMW10309	CAP002	*B. lutea*	*Vitis vinifera*	Portugal	A.J.L. Phillips

TABLE I. Continued.

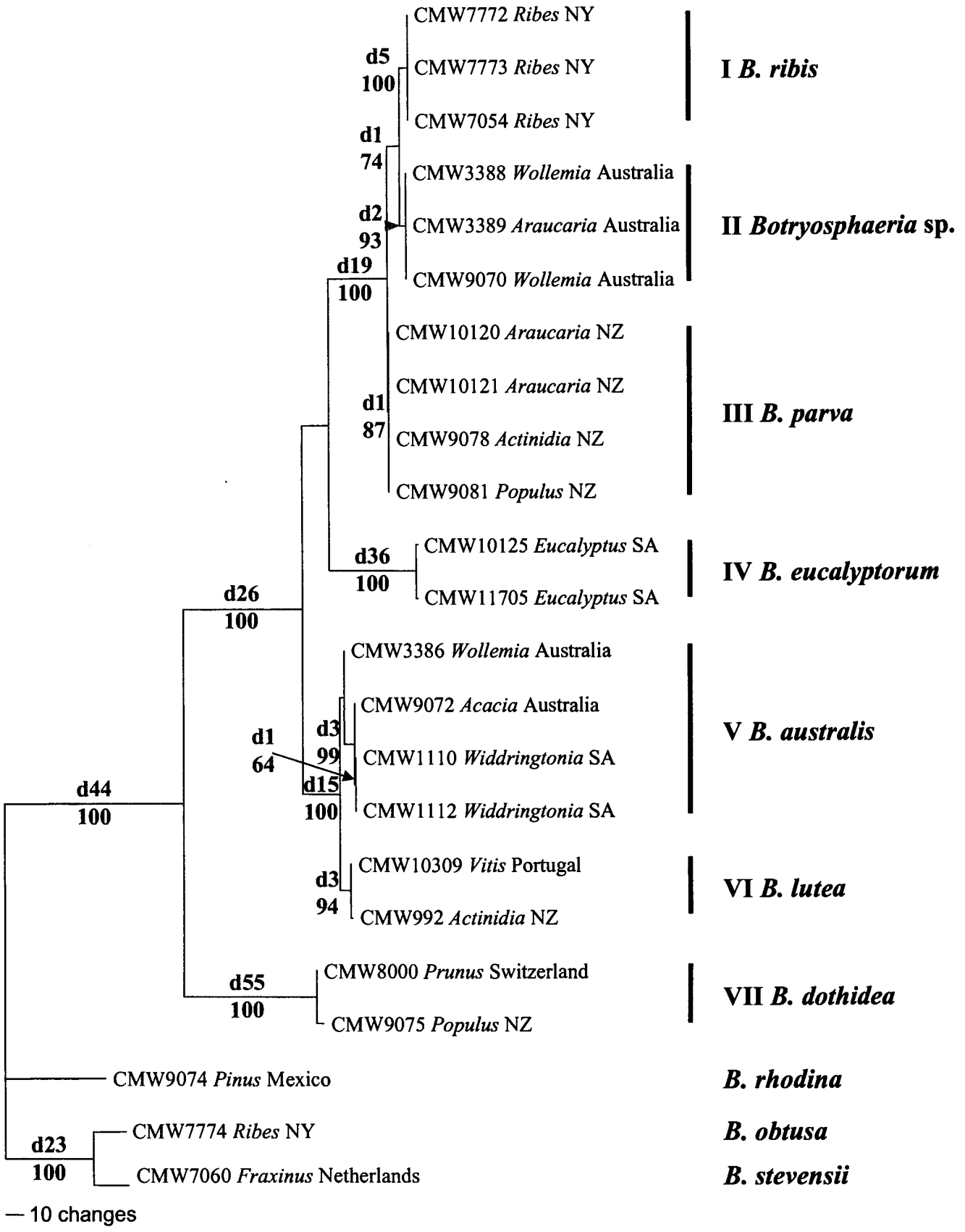
Culture no.[1]	Other no.[1]	Identity[2]	Host	Location	Collector
CMW992	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels
CMW10125		*B. eucalyptorum*	*E. grandis*	Mpumalanga, S Africa	H. Smith
CMW11705		*B. eucalyptorum*	*E. nitens*	S Africa	B. Slippers
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler
CMW7060	CBS431	*B. stevensii*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess

[1] Abbreviations for culture collections and isolates: CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ICMP = International Collection of Micro-organisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998).
[2] Identities as determined in this study.

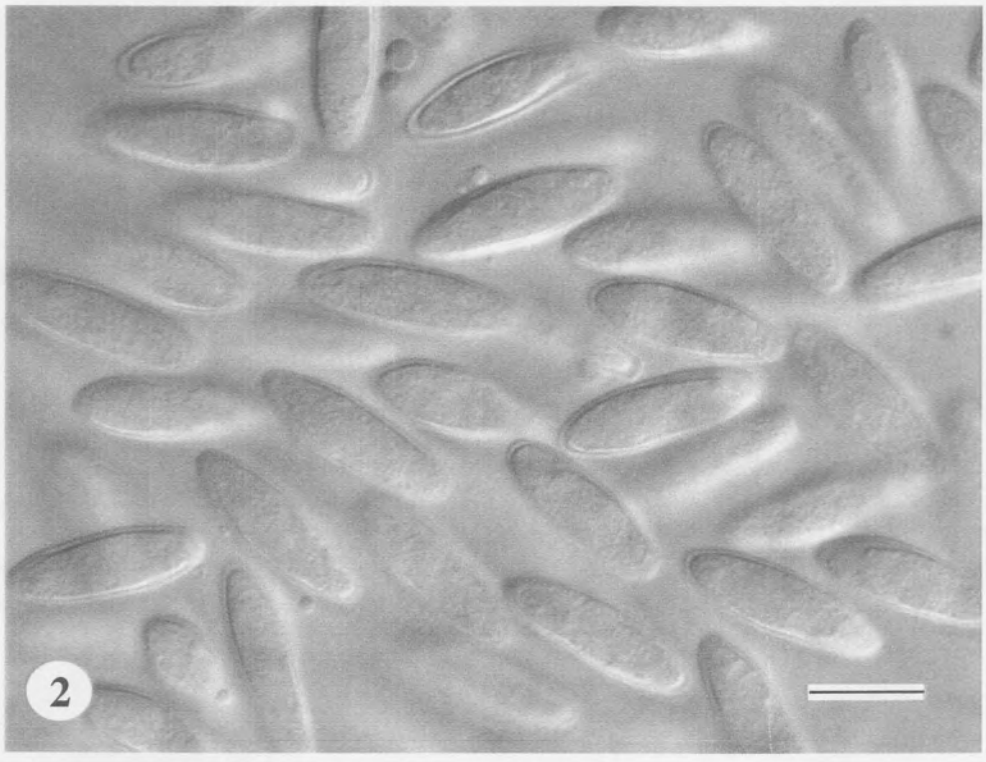
TABLE II. Polymorphic nucleotides (or alleles) from sequence data of the ITS rDNA, β-tubulin and EF-1α, that show the relationship between the unidentified *Botryosphaeria* sp. in Clade II of the phylogenetic analysis, and isolates of *B. ribis* and *B. parva*. Polymorphisms that are unique to the unidentified *Botryosphaeria* sp. are in bold type. All other polymorphisms that are shared between the isolates of the unidentified *Botryosphaeria* sp. and either *B. ribis* or *B. parva*, are highlighted.

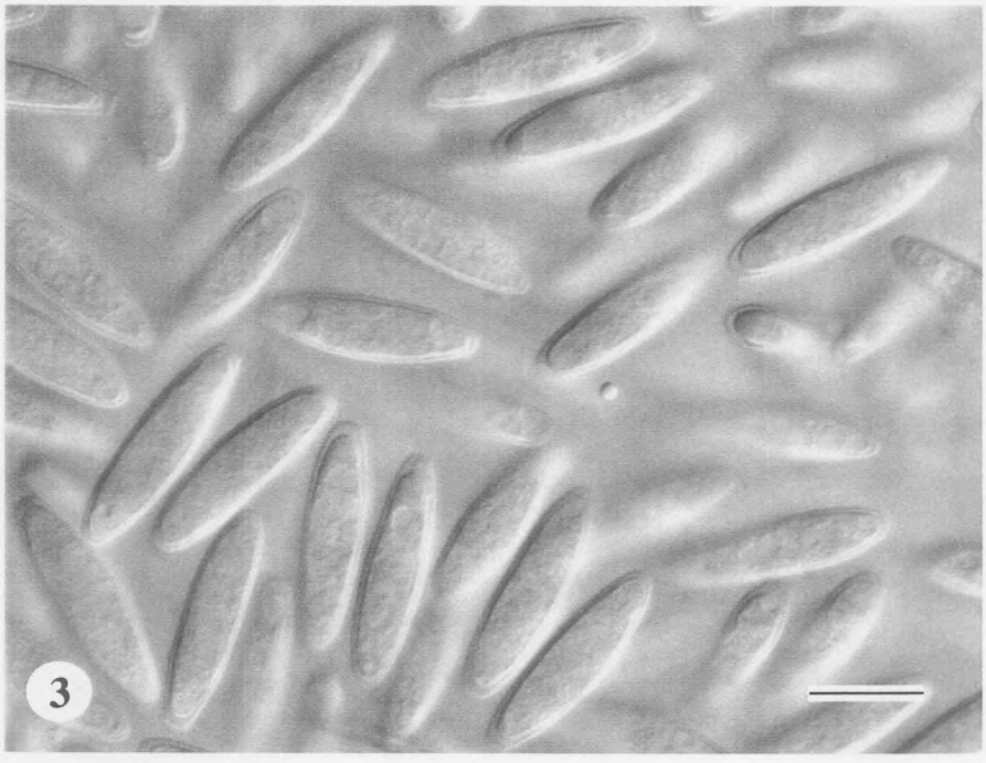
Identity	**Culture no.**	**β-tubulin**					**ITS**				**EF1-α**							
		96	129	188	419	437	513	585	797	864	1081	1082	1138	1145	1190	1216	1251	1309
B. ribis	CMW7772	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
	CMW7773	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
	CMW7045	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
Botryosphaeria sp.	CMW3388	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
	CMW3389	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
	CMW9070	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
B. parva	CMW9078	T	A	C	T	T	T	-	T	C	C	A	0	0	T	G	G	A
	CMW9081	T	A	C	T	T	T	-	T	C	C	A	0	0	T	G	G	A
	CMW10120	T	A	C	T	T	T	-	T	C	C	A	1	0	T	G	G	A
	CMW10121	T	A	C	T	T	T	-	T	C	C	A	0	0	T	G	G	A

FIG. 1. One of the most parsimonious trees obtained through heuristic searches of the combined datasets of ITS rDNA, β-tubulin and elongation factor 1-α. Branch supports are indicated as decay values above and bootstrap values below the branches. The tree is rooted to *Botryosphaeria rhodina*, *B. obtusa* and *B. stevensii*. These species all have *Diplodia*-like anamorphs, unlike the ingroup taxa that have *Fusicoccum*-like anamorphs. The host genus and geographic origin (SA = South Africa; NY = New York, USA; NZ = New Zealand) of each isolate are indicated.



FIGS. 2-3. Conidia of *Botryosphaeria* spp. produced in culture on pine needles and Water Agar. These figures correspond to isolates in Clades II and V, respectively, in the phylogenetic study. Bars = 10 µm.





7

A contribution to the taxonomy of *Botryosphaeria obtusa*, *B. stevensii* and *B. quercuum* based on molecular and morphological data

Abstract: The distinction between *B. quercuum, B. stevensii* and *B. obtusa* is mainly based on the morphological characters of their associated anamorphs. Morphological variation within these species, and confusion pertaining to the various published names, complicates identification of these taxa. This confusion is clearly evident from recent studies based on ITS rDNA sequence data, in which more than one well defined clade has contained isolates identified as either *B. quercuum*, *B. stevensii* or *B. obtusa*. All these species have *Diplodia* anamorphs. In the present study the anamorph morphology of these three species was re-considered. In order to interpret the morphological variation observed in each species, their morphology was considered in contrast to their phylogeny, derived from ITS rDNA sequence data. Conidial sizes and length to width ratios alone were insufficient to distinguish among isolates identified as *B. quercuum*, *B. stevensii* and *B. obtusa*. These species can be more reliably defined when these data are combined with their patterns of discoloration and septation that emerge as the conidia mature, as well as based on host association. *Botryosphaeria quercuum* is most common on *Quercus*, has thick, glassy conidial walls and forms 1--3 septa before or after discoloration. The ascospores of this species can also have 1--3 septa. *Botryosphaeria stevensii* rarely occurs on *Quercus*, and is more common on *Fraxinus, Malus* and other hosts. Conidia of this species also have thick, glassy walls that can be of similar size to *B. quercuum*, but they develop septa only after discoloration. Host ranges of *B. obtusa* and *B. stevensii* overlap, but their conidia generally discolor before septation and have thinner, pitted walls. From a survey of taxonomic literature and the study of type material, we also show that neither the names *Sphaeropsis malorum* or *D. malorum* can be used for the anamorph of *B. obtusa*. The anamorph of this species is currently best treated as a *Diplodia* sp.

INTRODUCTION

The genus *Botryosphaeria* Ces. & De Not was erected in 1863 to describe a group of 12 Ascomycetes with hyaline, ovoid ascospores (Cesati and De Notaris 1863). Since then, the concept of *Botryosphaeria* has been broadened and more than 140 species have been described (Denman et al 2000). Virtually since the time of its first description, the taxonomy of this genus has been problematic. One reason is that the morphological features of species overlap considerably and they also vary among individuals of the same species that occur on different hosts (von Arx and Müller 1954, Denman et al 2000, Slippers et al 2003). Furthermore, the teleomorph structures are not commonly encountered *in vivo* and not easily induced *in vitro*, reducing their taxonomic value.

Species concepts in *Botryosphaeria* have changed with time. Between 1863 and the 1950's, the host from which a specimen was collected, played an important role in distinguishing different species (Cesati and De Notaris 1863, De Notaris 1863, Saccardo 1882, Grossenbacher and Duggar 1911, Puterill 1919, Trotter 1928). Subsequently, it has been shown that a single *Botryosphaeria* species can occur on a large number of hosts (Stevens and Jenkins 1924, Punithalingam and Waller 1973). In contrast to many prior studies, von Arx and Müller (1954) synonymized many *Botryosphaeria* species solely based on the similarity of the ascocarp and ascospore morphology. In that study, a large number of species are placed in either *B. quercuum* (Schwein.) Sacc. (larger ascospores 24--42 × 10--18 µm) or *B. dothidea* (Moug.: Fr.) Ces. & De Not. (smaller ascospores 15--24 × 6--10 µm).

Due to the difficulty in distinguishing closely related *Botryosphaeria* spp. based on teleomorph features, species have often been distinguished by their anamorphs (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003). Based on the anamorph morphology, the genus *Botryosphaeria* can be divided into two groups, namely one with light to dark brown *Diplodia*-like anamorphs and one with hyaline *Fusicoccum*-like anamorphs (Denman et al 2000, Zhou and Stanosz 2001). Anamorph morphology also suggests that the species of *Botryosphaeria* synonymized by von Arx and Müller (1954), can be subdivided into several distinct taxa. This is especially true for the conglomeration of species residing under the name *B. quercuum sensu* Von Arx and Müller (1954), which is the focus of this study.

Shoemaker (1964) used conidial dimensions to re-describe species that were treated as *B. quercuum* by von Arx and Müller (1954). In his study, Shoemaker (1964) treated four species, namely *B. obtusa* (Schwein.) Shoemaker, *B. stevensii* Shoemaker, *B. quercuum* and *B. melanops* (Tul.) G. Winter. Shoemaker (1964) avoided assigning names to the anamorphs of the species he treated to avoid the 'nomenclatural problems involved in the conidial state'. Considerable progress has, however, since been made in defining some of these anamorphs (Sutton 1980, Crous and Palm 1999, Denman et al 2000, De Wet et al 2003).

Difficulties still exist in separating the *Botryosphaeria* spp. treated by Shoemaker (1964). This is evident from recent studies based on DNA sequence comparisons, which have shown that different clades accommodate isolates identified as *B. obtusa* or *B. stevensii* (Zhou and Stanosz 2001). In some studies, one clade contained isolates identified as *B. obtusa*, *B. stevensii* and *B. quercuum* (Jacobs and Rehner 1998, Zhou and Stanosz 2001).

The aim of this study was to re-examine the taxonomy of the species described by Shoemaker (1964) using partial rDNA gene sequence, as well as conidial morphology. Furthermore, an attempt is made to clarify some of the long-standing questions surrounding the anamorphs associated with these species of *Botryosphaeria*.

MATERIALS AND METHODS

Isolates and DNA based characterization.--Botryosphaeria spp. with *Diplodia* anamorphs were isolated from a *Ribes* sp., *Fraxinus* sp. and *Quercus* sp. in the USA and *Fraxinus* spp. in Europe (TABLE I). Additional isolates of *Botryosphaeria* spp. with *Diplodia* anamorphs from these and other hosts in Europe were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands (CBS) (TABLE I). Isolates with conidia resembling *Lasiodiplodia* from *Pinus* in Mexico and *Vitex* in Uganda were also used in the analyses (TABLE I). Isolates are maintained in the Culture Collection of the Tree Pathology Co-operative Programme (CMW) at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA, as described by Raeder and Broda (1985) and Smith et al (2001). The ITS rDNA region was amplified using the primers ITS1 and ITS4 (White et al 1990). PCR reaction mixtures, PCR conditions and visualization of amplicons were the same

as those described by Slippers et al (2003). The amplicons of all three DNA regions were also purified and sequenced as described in Slippers et al (2003), using the same primers described above. BLAST searches were used to detect related sequences from GenBank. Sequences arising from these searches were downloaded from GenBank or obtained from Slippers et al (2003) and included in the analyses (TABLE I). Trees were rooted to GenBank sequence data of the related taxa, *Guignardia bidwellii* (Ellis) Viala & Ravaz. and *Mycosphaerella africana* Crous & M.J. Wingf.

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. The ITS1 region contained a Cytosine repeat that was hypervariable. Analyses were done with and without this repeat. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), using heuristic searches with stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swopping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved.

Retention and consistency indices, a g1-value (Hillis and Huelsenbeck 1992) and bootstrap support (1000 replicates) (Felsenstein 1985) for branches were also determined in PAUP. Decay analysis of the branch nodes was done using Autodecay (Eriksson 1998).

Morphological characterization.--Conidial morphology was assessed from sporulating cultures on water agar, to which pine-, apple- or *Populus* sticks or pine needles had been added as substrate. The fungal cultures were exposed to 12 h cycles of near UV light and at 20--25 C for up to one month. Fruiting structures were mounted in clear lactophenol. Morphological observations were made with a light microscope and an Axiocam digital camera (Carl Zeiss, Germany).

In order to test for a possible link between *B. obtusa* and the anamorph name, *D. malorum*, type specimens (two samples both marked as types 1706) of the latter taxon was obtained from the Conservatoire et Jardin botaniques de la Ville de Genève, Genève, Switzerland (G). Conidia from these specimens were studied using the same equipment and techniques, described above.

RESULTS

DNA sequence comparisons.--A PCR product of approximately 600 bp was amplified using the primers ITS1 and ITS4. The final data set after alignment contained 561 characters. Of these, 12 characters representing a highly variable Cytosine repeat in the ITS1 region, were excluded. Of the remaining characters, 171 were parsimony-informative and used in the analyses. These characters contained significant phylogenetic signal compared to randomly sampled sets ($P < 0.01$; $g1 = -0.93$) (Hillis and Huelsenbeck 1992). Heuristic searches in PAUP found one most parsimonious tree of 339 steps (CI = 0.791, RI = 0.923) (FIG. 1). Trees obtained using the full dataset or distance analyses did not differ significantly from the trees obtained using parsimony.

The isolates sequenced in this study grouped into five distinct clades. These clades are identified as *B. quercuum*, *B. stevensii*, *B. obtusa*, *B. rhodina* and a clade containing one isolate of each of *Botryosphaeria subglobosa* (C. Booth) von Arx & E. Müller and *Botryosphaeria visci* (Kalchbrenner) von Arx & E. Müller. Identification of the clades was based on authentic isolates obtained from culture collections and verified based on morphology of isolates of each group (as discussed below) and host relationships.

Morphological characterization.--Not all the isolates used in the phylogenetic study sporulated in culture. However, representative isolates from each of the *B. obtusa*, *B. quercuum* and *B. stevensii* clades identified using the DNA sequence data sporulated after two weeks to two months. The needles and twigs, and pycnidia that formed on them were covered in a thick mat of grey mycelium. Pycnidia were globose, single to botryose, mostly superficial on the surface of the needles or twigs, and sometimes embedded in raised stromata.

The sizes and wall thickness of conidia were measured and L/W calculated for isolates considered to represent *B. quercuum*, *B. stevensii* and *B. obtusa*. These values differed from those reported in the description of the species by Shoemaker (1964) (TABLE II). The measurements of conidia of *B. quercuum* were longer, while those of *B. stevensii* were longer and wider than those previously reported (Shoemaker 1964). Conidia of *B. obtusa* were smaller than those reported by Shoemaker (1964).

The conidial walls of *B. quercuum* and *B. stevensii* were distinctly thick (1--2 μm) and glassy in appearance (FIGS. 2-6). The conidial walls of *B. obtusa* were thinner (0.5 μm) and appeared pitted or rough (FIGS. 7-9). Conidia of *B. quercuum* became discolored and 1--3-septate with age, while those of *B. stevensii* rarely discolored, and only did so after forming a septum (FIGS. 3, 4, 6). Conidia of *B. obtusa* discolor more regularly than those of the other species, and do so before becoming 1--3-septate (FIGS. 7-9).

Two samples of *D. malorum* marked as "*typus*" from the Fuckel collection (G) were studied. Both samples contained similar material of dried apple fruit containing numerous fruiting structures. Two types of conidia were, however, observed on the two samples (A and B). Conidia from both samples were discoloured and single septate. The walls of conidia from both samples were 0.8--1.2 μm thick, but those from the first sample (A) were smooth, while those from the second sample (B) were rough. Sample A had smaller conidia than those reported for *B. obtusa*, while sample B had larger conidia than *B. obtusa*. The conidia of the first sample (A) also contained depressions that appeared like vacuoles in the middle of each cell. This feature is likely due to the age and dehydrated state of these conidia. From the above data it appeared that two *Diplodia* species possibly co-occur on these samples.

DISCUSSION

In this study, isolates considered to represent *B. obtusa*, *B. quercuum* and *B. stevensii* were clearly separated based on DNA sequence data. These taxa all have *Diplodia*-like anamorphs. The conidial morphology of these taxa can be used to distinguish them when septation, size and shape are considered collectively.

Some isolates that group in the *B. quercuum* clade had been previously identified as either *B. stevensii* or *B. obtusa*. The confused identity of isolates in the *B. quercuum* clade, with other species has been noted previously (Zhou and Stanosz 2001). The taxonomic confusion in this group stems from the fact that all three species were synonymized under *B. quercuum* based on the similarity of their ascospores (von Arx and Müller 1954). However, Shoemaker (1964) showed that they can be distinguished based on their conidial characteristics. These characters do, however, also overlap between the species.

The distinction between *B. quercuum* and *B. stevensii* based on conidial size is complicated. Shoemaker (1964) used conidial sizes and L/W ratios to distinguish *B. quercuum* and *B. stevensii*. In this study, we have shown that the sizes and L/W ratios of *B. quercuum* and *B. stevensii* overlap more than was recognized, when initial distinctions between the species were determined. Other researchers (Tisserat 1988, Luque and Girbal 1989) have also reported a greater range of conidial sizes for *B. stevensii* than reported by Shoemaker (1964). Apart from overlap in size, the conidia of *B. stevensii* and *B. quercuum* are similar in general appearance, because both have thick glassy walls and granular contents.

Botryosphaeria quercuum and *B. stevensii* can be distinguished by combining conidial size measurements with the pattern of septation and discoloration. Conidia of *B. quercuum* regularly form one to three septa. These conidia can also discolor prior to septation. The conidia of *B. stevensii* rarely discolor and then only after septation. These conidia normally become 1-septate, and only rarely develop a second septum. Furthermore, the ascospores of *B. quercuum* sometimes become brown and 1--2 septate as they age (Shoemaker 1964).

There is little overlap in the host ranges of *B. quercuum* and *B. stevensii*. *Botryosphaeria quercuum* mainly infects *Quercus*, although it is also known from *Tsuga* and *Cercis*. In the present study, *B. quercuum* was isolated from *Quercus* in Europe and the USA. *Botryosphaeria stevensii* (anamorph = *D. mutila* (Fr.) Mont.) is reported in this study from *Fraxinus* and *Malus*. This taxon was initially described from *Fraxinus* and appears to be common on this host (Shoemaker 1964).

Two *Botryosphaeria* spp., *B. obtusa* and *B. stevensii*, with *Diplodia*-like anamorphs, commonly cause disease on pome and stone fruit trees (Shoemaker 1964, Laundon 1973, Sutton 1980). Both species have been regarded as synonyms of *B. quercuum* (von Arx and Müller 1954), before being described as separate taxa (Shoemaker 1964). On fruit trees, *B. obtusa* and *B. stevensii* have also been known as *Physalospora obtusa* (Schwein.) Cooke and *P. mutila* Stevens (Laundon 1973), respectively. The ascospores of these fungi are very similar and can easily be mistaken for one another. The conidia of *B. stevensii* and *B. obtusa* are more easily distinguished. Those of *B. stevensii* have thick, glassy walls, and become septate before discoloration. In contrast, the conidia of *B. obtusa* have thinner, rough walls, and discolour more commonly than *B. stevensii,* and this also occurs before septation.

Botryosphaeria obtusa has an extremely wide host range. Punithalingam and Waller (1973) listed 34 hosts for this pathogen. In the present study, *B. obtusa* was identified from species of *Malus, Prunus*, *Pyrus* and *Ribes*. This fungus can be mistaken for *D. pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf., because these fungi occasionally overlap in host range on *Pinus* and they all have similar conidial morphologies and ITS DNA sequences. It was for this reason that Hausner et al (1999) described isolates of this fungus from *Pinus* in Canada as the "I" morphotype of *D. pinea* (Burgess et al 2001, De Wet et al 2003). Where these species do co-infect *Pinus,* they can be distinguished using microsatellite markers or multiple gene sequences (Burgess et al 2001, De Wet et al 2003).

The appropriate taxon to accommodate the anamorph of *B. obtusa* has not been determined in this study. Some misconceptions regarding this fungus do, however, deserve discussion. The anamorph is generally referred to as a species of *Sphaeropsis* or *Diplodia*. The distinction between these two genera is supposedly found in the proliferation of the conidiogenous cells and the time of septation. Denman et al (2000), however, argued that representatives of both *Sphaeropsis* and *Diplodia* have percurrently proliferating conidia and that septation occurs widely and at varying stages among many anamorphs of *Botryosphaeria*, making this character inordinately variable to distinguish groups. We support the view that all anamorphs of *Botryosphaeria* with oval or ellipsoid, thick-walled conidia that regularly darken and become with age should be accommodated in *Diplodia*. Zhou and Stanosz (2001) also recognized this group of *Botryosphaeria* anamorphs and referred to them as section "Brunnea". The anamorph of *B. obtusa* has thick-walled, oval conidia, which regularly darken and become septate with age. This anamorph thus fits the description of *Diplodia.*

The illegitimate name *Sphaeropsis malorum* Peck is sometimes used to described the anamorph of *B. obtusa* (Shear et al 1925, Stevens 1925, Laundon 1973, Brown-Rytlewski and McManus 2000). Shoemaker (1964), as well as Punithalingam and Waller (1973) noted that Peck did not describe the name *S. malorum,* and that the older name, *S. malorum* (Berk.) Berk., is a synonym of *D. mutila.* This name should thus not be used for the anamorph of *B. obtusa.*

Diplodia malorum Fuckel is a more appropriate name for the anamorph of *B. obtusa* than *S. malorum*. This possibility is, however, rejected based on studies of the type material of *D. malorum* in the present study. *Diplodia malorum* was considered

to be the anamorph of *Physalospora cydoniae* Arn., which is now accepted as a synonym of *B. obtusa* (as *P. obtusa*) (Laundon 1973). Descriptions of *D. malorum* (Saccardo 1884, Grove 1937) are indistinguishable from those of the anamorph of *P. cydoniae* (= *Sphaeropsis malorum* 'Peck') (Stevens 1925) and *B. obtusa* (Shoemaker 1964, Punithalingam and Waller 1973). The type material of *D. malorum*, however, appears to contain spores of two species of *Diplodia*. The morphology of both these types of conidia differed from the anamorph of *B. obtusa* in size and wall texture. For the present, it would be most appropriate to use the genus *Diplodia* for *B. obtusa* and not to allocate a species name to it.

There is considerable variation in the ITS rDNA sequence data among isolates of *B. stevensii.* Isolates of this species from *Fraxinus* in North America and Europe group separately within the main *B. stevensii* clade. In contrast, *B. quercuum* from various hosts in Europe and North America had identical ITS rDNA sequences. The variation in the *B. stevensii* clade is similar to or greater than that found in this region for different species such as *B. parva* and *B. ribis*, and *B. obtusa* and *D. pinea* (De Wet et al 2003, Slippers et al 2003). This variation might, thus, be indicative of a species complex. Multiple gene genealogies are needed to resolve this question.

Botryosphaeria species with *Diplodia*-like anamorphs and those with *Fusicoccum*-like anamorphs grouped separately based on ITS rDNA sequences used in this study. This is similar to the results of previous studies (Denman et al 2000, Zhou and Stanosz 2001). The group that contained the four species with *Fusicoccum* anamorphs had high bootstrap support (100 %). The grouping of *B. rhodina* with *Botryosphaeria* species having *Diplodia* anamorphs, however, had no bootstrap support. Furthermore, support for the branch separating *B. visci* and *B. subglobosa* with other species having *Diplodia* anamorphs was low (64 % bootstrap). *Botryosphaeria quercuum*, *B. tsugae*, *B. stevensii*, *B. obtusa* and *D. pinea* grouped together with 100 % bootstrap support. The lack of strong bootstrap support for branches grouping all isolates with *Diplodia*-like conidia might indicate that this group is not as uniform as previously believed.

The anamorphs of *B. visci* and *B. subglobosa* are known as *Sphaeropsis visci* (Sollmann) Saccardo and *S. subglobosa* Cooke, respectively. These species, however, group most closely with *Botryosphaeria* species having *Diplodia* anamorphs, including the type species *D. mutila.* These species should be described as species of *Diplodia*, as suggested by Denman et al (2000).

In this study we show that the size and L/W ratio of conidia is probably a less valuable character to distinguish species of *Diplodia* than previously believed. In contrast, conidial wall characteristics and the pattern of septation and discoloration appear to be robust characters to identify these species. In the absence of DNA sequence comparisons, a combination of these characters will provide the best tool to distinguish species of *Diplodia*. The variation in morphology and sequence data among isolates of *B. quercuum*, *B. stevensii* and *B. obtusa*, as well as published data, requires a more in depth study of the taxonomy of these common and important species. Identification of authentic type material and ex-type isolates are needed to characterize these species, as was done for other important *Botryosphaeria* species (Slippers et al 2003).

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TABLE I. Isolates considered in the phylogenetic study.

Culture no.[1]	Other no.[1]	Identity[2]	Host	Location	Collector	GenBank[3]
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York USA	B. Slippers/G. Hudler	AY236935
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943
CMW7999		*B. dothidea*	*Ostrya* sp.	Crocifisso, Switzerland	B. Slippers	AY236948
CMW9076	ICMP7818	*B. lutea*	*Malus* sp.	New Zealand	S.R. Pennycook	AY236946
CMW7056	CBS448.91	*B. subglobosa*	Eye of man	UK	Unknown	
CMW7058	CBS218.25	*B. visci*	*Phoradendron* sp.	Unknown	A.W. Archer	
CMW7061	CBS118.39	*B. quercuum*	*Quercus borealis*	USA	R.W. Davidson	
	KJ93.29	*B. quercuum*	*Quercus* sp.	California, USA	E. Hecht-Poinar	AF027753
	KJ93.35	*B. quercuum*	*Q. suber*	North Eastern Spain	K.A. Jacobs	AF027753
	KJ93.58	*B. quercuum*	*Tsuga* sp.	North Carolina, USA	G.J. Samuels	
	KJ93.09	*B. quercuum*	*Cercis canadensis*	District of Colombia, USA	K.A. Jacobs	
CMW7062	CBS177.89	*B. quercuum*	*Q. cerris*	Italy	A. Vannini	AF243399
CMW8868		*B. quercuum*	*Quercus* sp.	California, USA	T. Gordon	
CMW8869		*B. quercuum*	*Quercus* sp.	California, USA	T. Gordon	
	ZS96-174	*B. tsugae*	*Tsuga heterophylla*	Canada	A. Funk	AF243405
	ZS94-6	*B. stevensii*	*Malus pumila*	New Zealand	N. Tisserat	AF243407
	ZS97-85	*B. stevensii*	*M. pumila*	Unknown	H.J. Boesewinkel	AF243406
CMW7776		*B. stevensii*	*Fraxinus excelsior*	Pusiona, Italy	B. Slippers	
CMW7779		*B. stevensii*	*F. ornus*	Pusiona, Italy	B. Slippers	
CMW7781		*B. stevensii*	*F. excelsior*	Porza, Switzerland	B. Slippers	
CMW7782		*B. stevensii*	*F. excelsior*	Grono, Switzerland	B. Slippers	
CMW1628	182	*B. stevensii*	*F. excelsior*	California, USA	T. Gordon	
CMW1632	183	*B. stevensii*	*F. excelsior*	California, USA	T. Gordon	
CMW7060	CBS431.82	*B. stevensii*	*F. excelsior*	Netherlands	H.A van der Aa	
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	
CMW1179	BO75	*B. obtusa*	*Populus* sp.	Cape, S Africa	A. Smith	
	ZS96-115	*B. obtusa*	*Prunus persica*	Georgia, USA	P.L. Pusey	AF243408
	KJ93.56	*B. obtusa*	Hardwood shrub	New York, USA	G.J. Samuels	AF027759
	KJ94.05	*S. sapinea* 'B'	*Pinus banksiana*	Wisconsin, USA	D.R. Smith	AF027757
	KJ94.07	*S. sapinea* 'A'	*P. resinosa*	Wisconsin, USA	D.R. Smith	AF027758

TABLE I. Continued.

Culture no.[1]	Other no.[1]	Identity[2]	Host	Location	Collector	GenBank[3]
	KJ93.31	*S. sapinea*	*Pinus* sp.	Netherlands	M. de Kam	AF027756
	KJ93.27	*B. rhodina*	*Quercus* sp.	California, USA	E. Hecht-Poinar	AF027761
	KJ93.40	*B. rhodina*	*Pistacia* sp.	California, USA	T.J. Michailides	AF027760
	KJ93.41	*B. rhodina*	*Pistacia* sp.	California, USA	T.J. Michailides	AF027762
CMW10130	BOT977	*B. rhodina*	*Vitex donniana*	Uganda	J. Roux	
	ZS96-112	*B. rhodina*	*P. radiata*	S Africa	W. Swart	AF243401
	ZS96-172	*B. rhodina*	*Theobromae cacao*	Sri Lanka	E. Muller	AF243400
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	
CMW7063	CBS447.68	*Guignardia philoprina*	*Taxus baccata*	Netherlands	H.A. van der Aa	

[1] Designation of isolates and culture collections: BO = *Botryosphaeria* collection, ARC Infruitec-Nietvoorbij, South Africa; BOT and CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection, Fairfax, VA, USA; TO = Ogata et al (2000); ZS = Zhou and Stanosz (2001).

[2] Identities as used in this study.

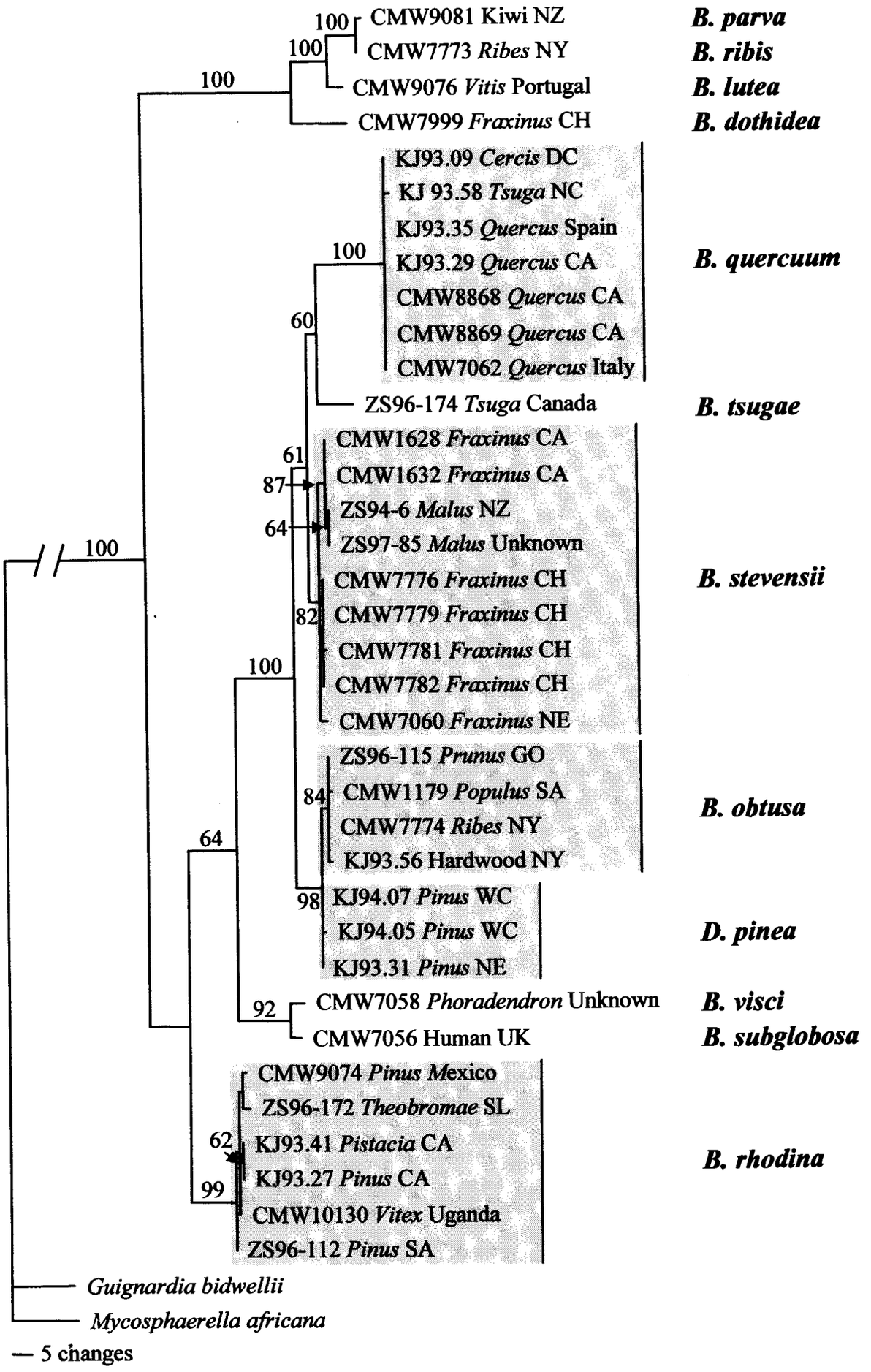
[3] Sequences determined in this study has not yet been deposited in GenBank.

TABLE II. Conidial measurements of selected species of *Botryosphaeria* with *Diplodia* anamorphs.

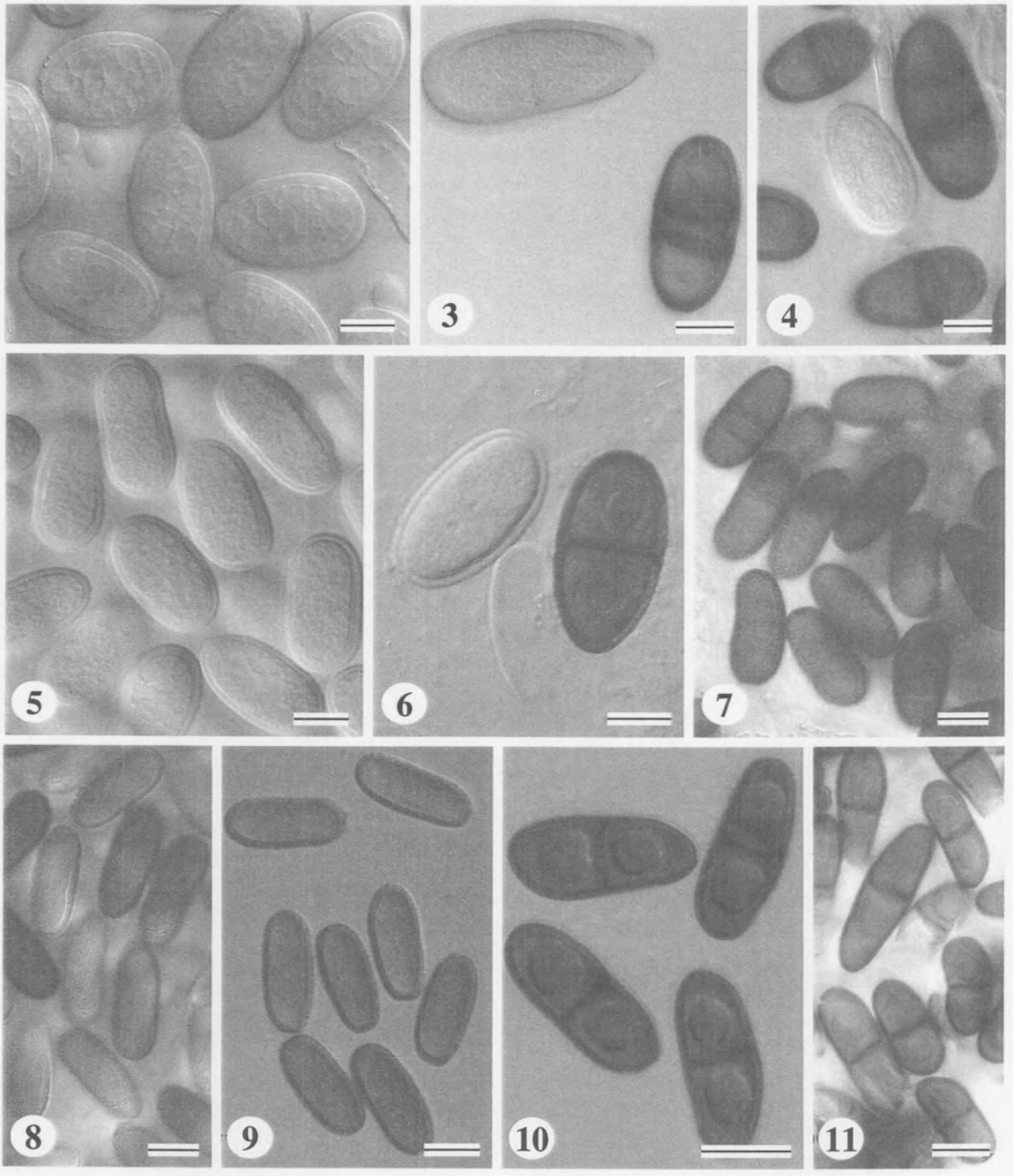
Identity	Culture No.	Conidial measurements[1] (μm)	L/W	Wall	Host	Location
B. quercuum	[2]CMW8868	(23--)27.7(--35) × (11--)14.2(--16)	2	1--1.5	*Quercus*	California, USA
	[2]CMW8869	(21--)26.4(--31) × (13--)14.8(--17)	1.8	1--2	*Quercus*	California, USA
	[2]CMW7062	(30--)31.8(--34) × (15--)16.1(--17)	2	1.5	*Q. cerris*	Italy
	Shoemaker 1964	(18--)21--24(--25) × (12--)15-16(--17)	1.5	1.5--2	*Quercus, Carya*	Canada, USA
B. stevensii	CMW7776	(23--)28.5(--32) × (12--)14.7(--17)	1.9		*Fraxinus excelsior*	Italy
	CMW7779	(26--)28.5(--33) × (12--)15.1(--17)	1.9		*F. ornus*	Italy
	CMW7780	(25--)29.2(--32) × (13--)15.1(--17)	1.9	1--1.5	*F. excelsior*	Switzerland
	Shoemaker 1964	(20--)25--27 × 10--12(--16)	2.3	1.5--2	*Fraxinus, Vitis*, etc.	Europe, Canada
B. obtusa	[2]CMW7774	(18--)21.2(--24) × (7--)9.6(--13)	2.2	1	*Ribes* sp.	New York, USA
	Shoemaker 1964	22--26 × 10--12	?	0.5	*Vitis, Malus, Pyrus, Ribes*, etc.	Europe, Canada, USA
D. malorum	G. 1706 (A)	(16.5--)21(--26) × (7.5--)8.5(--12)	2.5	1	*Malus*	Germany
	G. 1706 (B)	(21--)27.9(--35.5) × (9--)11.7(--14.5)	2.4	1	*Malus*	Germany

[1] Extreme measurements in brackets are actual ranges. Averages are given between extreme values, and are representative of 15-60 conidia.
[2] Conidia produced *in vitro* as described in Materials and Methods. Other isolates (CMW) are from field collected samples or from herbarium G (G). Measurements for the respective species reported by Shoemaker (1964) are also given.

FIG. 1. One of the most parsimonious trees obtained by heuristic searches of the full dataset of ITS rDNA sequence data. Bootstrap values (1000 replicates) for branches are indicated above the branches. The tree is rooted to sequences of *Guignardia bidwellii* and *Mycosphaerella africana*. Isolates' number, host and origin (CA = California, USA, CH = Switzerland, GO = Georgia, USA, NE = Netherlands, NY = New York, USA, NZ = New Zealand, SA = South Africa, UG = Uganda, WC = Wisconsin, USA) are indicated, as well as the taxonomic identities of the isolates or clades.



FIGS. 2-11. DIC compound-microscope micrographs of various anamorphs of species of *Botryosphaeria*. 2-4. Hyaline and pigmented, aseptate, as well as 1--3 septate, dark conidia with thick, glassy walls of the anamorph of *B. quercuum*. 5, 6. Hyaline, aseptate and pigmented, septate conidia of the anamorph of *B. stevensii*. 7-9. Hyaline and pigmented, aseptate conidia, as well as pigmented, 1--2 septate conidia with rough walls of the anamorph of *B. obtusa*. 10, 11. Dark, septate conidia from herbarium material of *Diplodia malorum* with dark, septate conidia. Bars = 10 µm.



8

Development of SSR and RFLP markers for *Botryosphaeria* spp. with *Fusicoccum* anamorphs

Species of *Botryosphaeria* are ascomycete (teleomorph, sexual) fungi that occur world-wide and on woody plants (von Arx 1987). The anamorphs (asexual) of these fungi reside in the genera *Diplodia* and *Fusicoccum*. Some species cause serious canker and die-back diseases, mostly following stress to plants (von Arx 1987). These fungi can, however, also exist as endophytes within seed or other living plant tissues, in the absence of symptoms (Fisher et al 1993, Smith et al 1996). In this way, they appear to have been moved around the world on various plants (Denman et al 2003, Chapter 5).

Botryosphaeria parva Pennycook & Samuels and *B. ribis* Grossenb. & Duggar, are closely related or cryptic species. These fungi are morphologically very similar and often not distinguishable, due to the overlapping of characteristics such as spore dimensions (Slippers et al 2003). ITS sequence data and PCR RFLP techniques also do not separate isolates of these two species, which can currently be distinguished only using multiple gene genealogies (Slippers et al 2003, Chapter 5).

Simple sequence repeat (SSR) or microsatellite markers have been developed for some Botryosphaeriaceous fungi, such as the anamorphs *D. pinea* (Desm.) J. Kickx., *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (anamorphic species of *Botryosphaeria*) and *B. rhodina* (Cooke) Arx (Burgess et al 2001, 2003). These co-dominant markers have been useful to characterize the structure of populations, and to understand diversity and movement of these fungi (Burgess et al 2001). These markers, together with multiple gene sequence data, have also defined boundaries between the cryptic species *D. pinea* and *D. scrobiculata* (Burgess et al 2001, De Wet et al 2003). However, the above primers were not useful for population studies in other important *Botryosphaeria* spp. such as *B. parva* and *B. ribis*.

The aim of this study was to develop polymorphic SSR markers that can be used in population studies of *B. parva* and related fungi, including some of the anamorphic species. It was hoped these markers might also be used to distinguish cryptic species residing in the *B. parva* – *B. ribis* complex. The same development strategy was followed as for the development of SSR markers for *D. pinea* and *L. theobromae* (Burgess et al 2001, 2003).

Repeat regions were identified and primers designed using isolate CMW10122 of *B. parva*. This isolate grouped in a *B. parva* clade using a multiple gene genealogical approach, but showed some sequence divergence from most other *B. parva* isolates used in that study (Slippers et al 2003). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

The Inter Simple Sequence Repeat (ISSR) primers DV$(CT)_8$, DB$(CA)_8$, VH$(TG)_8$, HVH$(GTG)_5$, DHB$(CGA)_5$, DBD$(CAC)_5$ and DBV$(CAT)_5$, as well as the combinations DHB$(CGA)_5$ / HVH$(GTG)_5$, BDB$(ACA)_5$ / HBDB$(GACA)_5$ and DBV$(CAT)_5$ / VH$(TG)_8$ were used to amplify multiple fragment fingerprints. The PCR reaction mixtures consisted of 0.4 mM of each dNTP, 1x PCR buffer containing $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA), 10 pM of each primer, 0.5 U Expand High Fidelity *Taq* Polymerase (Roche Molecular Biochemicals) and 1-10 ng of genomic DNA. PCR conditions included 35 cycles of denaturation at 95 C (30 s), annealing at 48 C (45 s) and elongation at 72 C (2 min), followed by a final elongation step of 7 min. Amplified products were separated on 1.5 % agarose gels that were stained with ethidium bromide and visualized under UV light. The PCR products were cleaned (Roche Molecular Biochemicals), cloned (Promega Corp. Madison, WI) and sequenced (Perkin Elmer Applied Biosystems Inc., Foster City, CA).

Sequences containing microsatellite repeats were identified and forward and reverse primers were designed to flank these sequences. Some fragments contained microsatellite repeats at the ends of the fragments. For these fragments, two reverse primers were developed on the 3' side of the repeat. These primers were used to 'genome walk' across the fragment following the protocol described by Siebert et al (1995) and as applied by Burgess et al (2001).

Twenty primer sets were designed and used to amplify the fragments from isolate CMW10122, as well as from isolates CMW1239, CMW2283, CMW7885 and CMW2387. PCR reactions were conducted using the Expand High Fidelity *Taq* polymerase enzyme and accompanying chemicals, following to the instructions of the manufacturer (Roche Molecular Biochemicals). Initial amplification was done at 54 C annealing temperature. Where multiple bands were obtained, annealing temperature was increased stepwise to 60 C until single bands were obtained. PCR amplicons were subjected to electrophoresis on 1.5 % agarose gels, stained with ethidium bromide, and visualized under UV illumination. Where

size polymorphism of amplicons was not already visible on the agarose gels, the products were cleaned and sequenced, using the same primers as those used for amplification.

Of the twenty primer pairs designed, eight amplified length polymorphic regions and were selected for further investigation (TABLE I). The twelve other primer pairs either did not amplify single fragment, were monomorphic or did not amplify any fragment. Some of the length monomorphic primers contained single base pair substitutions or indels that differed among the isolates. These data were analyzed in Webcutter 2.0 (www.firstmarket.com/cutter.cut2) to identify polymorphisms of restriction enzyme sites. Four regions with polymorphic restriction sites were identified, including two fragments that were previously included with the length polymorphic sites (TABLE I). These fragments were digested with the restriction enzymes (RE), *Hae*III, *Sau*3AI and *Msp*I (Roche Diagnostics, Indianapolis, USA). Each RFLP reaction consisted of 20 µl PCR reactions with ITS DNA template, 0.2 µl RE, 2.2 µl matching enzyme buffer and 2.5 µl sterile Sabax water. The reaction mixture was incubated at 37 C for 3 hours or overnight. Restriction fragments were separated on 1.5 or 2 % agarose gels as described for PCR products.

The SSR markers were tested on various *Botryosphaeria* and *Fusicoccum* species (where sexual states are not known), for their ability to amplify the same regions as in isolate CMW10122. These included *B. parva* (CMW9081), *B. ribis* (CMW7772), *F. indigoticum* (CMW62), *F. bacilliforme* (CMW90), *F. mangiferum* (CMW7797), *B. eucalyptorum* (CMW6551), *B. irregularis* (CMW6222), *B. lutea* (CMW992), *B. australis* (CMW6836), *B. dothidea* (CMW8000). Most primer pairs amplified the same fragment as that of CMW10122, although these varied in size (TABLE II). In some cases, using primer pairs BOT11&12, BOT15&16, BOT17&18 and BOT 35&36, different fragments, multiple bands were amplified or no amplicons were obtained (TABLE II). Primer pair BOT37 and BOT38 amplified only fragments from three species, including *B. parva* (TABLE II). The annealing temperatures were adapted in cases where single bands were not obtained at 54 C (TABLE III). Despite efforts to optimize the annealing temperatures, different fragments were amplified in five cases, multiple fragments in three cases, and in one case no amplicons was produced.

Species of *Botryosphaeria* belong to two groups based on their asexual states, namely those with fusicoccum-like and those with diplodia-like asexual spores (Denman et al 2000). *Botryosphaeria parva,* and most of the other species tested in this study, belongs to the *Botryosphaeria* group that has *Fusicoccum* anamorphs. When markers were tested for

their ability to amplify polymorphic regions in *Diplodia pinea* (CMW2389) and *B. rhodina* (CMW2388), fragments of similar size to those found in *B. parva* (CMW10122) were amplified by only three of the eight primer pairs (TABLE II). These fragments were not sequenced.

All the fragments amplified using the primers designed in this study contained some SSR repeats. However, not all the size polymorphisms were found in these regions. These primers are thus better described as polymorphic SSR containing markers, rather than pure SSR markers. These primer pairs, however, still provide co-dominant markers that will be useful in studying population structures, diversity, gene flow and for identifying reproductive strategies and barriers in *B. parva* and other species of *Botryosphaeria* with *Fusicoccum* anamorphs.

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TABLE I. Primers designed in this study to amplify SSR containing polymorphic loci from *Botryosphaeria* spp.

SSR Primers[1]	Primer sequence (5'-3')	Core sequence[2]	Fragment length (bp)	Annl Temp C	Melt Temp C	Comments[3]
BOT 1	CGG ACA GGT CGC ATT CGC G	*$(GAAG)_2$ *GACA rich *GC rich	187	54	64	Monomorphic
BOT 2	CTT GCC GTT GCC CTT GAG CG	*(CAA)x8(GAA)x6 interrupted			66	RFLP - *Hae*III
BOT 11	CGG CAT GGT CTG CCG CTC C	*(GCAT)$(GCAC)_3(GC)_3$(GCAT)	429	54	66	Polymorphic
BOT 12	GCA TCT CCG GCT ACC AAC CG	*(GA)(CA)(GT)(CT) alternating			66	RFLP - *Sau*3AI
BOT 15	CTG ACT TGT GAC GCC GGC TC	*$(TCTTCG(T/A)GGCGG)_4$	374	54	66	Polymorphic
BOT 16	CAA CCT GCT CAG CAA GCG AC	*$(CT)_{13}(CTT)_8(CTTT)_2$(CTTTT) alternating			64	RFLP – *Cfo*I
B-GW2	CAC CGA AGG CTC AAA GCA CC	*$(CTT)_{1-2}$x9$(CTTT)_{1-2}$x5$(CTTTTT)_2$ interrupted			64	
BOT 17	GGC GCA ATC TCG ATT CGA GC	*$(CAT)_{12}$	362	54	64	Polymorphic
BOT 18	CCA CGA TGT CCG TTC ATC G				64	
BOT 19	GGC GGT CGC AGA TGC GGT C	*$(GCT)_7$T(GCT) *$(GCT)_{1-3}$ and $(GC)_{1-3}$	274	54/58	66	Polymorphic
BOT 20	GCC CTA TTC TGC GTG CCT CC	scattered throughout			66	
BOT 21	CGC CAC CTG CCT CGC AGC AG	*$(GAC(AorG))_8$(GATA) *$CT(GT)_5GG$	211	58	70	Polymorphic
BOT 22	GAC AGG AAC GTA ACT GCG ATC C				68	
B-GW3	GGC TGT ACG TAC CCT TCA AGC C				70	
BOT 23	CAT CGC ACA GGA GCC GAT TCT	*$(T)_{1-6}$*$(A)_{1-8}$ interrupted *$(CT)_3$*$(CCT)_2$	415	54	66	Polymorphic
BOT 24	CAT ACA TCG AGC TTT CTT GAG GG				68	
BOT 27	GCC GCA GCG GAA CGG TGT CGC	*(TCA)3TCG*(TC)4(CAC)2*(TC)x4(TCC)x3(	253	58	70	Monomorphic
BOT 28	GAC GGC CTG TCG CAA CTC GG	TTC)x4 interspersed			68	RFLP – *Msp*I
BOT 35	CTC CAT CCT GAT CCA GGG TCC	*$CACATCT(CAT)_4(CAG)_2CGG(CAG)_8(CAT)_2$	261	54	68	Polymorphic
BOT 36	GAC GAA TCA AGC GGG CTG CCC	$CAG(CAA)_3$			70	
B-GW7	CCG AGA CCG AAG GCT GCG CG				66	
BOT 37	GGC GTA GCG TGG GCG ACT GG	*$(GCCC)_2GC$*$(GAT)_4C(GAT)_4$	317	54	70	Polymorphic
BOT 38	CCC ATC GCC CAC TCA ACC CG				68	
B-GW8	CGT GGT GCT CCG GGC AAG GG				70	

[1] BOT primer numbers are those used for amplification. B-GW primers were used for genome walking.
[2] Core sequences are as observed in amplicons of CMW10122. Subscript numbers refer to the number of uninterrupted repeats, while 'x' refers to the number of times that a motive is found, interrupted, throughout the fragment.
[3] The morphism refers to the size of the fragments. Some loci that did not vary in length, however, had single point mutations that were useful for RFLP analysis.

TABLE II. Amplicons of various *Botryosphaeria* spp. with *Fusicoccum* anamorphs, using the SSR primers developed in this study. Approximate fragment sizes are indicated. '+++' indicates multiple bands and '-' indicates no amplification. All fragments, except those of *Diplodia pinea* and *B. rhodina*, were sequenced and compared to the sequence of the original fragments in *B. parva*. Shaded fragments are not analogous to the same locus in *B. parva*.

	CMW	**BOT11&12**	**BOT15&16**	**BOT17&18**	**BOT19&20**	**BOT21&22**	**BOT23&24**	**BOT35&36**	**BOT37&38**
Botryosphaeria parva	9080	500 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	250 bp
B. ribis	7772	500 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	250 bp
B. lutea	992	500 bp	500 bp	500 bp	275 bp	220 bp	400 bp	200 bp	-
B. australis	6836	500 bp	400 bp	500 bp	275 bp	220 bp	400 bp	200 bp	-
B. dothidea	8000	+++	+++	250 bp	275 bp	220 bp	400 bp	-	-
B. irregularis	6222	1000 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	-
B. eucalyptorum	6551	1000 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	-
Fusicoccum bacilliforme	90	200 bp	+++	250 bp	275 bp	220 bp	400 bp	180 bp	-
F. indigoticum	62	500 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	-
F. mangiferum	7797	500 bp	400 bp	250 bp	275 bp	220 bp	400 bp	200 bp	250 bp
Diplodia pinea	2387	200 bp	600 bp	250 bp	-	-	400 bp	200 bp	-
B. rhodina	2388	-	+++	250 bp	-	+++	400 bp	200 bp	-

TABLE III. Temperatures used to amplify single fragments for the species of *Botryosphaeria* using the primers developed in this study.

	CMW	BOT11&12	BOT15&16	BOT17&18	BOT19&20	BOT21&22	BOT23&24	BOT35&36	BOT37&38
Botryosphaeria parva	9080	54	54	54	62	58	54	60	54
B. ribis	7772	54	54	54	62	58	52	60	54
B. lutea	992	60	52-64	54	62		54	60	-
B. australis	6836	62	54	54	62	62	54	60	-
B. dothidea	8000	52-64	52-64	62	62	62	52	-	-
B. irregularis	6222	54	54	60	62	62	52	60	-
B. eucalyptorum	6551	54	54	62	62	62	54	60	-
Fusicoccum bacilliforme	90	54	52-64	54	62	62	54	54	-
F. indigoticum	62	54	54	54	54	58	54	60	54
F. mangiferum	7797	62	54	54	62	58	54	60	-
Diplodia pinea	2387	54	54	54	-	-	54	54	-
B. rhodina	2388	-	-	54	-	-	54	54	-