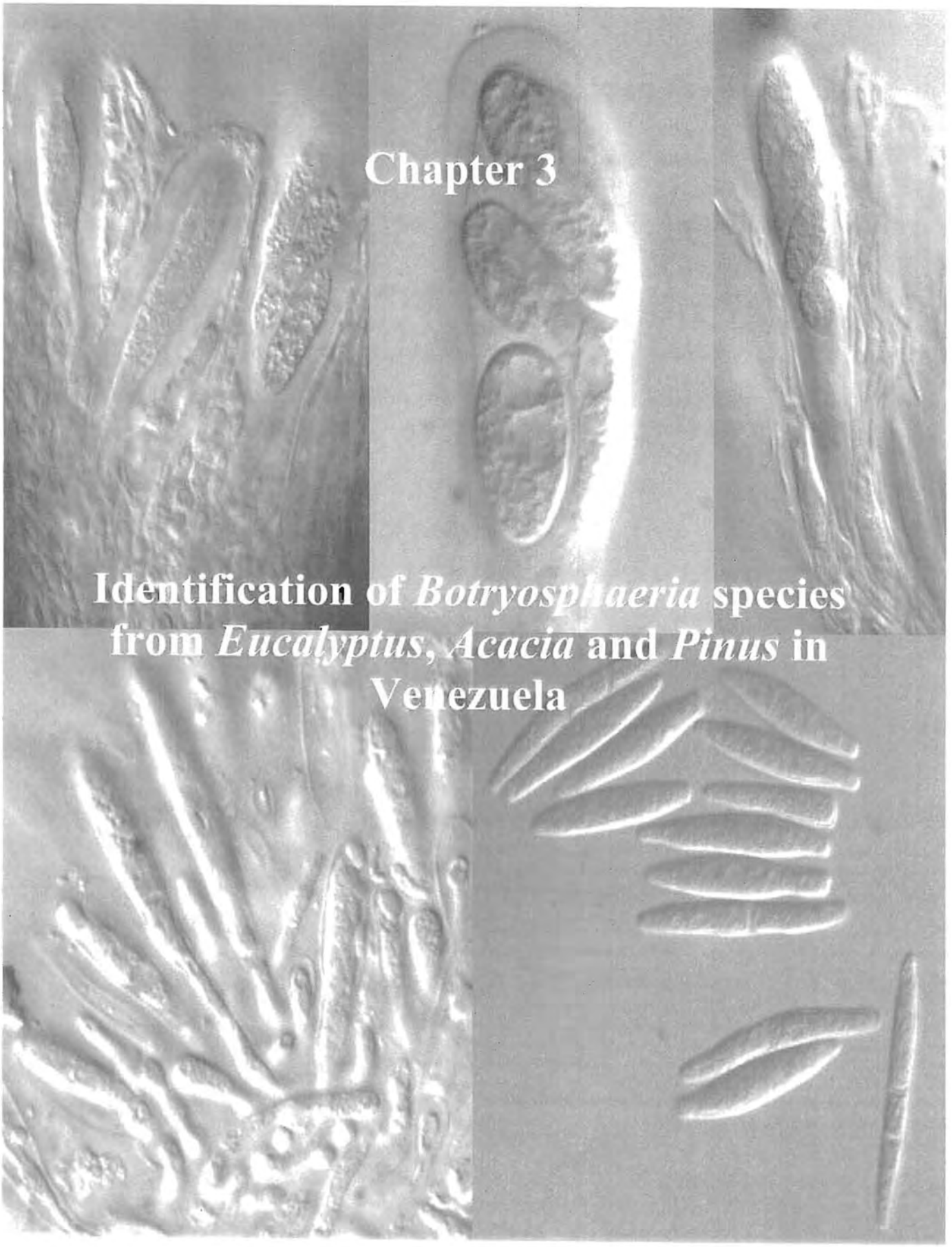

Chapter 3

Identification of *Botryosphaeria* species from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela

Identification of *Botryosphaeria* species from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela

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Botryosphaeria spp. are pathogens of many plantation trees, including species of *Eucalyptus*, *Pinus* and *Acacia*. Some *Botryosphaeria* anamorphs have been reported from Venezuela, but their identification is not certain. The aim of this study was to identify *Botryosphaeria* spp. affecting plantations of *Eucalyptus*, *Acacia* and *Pinus* in Venezuela. Identifications were made using a combination of morphological characteristics and DNA based molecular techniques, namely comparisons of DNA sequence data and restriction digestion (PCR-RFLP) patterns of ITS rDNA amplicons. From a total of 204 isolates from Venezuela, *B. mamane*, the *B. ribis* / *B. parva* complex, *B. dothidea*, *B. rhodina*, *Fusicoccum andinum prov. nom.* and *F. stromaticum prov. nom.* were identified. To discriminate between isolates residing in the *B. ribis-B. parva* complex, PCR-RFLP patterns for an unidentified DNA region, that were characterised previously, were used. This technique showed that both these species are present in Venezuela. This study represents the first report of *B. mamane* outside Hawaii and the first records of *B. dothidea*, *B. parva* and *B. ribis* in Venezuela.

INTRODUCTION

Botryosphaeria spp. have a cosmopolitan distribution and occur on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts, as well as on lichen thalli (von Arx, 1987; Barr, 1987). These fungi are associated with different symptoms such as shoot blights, stem cankers, fruit rots, die-back and gummosis (Ciesla *et al.*, 1996). *Botryosphaeria* spp. are generally regarded as weak pathogens that infect stressed or wounded plants after drought, hail, wind, frost or insect damage (Crist & Schoeneweiss, 1975; Smith *et al.*, 1994; Crous *et al.*, 1989; Ciesla *et al.*, 1996). It has also been shown that *Botryosphaeria* spp. occur in asymptomatic tissue as latent pathogens in trees such as *Eucalyptus*, *Pinus* and *Syzigium* (Swart & Wingfield, 1991; Smith *et al.*, 1996; Pavlic *et al.*, 2004).

The genus *Botryosphaeria* has been known for more than a century and its taxonomy has been confused for much of this time. This confusion arises largely from overlapping morphological characteristics, particularly those of the teleomorph structures. In some instances, and particularly prior to the common use of artificial cultures for the study of these fungi, names were assigned to taxa based on the hosts on which they have been found (Cesati & De Notaris, 1863; De Notaris, 1863; Saccardo, 1877, 1882; Putterill, 1919; Trotter, 1928). The resulting taxonomic confusion has also had a negative impact on the understanding of diseases caused by *Botryosphaeria* spp.

Identification of *Botryosphaeria* spp. causing diseases has largely been dependent on the taxonomy of the anamorphs, which represent the most frequently found state (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Smith & Stanosz, 2001). The morphological characteristics of the anamorphs that are considered useful for identification include conidial size, shape, color and septation (Pennycook & Samuels,

1985; Jacobs & Rehner, 1998; Denman *et al.*, 2000, Phillips *et al.*, 2002; Slippers *et al.*, 2004b). However, conidial characteristics are also variable within species and change with age of the conidia (Sivanesan, 1984; Pennycook & Samuels, 1985).

In recent years, significant advances have been made in the identification of *Botryosphaeria* spp. using DNA-based techniques. Specifically, comparisons of sequence data for the nuclear ribosomal DNA internal transcribed spacer (ITS1 and ITS2) region have been used to analyse intraspecific and interspecific relationships in *Botryosphaeria* (Jacobs & Rehner, 1998; Smith *et al.*, 2001; Smith & Stanosz, 2001; Denman *et al.*, 2000, Zhou & Stanosz, 2001; Slippers *et al.* 2004b). Thus, for the first time, a relatively robust taxonomy is emerging for *Botryosphaeria* and this is already leading to a deeper understanding of host pathogen relationships and geographic distribution of species.

Very little is known regarding *Botryosphaeria* spp. in Venezuela. A number of *Botryosphaeria* anamorphs are known to occur in this country and they include *Lasiodiplodia theobromae* (Pat.) Griffon & Maublanc., *Diplodia pinea* (Desm.) Kickx (=*Sphaeropsis sapinea* (Fr.) Dyko & Sutton), *D. mutila* Fr. Apud Mont., and a species of *Dothiorella* Sacc. (Cedeño & Palacios-Pru, 1992; Cedeño *et al.*, 1994, 1995, 1996; Mohali, 1993, 1997; Mohali & Encinas, 2001; Mohali *et al.*, 2002; De Wet *et al.*, 2003). Identifications of these fungi, originating from disease symptoms on both agricultural crops and forest trees, were based on conidial morphology. Many of these *Botryosphaeria* spp. are thought to be important pathogens in Venezuela and their correct identification is desired.

The aim of this study was to characterise *Botryosphaeria* spp. and their anamorphs found on important forest plantation trees in Venezuela. The *Botryosphaeria* spp. were isolated from *Acacia mangium* Willd., *Eucalyptus* spp. and *Pinus caribaea*

Morelet var. *hondurensis* (Sénécl.) W.H.G. Barrett et Golf in different regions of the country where they have been extensively propagated. Identifications were made using morphological characteristics, as well as comparisons of DNA sequence data from the internal spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and PCR-RFLPs.

MATERIALS AND METHODS

Isolates and Morphology

Botryosphaeria spp. were isolated from stems and branches of three main tree hosts (Table 1). Seventy-eight isolates from *Eucalyptus urophylla* S.T. Blake x *E. grandis* W. Hill ex Maiden hybrids and sixteen from *Acacia mangium* growing in plantations in Portuguesa state. Thirty-two isolates from different *Eucalyptus*-hybrids and twenty-five from *A. mangium* were likewise obtained in plantations in the Cojedes state. Twenty isolates from *Eucalyptus* sp. were also obtained in Los Andes Cordillera, in Mérida state, thirty two isolates were isolated from *Pinus caribaea* var. *hondurensis* in a seedling orchard in Falcon state, and one isolate from *Psidium guajava* L. (Guava) in Zulia state (Table 1). Isolates were collected from asymptomatic plant tissue, as well as from trees exhibiting blue stain or die-back, and from entirely dead trees. Plant tissue was surface disinfested in 70 % ethanol for 30 s, after which it was rinsed in sterile water for 1 min. Pieces of tissue were cut from the specimens and placed on 2 % malt extract agar (MEA) (2% DIFCO, Detroit, MI, USA) at 25 °C and stored on this medium at 4 °C. Isolates were characterised based on colony morphology and anamorph structures.

Isolates were induced to produce anamorph structures in culture by transferring them to water agar (WA) (2 % Biolab agar, Midrand, South Africa) with sterilized pine needles placed on the agar surface. These cultures were incubated at 25 °C under near UV-light until fungal structures appeared on the pine needles. Ascospore morphology was based on structures found on the plant tissue originally collected in the field. Conidial and ascospore morphology was studied using a light microscope with an Axiocam digital camera and software to analyse photographs (Carl Zeiss, Germany). Sections through some of the pycnidia and stromatal structures were made using an American Optical Freezing Microtome. Length, width, shape and color of the conidia was recorded after mounting these structures in lactophenol.

Single-conidial and ascospore isolates of *Botryosphaeria* spp. were isolated and used for DNA extraction. 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.

DNA isolation

A modification of the method of Reader and Broda (1985) was used to isolate DNA from all the fungi. The method is similar as described in chapter 2. Cultures were grown in liquid MEA (3 %) medium in 1.5 ml Eppendorf tubes at 25 C for 7-10 days. Mycelium was harvested by centrifugation, then homogenised and incubated. Nucleic acids were quantified using a spectrophotometer with an absorbance at 260 nm and 280 nm (OD_{260}: OD_{280}).

DNA amplification

The extracted DNA was used as template to amplify a part of the nuclear rRNA operon in PCR reactions using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.*, 1990). The PCR reaction was the same as described in chapter 2. The size of the PCR amplicons was estimated using DNA molecular weight marker XIV (100 bp ladder) (Roche Molecular Biochemicals, Mannheim, Germany).

Sequence comparisons and analysis

Twenty-three of the 204 isolates from Venezuela were selected for DNA sequence as representative of the morphological groups, hosts and geographic origins. Sixteen sequences obtained from previously published work deposited in GenBank (Slippers *et al.*, 2004b) were included in the analyses (Table 2) to appropriately characterise the Venezuelan *Botryosphaeria* spp. Sequences were also compared with those in GenBank by BLAST to determine whether they had a closer relationship to any other sequences than those already selected for the phylogenetic analyses. The trees were rooted with the ITS sequence data of a *Bionectria* sp.

All PCR amplicons were purified prior to sequencing using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, California, USA) following the manufacturer's specifications. The PCR products were sequenced in both directions using the primers ITS1 and ITS4. Sequencing reactions were performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied BioSystems, Foster City, CA) as recommended by the manufacturer and run on an ABI PRISM 3100 autosequencer (Perkin-Elmer Applied BioSystems, Foster City, CA).

Sequence data were analysed using Sequence Navigator version 1.0.1™ (Perkin-Elmer Applied BioSystems, Foster City, California, USA) and manually aligned by inserting gaps. Phylogenetic analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford, 1999). Gaps were treated as a fifth character and all characters were given equal weight. The heuristic searches were done using random stepwise addition tree bisection and reconstruction (TBR) as branch swapping algorithm to obtain maximum parsimonious trees. Bootstrap analysis (1000 replicates) (Felsenstein, 1985) was used to determine the confidence intervals of branch points on the shortest tree. Branches with a length of zero were collapsed and all multiple equally parsimonious trees were saved. Levels of homoplasy (retention and consistency indices) (Hillis & Huelsenbeck, 1992) were determined.

Restriction analysis

A computer simulation analysis was made based on the sequence data described above to determine polymorphisms within the restriction sites for restriction endonuclease (RE) *Cfo*I. This analysis suggested that the enzyme could separate all the species of *Botryosphaeria* from *Eucalyptus* and *Acacia* in Venezuela, except *B. parva* Pennycook & Samuels and *B. ribis* Grossenb. & Duggar. Therefore, an empirical study was undertaken.

Restriction analysis of the amplified ITS regions was consequently done using the RE *Cfo*I (Roche Molecular Biochemicals, Mannheim, Germany). The RFLP reaction consisted of 20 µl PCR reactions of the amplicons, 0.25 µl RE, 2.75 µl matching enzyme buffer and 2.0 µl sterile Sabax water. The ITS PCR amplicons of all 204 isolates (Table 1) were digested overnight at 37 °C. The resulting restriction fragments were separated on 3 % (w/v) agarose gels, stained with ethidium bromide and

visualized under UV light. The fragment sizes were estimated using DNA molecular weight marker XIV (100 bp ladder) (Roche Molecular Biochemicals, Mannheim, Germany).

RFLP analysis of B. ribis-B. parva complex

Restriction patterns for an unidentified DNA region-Locus *BotF15* (Slippers *et al.*, 2004a) were used to distinguish cryptic species residing in *B. ribis-B. parva* complex, which could not be separated using ITS sequences (Slippers *et al.* 2004b). The DNA fragment was amplified using the primers BOT 15 and BOT 16 (Slippers *et al.* 2004a). The PCR reaction mixtures were made as described in Slippers *et al.* (2004a). The amplified fragments were digested with the RE *Cfo*I as described above. The RFLP reaction was incubated at 37 °C overnight and the restriction fragments separated on 2 % (w/v) agarose gels stained with ethidium bromide and visualized under UV light. The sizes of the PCR fragments were estimated as described above.

RESULTS

Isolates and Morphology

All isolates produced conidiomata on sterilized pine needles on WA after two weeks. The *Botryosphaeria* isolates from Venezuela could be separated into groups based on length, width and shape of the conidia (Table 3). Teleomorph structures were found for only one of these groups on the original plant material, and cultures were made from the ascospores. Teleomorph names are, however, used preferentially in this study where the holomorph connection is known. Isolates were thus identified as belonging to *B. mamane* Gardner (Figs 1-5), *B. rhodina* (Berk. & M.A. Curtis) Arx, the *B. ribis-B.*

parva complex (Slippers *et al.* 2004b) (Figs 6-10), *B. dothidea* (Moug. ex Fries) Ces. & de Not. (Figs 11-15), *Fusicoccum andinum prov. nom.* Mohali, Slippers & M. J. Wingf., and *F. stromaticum prov. nom.* Mohali, Slippers & M. J. Wingf. (Mohali *et al.*, 2005).

Phylogenetic analyses

ITS sequence data (after alignment 562 characters) were obtained from 39 isolates (Table 2). Of the total data set, 317 characters were constant, and of the variable characters 91 were parsimony informative. Heuristic search analysis of the sequence data resulted in one tree [Consistency Index (CI) = 0.893; Retention Index (RI) = 0.931; Homoplasy Index (HI) = 0.107] (Fig. 16). Nine principal clades (I to IX) were obtained by comparing isolates from Venezuela with sequences from the GenBank. Venezuelan (VZLA) isolates grouped in six clades (II, IV, VI, VII, VIII, IX), which corresponded to the morphological groups noted above. The ITS rDNA sequence data analysis could not distinguish between isolates belonging to the *B. ribis* / *B. parva* complex, which grouped in clade II. *Botryosphaeria dothidea*, *B. mamane* and *B. rhodina* grouped in clades supported a bootstrap value ≥ 90 %. *Fusicoccum andinum prov. nom.* (Clade IV) and *F. stromaticum prov. nom.* (Clade VI) recently described from Venezuela (Mohali *et al.*, 2005) were also strongly supported by bootstrap values of 100 % (Fig. 16).

PCR-RFLP analysis

All *Botryosphaeria* spp., except *B. ribis* and *B. parva*, from Venezuela could be identified using the RE *Cfo*I (Fig. 17) and restriction maps were determined for them (Fig. 18). Restriction fingerprints and maps using the RE *Cfo*I showed different restriction patterns for the isolates of *B. mamane* (CMW 13432 / 13429), *B. ribis* / *B. parva* (CMW 13409 / 13418) as a complex, *B. dothidea* (CMW 13373 / 13390),

Fusicoccum stromaticum prov. nom. (CMW 13434 /13435) and *Fusicoccum andinum prov. nom.* (CMW 13446 / 13455).

RFLP analysis of B. ribis-B. parva complex

The DNA locus *BotF15*, amplified with the primers BOT 15 and BOT 16 for the isolates grouping in the *B. ribis-B. parva* complex (Clade II), was polymorphic, with a restriction site for the enzyme *Cfo*I in the isolates of *B. ribis*, but not in the *B. parva* isolates (Fig. 19). It was, therefore, possible to distinguish isolates belonging to these two species from each other, as previously noted by Slippers *et al.* (2004a).

DISCUSSION

In this study, seven different *Botryosphaeria* spp. were identified and characterized from Venezuela. These identifications were supported by morphological characteristics, as well as by comparisons of DNA sequence data and analyses of RFLP patterns. The majority of these fungi are recognised from Venezuela for the first time, and some include important plant pathogens. The results thus represent an important contribution towards understanding the world-wide distribution of *Botryosphaeria* spp. and they will also facilitate studies of the diseases associated with them.

One of the more intriguing results of this study was the discovery of *B. mamane* in Venezuela. Previously, this fungus was known only from the native leguminous forest tree, *Sophora chrysophylla* (Salisb.) Seem. in Hawaii (Gardner, 1997). In that respect, it might have been considered a curiosity. Its presence in Venezuela on the stems and branches of *Eucalyptus* spp. and *Acacia mangium* suggests that this fungus has a greater importance than was previously recognised. In Hawaii, *B. mamane* was

associated with witches'-broom on *Sophora* (Gardner, 1997), but these symptoms were not present in Venezuela. Our isolates of the fungus originated from twig die-back symptoms as well as from asymptomatic tissue. It thus appears to be an endophyte, which is similar to many other *Botryosphaeria* spp. (Swart & Wingfield, 1991; Smith *et al.*, 1996; Pavlic *et al.*, 2005). Its role in causing disease on *Eucalyptus* is not known and will need to be evaluated through pathogenicity tests.

The morphology of *B. mamane* and its anamorph, *F. mamane* (Figs 1-5), differs somewhat from the description of the fungus from Hawaii (Gardner, 1997). The macroconidia of the Venezuelan specimens sometimes have septa, but these were not reported previously. The asci and ascopores in the Venezuelan isolates of this fungus were also smaller than those found in Hawaii (Gardner, 1997). Because the fungi share identical sequences, these morphological differences should best be viewed as representing variation within the species.

Both *B. ribis* and *B. parva* (Figs 6-10) are well-known pathogens of forest tree species, including *Eucalyptus* spp. (Frezzi, 1952; Davison & Tay, 1983; Shearer *et al.*, 1987; Crous *et al.*, 1989; Slippers *et al.*, 2004b, c; Ahumada, 2003; Rodas, 2003) and their presence on forest tree species in Venezuela is not surprising. Both these species have been associated with disease symptoms on *Eucalyptus* previously (Frezzi, 1952; Davison & Tay, 1983; Shearer *et al.*, 1987; Crous *et al.*, 1989). However, the use of these species names in the disease reports emerged from identifications based on morphology. Thus, the importance of these species as pathogens is unclear and pathogenicity tests with the fungi in Venezuela will be needed to resolve this question.

Considerable problems have been experienced in distinguishing between *B. ribis* and *B. parva* based on morphology (Zhou & Stanosz, 2001; Slippers *et al.*, 2004b) and single locus DNA sequence comparisons (Slippers *et al.*, 2004a). It was, therefore, not

surprising that we experienced similar difficulty in distinguishing between these fungi (Clade II) in this study. Slippers *et al.* (2004b) separated these fungi based on conidial morphology, but expressed caution in using these characters alone. In that study, phylogenetic evidence from various gene regions combined (ITS rDNA, partial β-tubulin and translation elongation factor (EF) 1- α) were used to distinguish between isolates of these species.

We used an RFLP method to distinguish between *B. ribis* and *B. parva*. This method, using primers that amplify a microsatellite-containing region in both species (Slippers *et al.*, 2004a), has a unique restriction site for *B. ribis*. We consider our isolates distinguished in this way as representing *B. ribis sensu lato* and *B. parva sensu lato* (Slippers, 2003). This is because uncertainty remains as to whether the variation within these groups represents speciation events or population variation within species as discussed by Slippers (2003).

Botryosphaeria dothidea and its anamorph *F. aesculi* (Figs 11-15) is one of the most commonly reported species in *Botryosphaeria* (Smith *et al.*, 1994; Smith *et al.*, 1996; Ciesla *et al.*, 1996). *Dothiorella dothidea* has been reported as the anamorph of *B. dothidea* in Venezuela causing brown rot disease on peaches (*Prunus persica* (L.) Bastch. (Cedeño *et al.*, 1994). The conidial morphology for the isolates from peach was reported as fusoid to navicular and unicellular, which are characteristics very similar to those of isolates in the present study. *Botryosphaeria dothidea* was isolated only from *E. urophylla* and *Eucalyptus*-hybrids, where it originated from dieback symptoms and asymptomatic tissue. Like various other *Botryosphaeria* spp., it has been associated with diseases on a wide range of hosts (Smith *et al.*, 1994; Smith *et al.*, 1996), but the identification of the species prior to the use of DNA comparisons is uncertain.

Fusicoccum andinum prov. nom. and *F. stromaticum prov. nom.* were isolated and originally characterized from Venezuela (Mohali *et al.*, 2005). These fungi originate from branches and stems on *Eucalyptus*-hybrid, *E. urophylla* x *E. grandis* hybrids and *Acacia mangium*. Despite the fact that these fungi were isolated on these hosts, there is a possibility that they are present on other plants and that they have a wider distribution (Mohali *et al.*, 2005). These fungi appear to be adapted to the environmental conditions under which they occur in Venezuela and are absent from other studies on *Eucalyptus* and the other hosts studied here (Slippers *et al.*, 2004b, c).

Botryosphaeria rhodina was identified in this study from *Pinus*, *Acacia* and *Eucalyptus*. This fungus is well known in Venezuela and has been isolated from *Pinus caribaea* var. *hondurensis* Barret & Golfani, *Pinus oocarpa* Schiede, *Azadirachta indica* A. Juss, *Citrus aurantiifolia* L., *Citrus sinensis* (L.) Osbeck, and *Passiflora edulis* Sims f. *flavicarpa* Deg. It poses a serious threat to wood production in the country because it has been shown to cause blue stain, shoot blight and dieback on Caribbean pine (Cedeño & Palacios-Pru, 1992; Cedeño *et al.*, 1995, 1996; Mohali, 1993; Mohali & Encinas, 2001; Mohali *et al.*, 2002). It also causes mechanical damage and weakens the anatomical structures on wood of *Pinus caribaea* (Mohali, 1993; Cedeño *et al.*, 1996) and reduces the strength in tropical hardwoods of low density (Findlay & Petiffor, 1939; Findlay, 1959). Furthermore, *B. rhodina* produces cankers and kino exudation on *Eucalyptus grandis* Hill ex Maid plantations in Uganda (Roux *et al.*, 2001), and has been associated with root and stem diseases of *Eucalyptus* spp. in the Congo (Roux *et al.*, 2000). In young *Eucalyptus* plantations in the India, *B. rhodina* infects the host through wounds produced by termites, causing cankers during the warm and dry weather with high temperature (Sharma *et al.*, 1984). Unlike other species of *Botryosphaeria*, this fungus can easily be identified based on its very distinctive striated

conidia and this characteristic is also consistent with DNA based comparisons. It is probable that it is an important pathogen of the trees from which it was isolated in this study.

Botryosphaeria rhodina was the most abundant species identified during this study, and was isolated from all three hosts considered. The next most common species isolated, in order of decreasing abundance were, *B. mamane*, *B. dothidea*, *B. ribis*, *B. parva* and *Fusicoccum stromaticum prov. nom.*, *Fusicoccum andinum prov. nom.* was isolated from all the sampled *Eucalyptus* trees in the mountainous areas of Venezuela, but was absent from all other areas (Table 4).

The *Botryosphaeria* species composition on *Eucalyptus* in Venezuela is unique, compared with other recent studies on this host from South America, South Africa and eastern Australia, which used similar techniques. In Colombia, only *B. ribis* and *B. dothidea* were found to cause *Eucalyptus* diseases (Rodas, 2003). In Chile, South Africa and Australia, *B. parva*, *B. eucalyptorum* and *B. eucalypticola* are the most dominant species associated with *Botryosphaeria* canker and die-back diseases of *Eucalyptus* (Ahumada, 2003; Slippers *et al.*, 2004b, c). Thus, *Eucalyptus* in Venezuela share some pathogens with other *Eucalyptus* growing countries (e.g. the *B. parva* - *B. ribis* complex with all areas, and *B. dothidea* with Colombia). However some *Botryosphaeria* spp. from native *Eucalyptus* (e.g. *B. eucalyptorum* and *B. eucalypticola*) were not found; and the trees are infected by some unique species (e.g. *B. mamane*, *F. stromaticum prov. nom.* and *F. andinum prov. nom.*), which are probably native to Venezuela. These results confirm the importance of ongoing monitoring of *Botryosphaeria* spp. involved in causing specific disease symptoms in different areas and at different times, rather than extrapolating data from unrelated studies. It also shows that *Eucalyptus* plantations

across the world are at risk from both introduced pathogens on germplasm, as well as native pathogens that might expand their host range.

In this study, we have shown that there is a large number of *Botryosphaeria* spp. present on forest tree species in Venezuela. Given the fact that the study arose from a relatively limited sampling of woody host plants in the country, it seems likely that additional species will emerge as collections are expanded. Our results extend the geographic distribution of some *Botryosphaeria* spp. considerably and they will be used as a foundation to re-evaluate the importance of diseases associated with these fungi in Venezuela.

KEY TO ***BOTRYOSPHAERIA*** SPP. FROM VENEZUELA OCCURRING ON *EUCALYPTUS*, *ACACIA* AND *PINUS*

All *Botryosphaeria* spp. that have been described from Venezuela are included in this key. Although the key is based on anamorph morphology, teleomorph names are used where they are known. *Diplodia mutila* (Fries) Mont. (teleomorph: *B. stevensii* Shoemaker) is included because it was isolated in Venezuela and might resemble some of the species treated here. Data for *B. stevensii* are from previous studies (Mohali and Encinas, 2001; Alves *et al.*, 2004).

1. Conidia produced in culture thick-walled and often pigmented and/or striated with age; *Diplodia*-like anamorphs.....**2**

1. Conidia produced in culture mostly thin-walled and hyaline, only rarely pigmented and with slightly thickened walls; *Fusicoccum*-like anamorphs.....**3**

2. Conidia oblong, broadly rounded at apex, truncate at base, with irregular longitudinal striations when aged, 20-30 x 10-15 µm (average 24.5 x 12.8).....***B. rhodina***

2. Conidia smooth, cylindrical with broadly rounded ends, some with a large central guttule, with a thick glassy wall, 28-32 x 13-15 µm (average 25.3 × 13.2)....***B. stevensii***

3. Conidia in culture with average length ≥ 27 µm **4**

3. Conidia in culture with average length ≤ 25 µm **5**

4. Conidia fusiform, hyaline, aseptate to two septate, 21-52 x 4-8 µm (average 35.5 x 6.1), l/w 5.8 .....***B. mamane***

4. Conidia clavate to slightly navicular, hyaline, aseptate to one septate, 19-40 x 4-8 µm (average 27.1 x 5.6), l/w 4.84 .....***F. andinum***

5. Conidia fusiform to bacilliform, l/w ≥ 4.....**6**

5.Conidia ellipsoidal, l/w < 4.....7

6. Conidia narrowly fusiform with subobtuse apex, base subtruncate, hyaline, aseptate to two septate, 18-32 x 3-6 µm (average 23.4 x 4.9), l/w 4.7.....***B. dothidea***

6. Conidia mainly bacilliform, hyaline, slightly thickened walled, apex and base both bluntly rounded or just blunt, 19-24 x 4-6 µm (average 21.7 x 5.4), l/w 4.....***F. stromaticum***

7. Conidia 12-22 x 5-7 µm (average 17.2 x 5.6), l/w 3.7 .....***B. ribis***

7. Conidia 16-22 x 5-7 µm (average 18.6 x 5.8), l/w 3.2 .....***B. parva***

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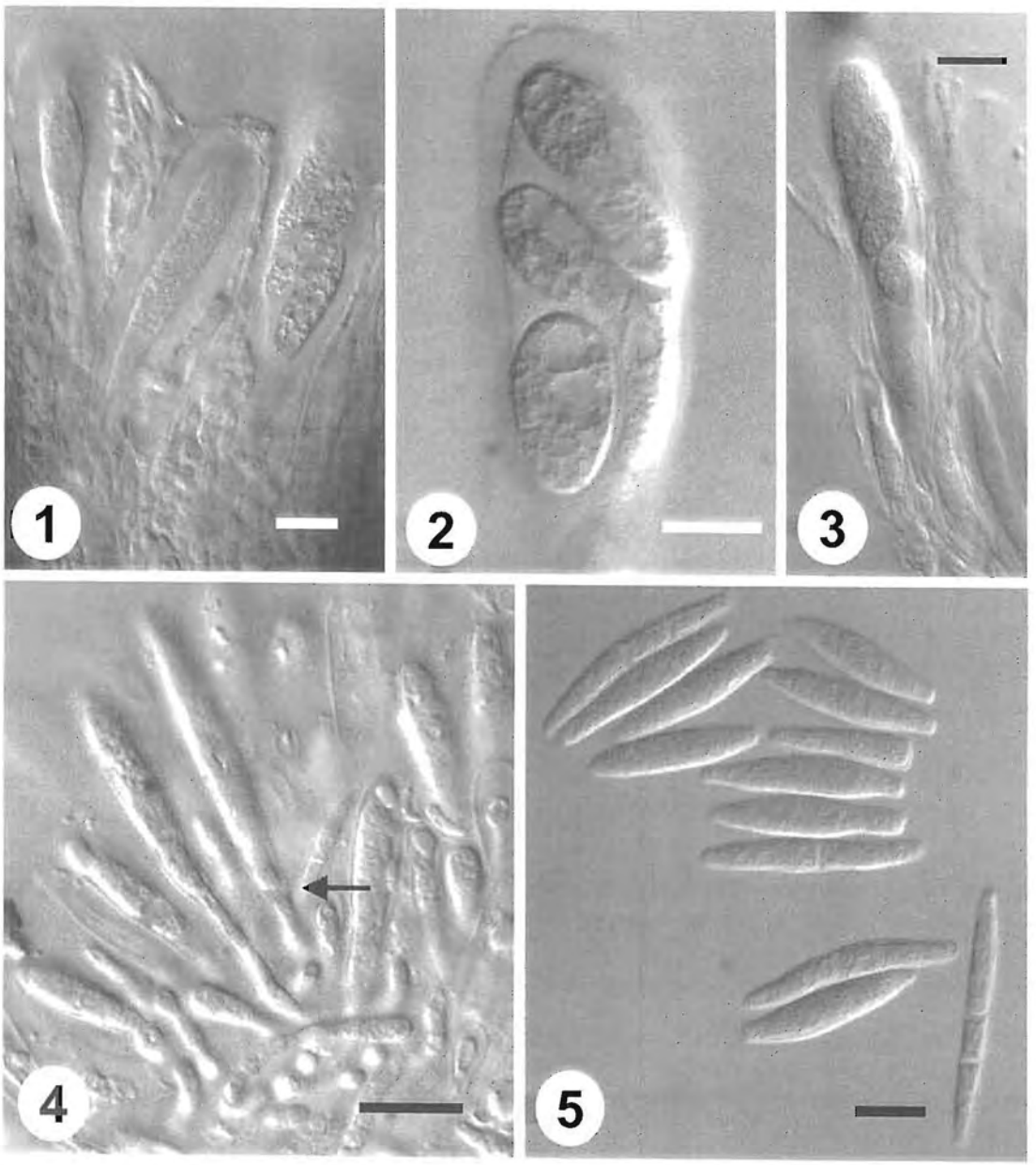
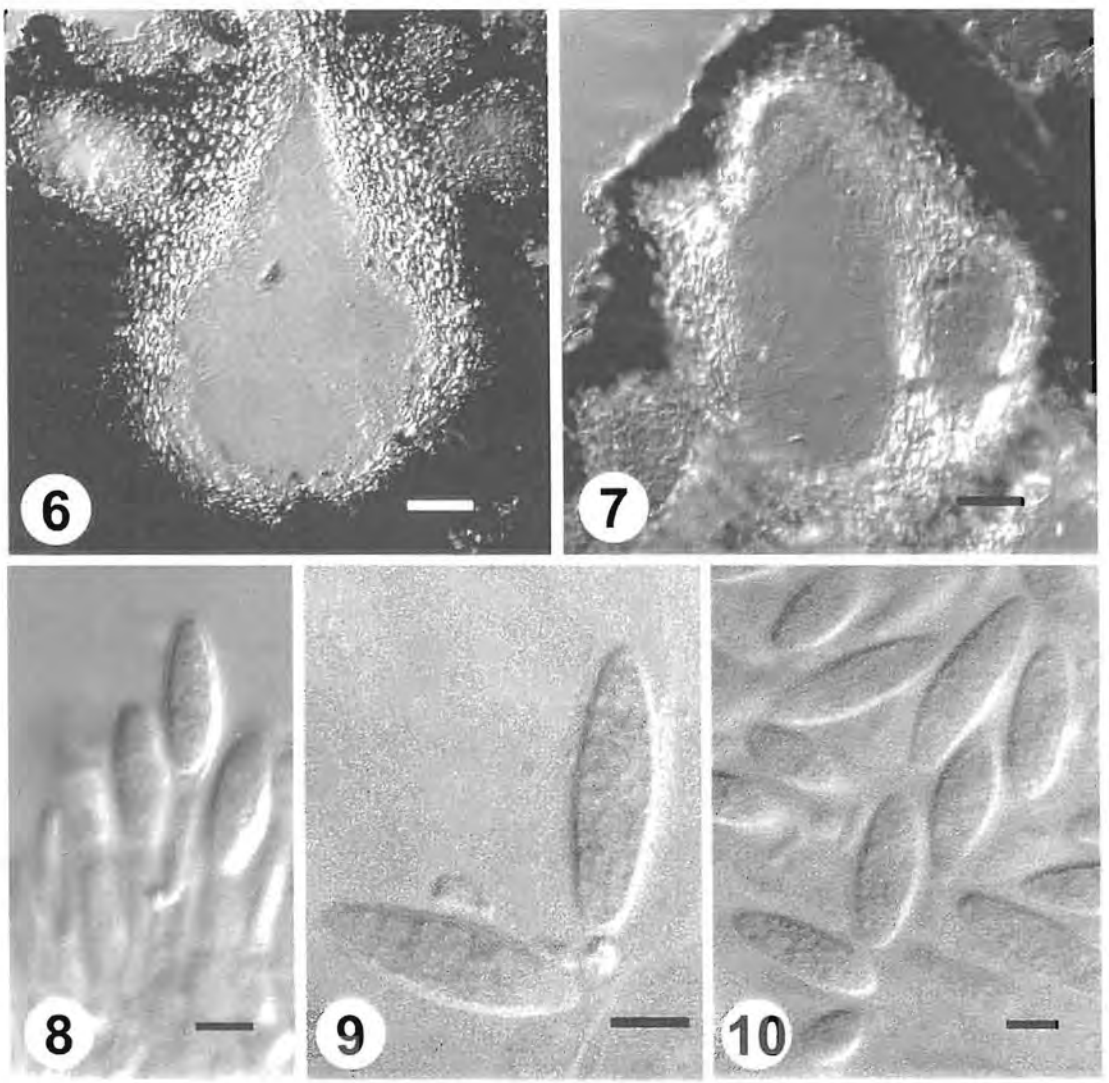
Figs 1-5. Microscopic characteristics of *Botryosphaeria mamane*. **Fig. 1.** Immature and mature asci. **Figs 2-3.** Mature ascus and ascospores with granular, textured contents. **Fig. 4.** Conidiogenous cells (arrow) with macroconidium. **Fig. 5.** Macroconidia with 0-2 septa. Bars = 10 µm.



Figs 6-10. Micrographs of structures for isolates in the *Botryosphaeria ribis-B. parva* complex. **Fig. 6.** Pyriform pycnidium with a short and acute papilla. **Fig. 7.** Globose pycnidium. Bars = 50 µm. **Figs 8-9.** Conidiogenous cells and macroconidia produced in culture on MEA (2 %) with pine needles. **Fig 10.** Macroconidia. Bars = 5 µm.



Figs 6-10. Micrographs of structures for isolates in the *Botryosphaeria ribis-B. parva* complex. **Fig. 6.** Pyriform pycnidium with a short and acute papilla. **Fig. 7.** Globose pycnidium. Bars = 50 µm. **Figs 8-9.** Conidiogenous cells and macroconidia produced in culture on MEA (2 %) with pine needles. **Fig 10.** Macroconidia. Bars = 5 µm.

Figs 11-15. Micrographs of structures of *Botryosphaeria dothidea* from Venezuela. **Fig. 11.** Globose pycnidium. Bar = 50 µm. **Fig. 12.** Section through pycnidium with conidia. Bar = 10 µm. **Figs 13-15.** Conidia with 0-2 septa produced in culture on MEA (2 %) with pine needles. Bars = 5 µm.

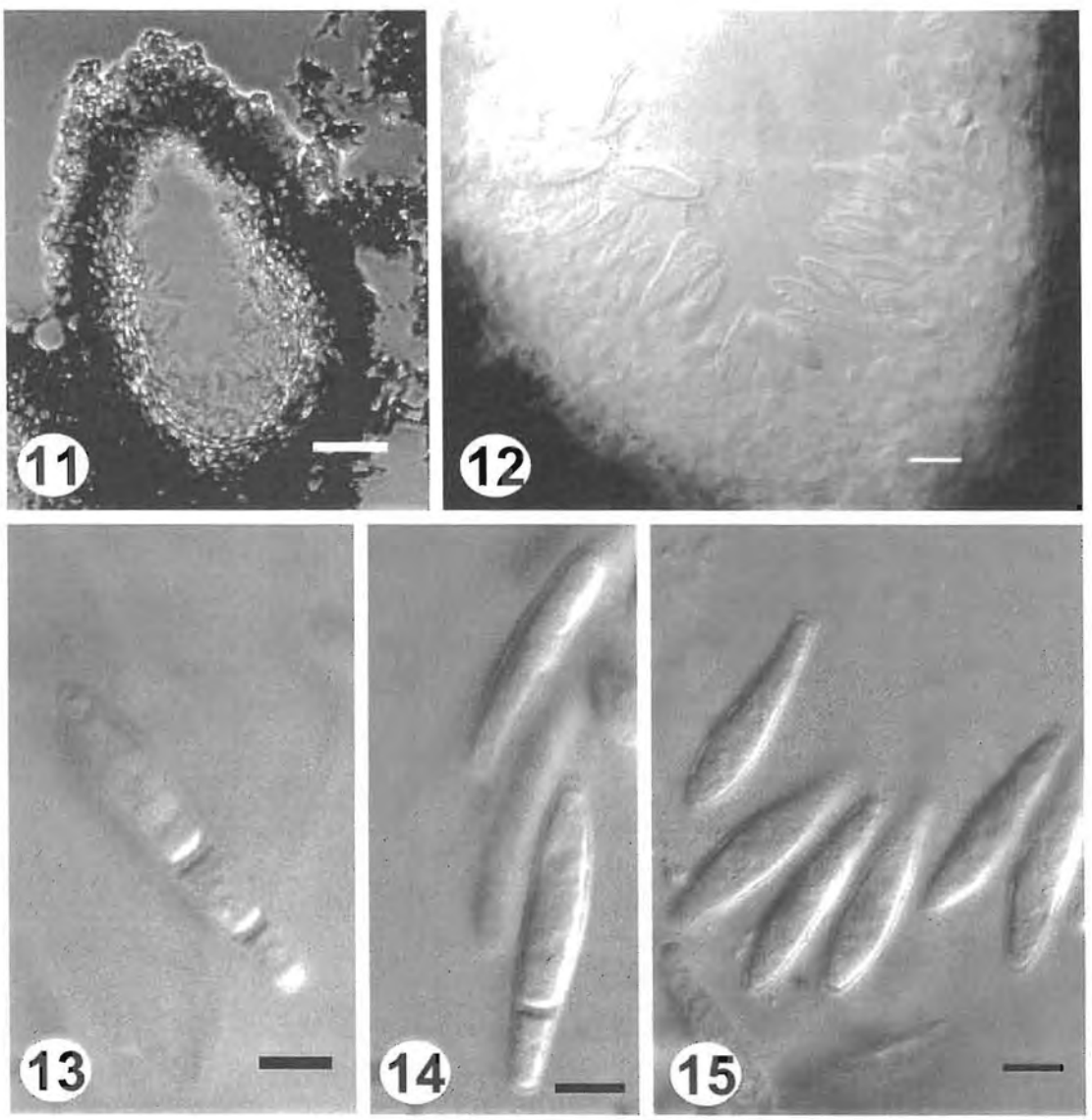


Fig. 16. Phylogenetic relationships among *Botryosphaeria* spp. from Venezuela and elsewhere (GenBank data) based on maximum parsimony analysis of ITS-1 and ITS-2 and 5.8S rDNA sequence data. The phylogram is rooted to the outgroup *Biomectria* sp. Bootstrap values greater than 50 % from 1000 replications of a heuristic search are indicated below internodes. Branch lengths proportional to the number of steps are indicated above internodes. Roman numerals indicate grouping of the different strains.

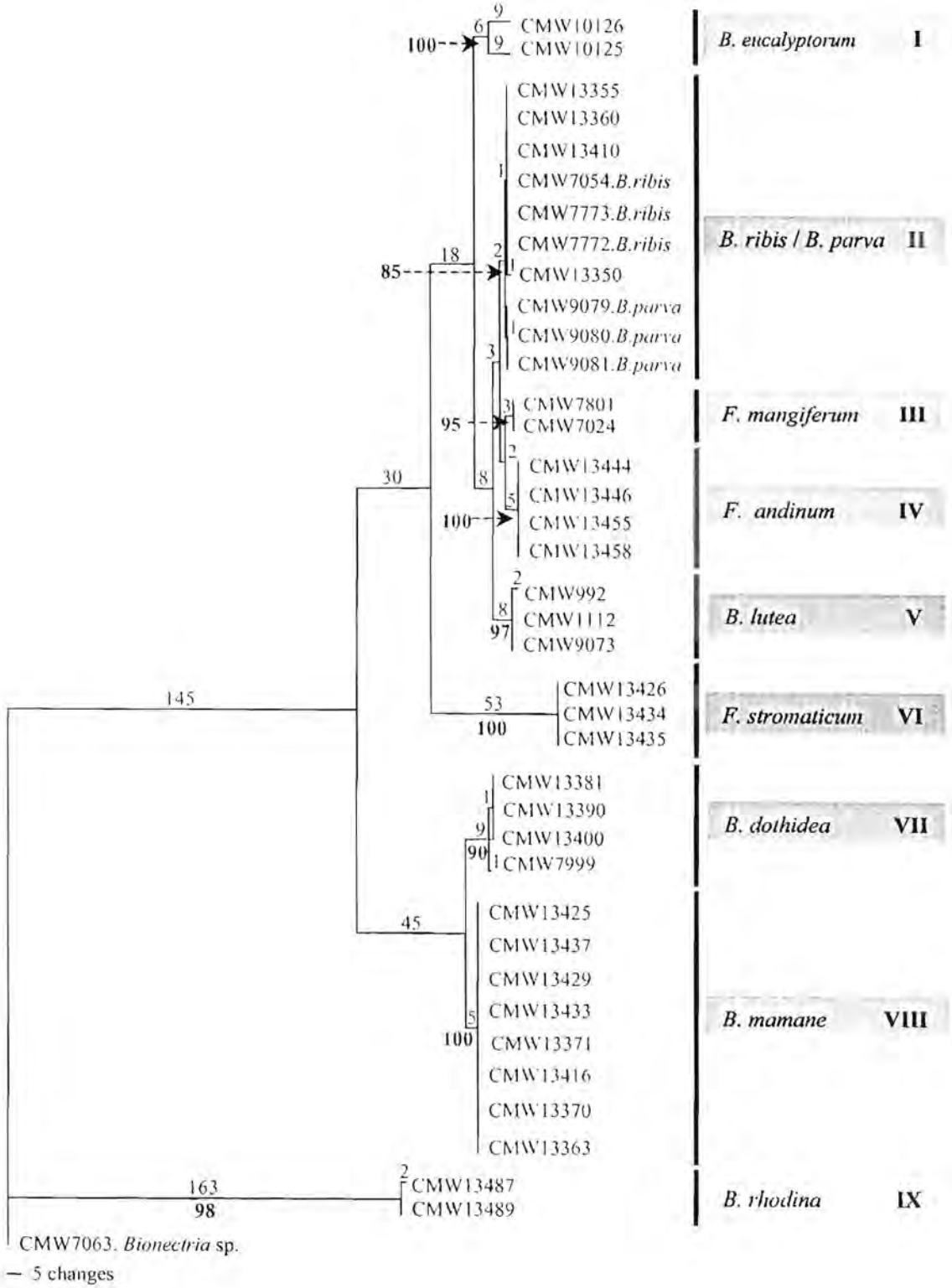


Fig. 17. Restriction fragments for the restriction enzyme *Cfo*I of the ITS-PCR products of different species of *Botryosphaeria* and its anamorphs (*Fusicoccum*) from Venezuela on a 3% agarose gel stained with ethidium bromide. Lane one contains a 100 bp size marker.

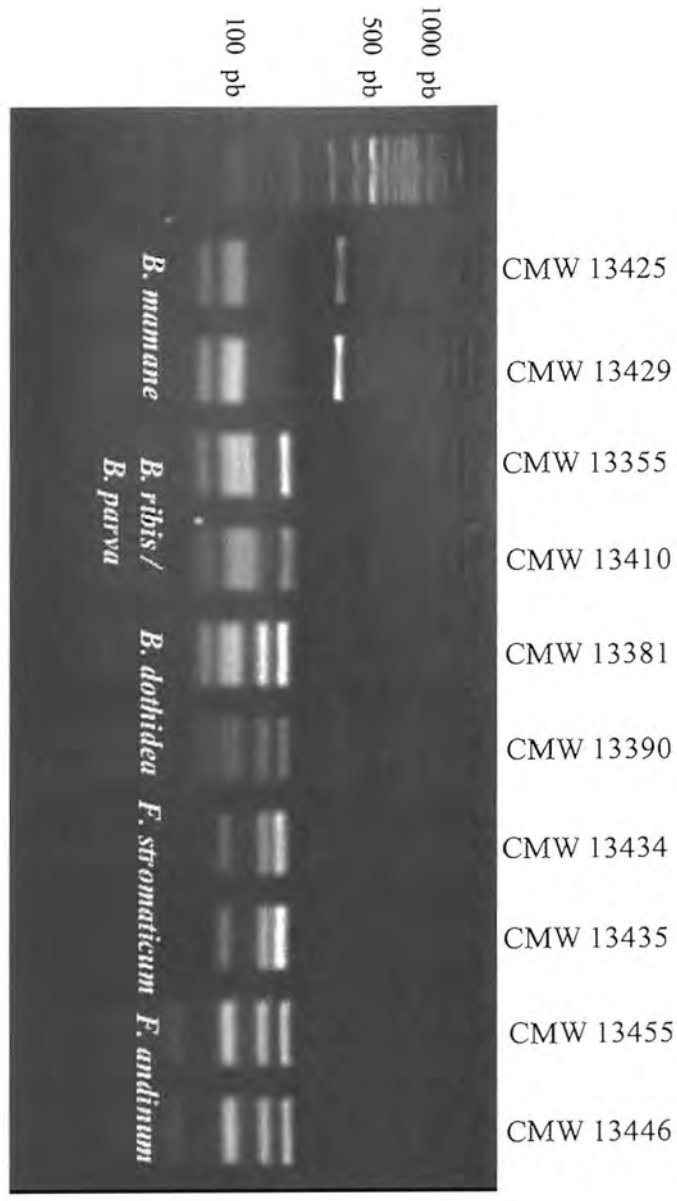


Fig. 18. Restriction maps for the restriction enzyme *Cfo*I to the ITS rDNA regions of different species of *Botryosphaeria* from Venezuela. Fragment sizes (numbers indicate sizes in bp) were inferred from sequence data.

***Cfo*I**

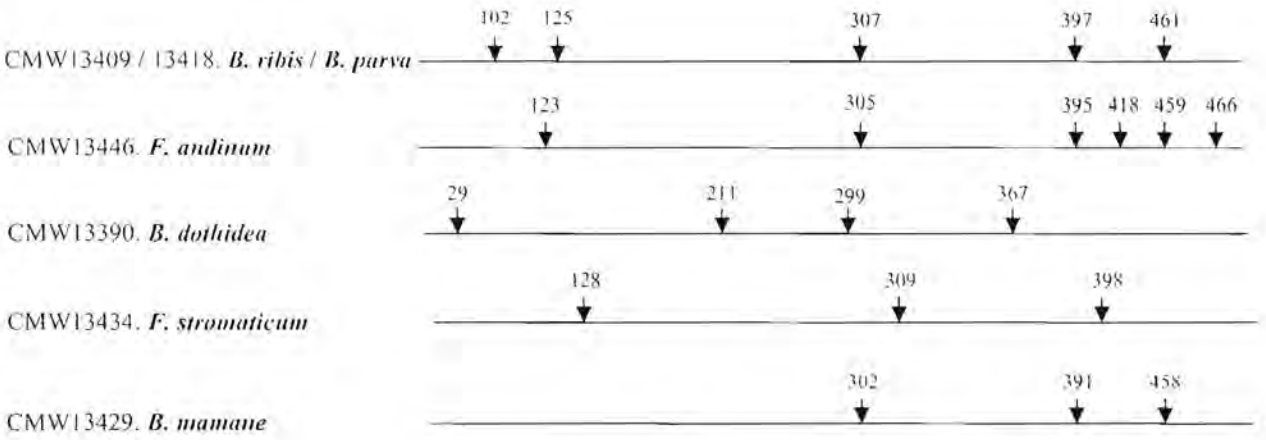


Fig. 19. PCR products (obtained with primers BOT15 & BOT16 (Slippers *et al.* 2004a) for isolates of *B. parva* and *B. ribis* that were digested with the restriction enzyme *Cfo*I, visualized on 2% agarose gel stained with ethidium bromide. The last lane represents a 100 bp size marker.

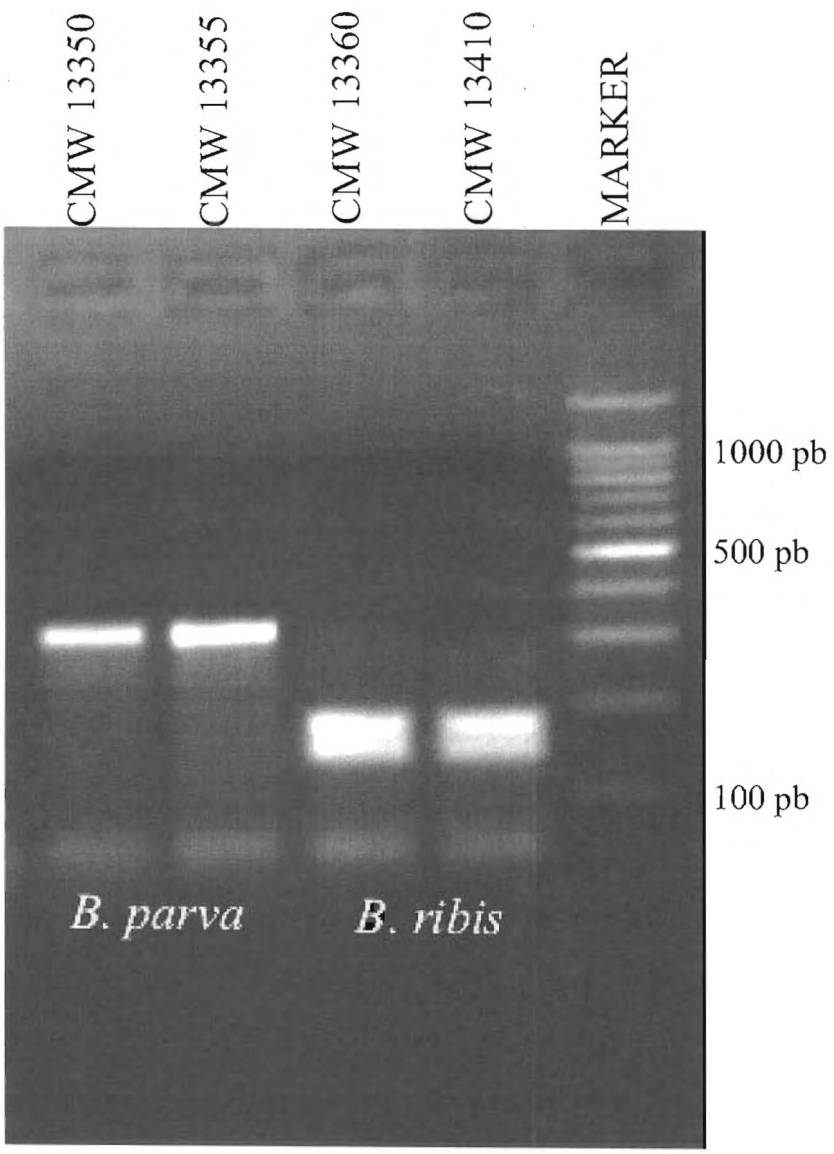

CMW 13350
CMW 13355
CMW 13360
CMW 13410
MARKER
1000 pb
500 pb
100 pb
B. parva
B. ribis

Table 1. Isolates of *Botryosphaeria* spp. from Venezuela considered in this study.

Host	Number of isolates	Location
Eucalyptus urophylla x *E. grandis*	78	Portuguesa state
Acacia mangium	16	Portuguesa state
Eucalyptus-hybrids	32	Cojedes state
A. mangium	25	Cojedes state
Eucalyptus sp.	20	Mountain range, Mérida state
Pinus caribaea var. *hondurensis*	32	Falcon state
Psidium guajava L.	1	Zulia state

Table 2. Isolates considered in the phylogenetic study in *Botryosphaeria*.

Culture No[1]	Other No[1]	Identity	Host	Location	Collector	GenBank[2]
CMW10126	BOT16	*Botryosphaeria eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, South Africa	H. Smith	AF283687
CMW10125	BOT24	*B. eucalyptorum*	*E. grandis*	Mpumalanga, South Africa	H. Smith	AF283686
CMW992		*B. lutea*	*Actinidia deliciosa*	New Zealand	G. J. Samuels	AF027745
CMW1112		*Fusicoccum luteum*	*Widdringtonia nodiflora*	South Africa	W. Swart	
CMW9073		*F. luteum*	*Acacia mearnsii*	Australia	J. Roux	
CMW13355	CBS117915	*B. parva*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW9079	ICMP7933	*B. parva*	*A. deliciosa*	New Zealand	S. R. Pennycook	AY236941
CMW13350	CBS117923	*B. parva*	*Psidium guajava*	Zulia state, Venezuela	L. Cedeño	
CMW9080	ICMP8002	*B. parva*	*Populus nigra*	New Zealand	G. J. Samuels	AY236942
CMW9081	ICMP8003	*B. parva*	*P. nigra*	New Zealand	G. J. Samuels	AY236943
CMW13360	CBS117916	*B. ribis*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13410	CBS117443	*B. ribis*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW7054	CBS121	*B. ribis*	*Ribes rubrum*	New York, USA	N. E. Stevens	AF241177
CMW7772		*B. ribis*	*Ribes sp.*	New York, USA	B. Slippers/ G. Hudler	AY236935
CMW7773		*B. ribis*	*Ribes sp*	New york, USA	B. Slippers/ G. Hudler	AY236936
CMW7801	BRIP23396	*F. mangiferum*	*Mangiferae indica*	Australia	G. I. Johnson	
CMW7024	BRIP24101	*F. mangiferum*	*M. indica*	Australia	G. I. Jonson	
CMW13444	CBS 117451	*F. andinum*	*Eucalyptus* sp.	Mérida state, Venezuela	S. Mohali	
CMW13446	CBS 117452	*F. andinum*	*Eucalyptus* sp	Mérida state, Venezuela	S. Mohali	
CMW13455	CBS 117453	*F. andinum*	*Eucalyptus* sp	Mérida state, Venezuela	S. Mohali	AY693976
CMW13458	CBS117921	*F. andinum.*	*Eucalyptus* sp.	Mérida state, Venezuela	S. Mohali	
CMW13381	CBS117918	*B. dothidea*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13390	CBS117919	*B. dothidea*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13400	CBS 117442	*B. dothidea*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW7999		*B. dothidea*	*Osteya* sp.	Crocifisso, Switzerland	B. Slippers	AY236948
CMW13425	CBS 117445	*B. mamane*	*Acacia mangium*	Portuguesa state, Venezuela	S. Mohali	
CMW13437	CBS 117450	*B. mamane*	*A. mangium*	Cojedes state, Venezuela	S. Mohali	
CMW13429	CBS 117446	*B. mamane*	*Eucalyptus-hybrid*	Cojedes state, Venezuela	S. Mohali	
CMW13433	CBS 117447	*B. mamane*	*Eucalyptus-hybrid*	Cojedes state, Venezuela	S. Mohali	

Table 2. Continued.

Culture No[1]	Other No[1]	Identity	Host	Location	Collector	GenBank[2]
CMW13416	CBS117444	*B. mamane*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13370	CBS117917	*B. mamane*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13363	CBS118624	*B. mamane*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13371		*B. mamane*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13487		*B. rhodina*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13489	CBS117922	*B. rhodina*	*E. urophylla* x *E. grandis*	Portuguesa state, Venezuela	S. Mohali	
CMW13426	PREM58513	*Fusicoccum stromaticum*	*A. mangium*	Portuguesa state, Venezuela	S. Mohali	
CMW13434	CBS 117448	*F. stromaticum*	*Eucalyptus-hybrid*	Cojedes state, Venezuela	S. Mohali	AY693974
CMW13435	CBS 117449	*F. stromaticum*	*Eucalyptus-hybrid*	Cojedes state, Venezuela	S. Mohali	
CMW7063		*Bionectria* sp.	Unknown	Netherlands	H. A. van der Aa	AY236956

[1] Culture collection and isolate abbreviations: CMW = Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; BRIP = Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia.

[2] Sequences obtained from GenBank.

Table 3. Conidia (*in vitro*) measurements and descriptions for *Botryosphaeria* species from Venezuela.

	B. mamane	*B. ribis*	*B. parva*	*B. dothidea*	*B. rhodina*	*F. andinum*	*F. stromaticum*
Size	(21-) 28-43 (-52) x (4-) 5-7 (-8)	(12-) 15-20 (-22) x 5-6 (-7)	(16-) 17-20 (-22) x 5-6 (-7)	(18-) 21-26 (-32) x (3-) 4-6	(20-) 23-27 (-30) x (10-)12-15	(19-) 23-31 (-40) x (4-) 5-6 (-8)	(19-) 20-23 (-24) x (4-) 5-6
Average	35.5 x 6.1	17.2 x 5.6	18.6 x 5.8	23.4 x 4.9	24.5 x 12.8	27.1 x 5.6	21.7 x 5.4
L/W	5.8	3.7	3.2	4.7	1.9	4.84	4.01
Shape	Fusiform, straight or lightly curved, with truncated base.	Fusiform to ellipsoidal with apex round and base subtruncate.	Fusiform to ellipsoidal with apex round and base subtruncate	Narrowly fusiform with subobtuse apex, base subtruncate or round base.	Ellipsoid or oblong, straight.	Clavate to slightly navicular, apex obtuse and base truncate.	Mainly bacilliform, straight to slightly curved, apex and base both bluntly rounded or just blunt.
Description	0-2 septa and hyaline	Unicellular, hyaline, smooth with granular contents	Unicellular, hyaline, smooth with granular contents	0-2 septa, hyaline, smooth with granular contents	At first hyaline and aseptate becoming dark brown and one-septate with irregular longitudinal striations, broadly rounded at apex, truncate at base.	0-1 septa, hyaline and granular contents.	Aseptate, hyaline, thin to slightly thickened walled, granular contents.

Table 4. Identification of *Botryosphaeria* species in Venezuela, based on the combined results of the morphological, DNA sequence and RFLP analysis of all the 204 isolates.

Fungi	Host	No of Isolates	Location
Botryosphaeria mamane	*E. urophylla* x *E. grandis*, *Eucalyptus*-hybrid, *Acacia mangium*, *Dipterix punctata*.	41	Portuguesa, Barinas and Cojedes states.
B. dothidea	*E. urophylla* x *E. grandis*, *Eucalyptus*-hybrid	24	Portuguesa and Cojedes states.
B. ribis	*E. urophylla* x *E. grandis*	16	Portuguesa state
B. parva	*E. urophylla* x *E. grandis*, *Psidium guajava*	07	Portuguesa and Zulia states
B. rhodina	*Pinus caribaea* var. *hondurensis*, *E. urophylla* x *E. grandis*, *A. mangium*.	89	Falcon, Portuguesa and Cojedes state
Fusicoccum andinum	*Eucalyptus* sp.	20	Mountain range, Mérida state
F. stromaticum	*E. urophylla* x *E. grandis*, *Eucalyptus*-hybrids, *A. mangium*.	07	Portuguesa and Cojedes state