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## Identification of *Botryosphaeriaceae* from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela

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Species of the *Botryosphaeriaceae* are pathogens of many plantation trees, including species of *Acacia*, *Eucalyptus* and *Pinus*. Some anamorphs associated with the *Botryosphaeriaceae* have been reported from Venezuela, but their identification is not certain. The aim of this study was to identify *Botryosphaeriaceae* affecting plantations of *Acacia*, *Eucalyptus* 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, *Botryosphaeria dothidea*, *B. mamane*, the *Neofusicoccum ribis* / *N. parvum* complex, *N. andinum*, *Lasiodiplodia theobromae*, and *Pseudofusicoccum stromaticum* were identified. To discriminate between isolates residing in the *Neofusicoccum ribis*-*N. parvum* 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 *Botryosphaeria mamane* outside Hawaii and the first records of *B. dothidea*, *Neofusicoccum parvum* and *N. ribis* in Venezuela.

**Key words:** *Acacia mangium*, *Botryosphaeria*, *Eucalyptus urophylla*, *Lasiodiplodia*, *Neofusicoccum*, *Pinus caribaea* var. *hondurensis*, *Pseudofusicoccum*.

### Introduction

Species of the *Botryosphaeriaceae* have a cosmopolitan distribution and occur on a wide range of monocotyledonous, dicotyledonous and gymnospermous 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). Species of the *Botryosphaeriaceae* are generally regarded as weak pathogens that invade

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stressed or wounded plants after drought, hail, wind, frost or insect damage (Crist and Schoeneweiss, 1975; Smith *et al.*, 1994; Crous *et al.*, 1989; Ciesla *et al.*, 1996). It has also been shown that the *Botryosphaeriaceae* occur in asymptomatic tissue as latent pathogens in trees such as *Eucalyptus*, *Pinus* and *Syzygium* (Swart and Wingfield, 1991; Smith *et al.*, 1996a, b; Pavlic *et al.*, 2004).

The taxonomy of the *Botryosphaeriaceae* has been confused for many years. 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 had been found (Cesati and De Notaris, 1863; De Notaris, 1863; Saccardo, 1877, 1882; Putterill, 1919; Trotter, 1928). The resulting taxonomic confusion has also impacted negatively on the understanding of diseases caused by the *Botryosphaeriaceae*.

Identification of species in the *Botryosphaeriaceae* causing diseases has largely been dependent on the taxonomy of the anamorphs, which represent the most frequently found state (Jacobs and Rehner, 1998; Denman *et al.*, 2000; Smith and Stanosz, 2001). The morphological characteristics of the anamorphs that are considered useful for identification include conidial size, shape, color and septation (Pennycook and Samuels, 1985; Jacobs and 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 and Samuels, 1985).

Since the 1990's significant advances have been made in the identification of species in the *Botryosphaeriaceae* 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 the *Botryosphaeriaceae* (Jacobs and Rehner, 1998; Smith *et al.*, 2001; Smith and Stanosz, 2001; Denman *et al.*, 2000, Zhou and Stanosz, 2001; Slippers *et al.*, 2004b; Alves *et al.*, 2006; Phillips *et al.*, 2006). Thus, for the first time, a relatively robust taxonomy is emerging for the *Botryosphaeriaceae* and this is already leading to a deeper understanding of host pathogen relationships and geographic distribution of species.

Very little is known regarding the *Botryosphaeriaceae* in Venezuela. A number of *Botryosphaeriaceae* 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 and

Palacios-Pru, 1992; Cedeño *et al.*, 1994, 1995, 1996; Mohali, 1993, 1997; Mohali and Encinas, 2001; Mohali *et al.*, 2002). Identifications of these fungi, originating from disease symptoms on both agricultural crops and forest trees, were based on conidial morphology. Many of these *Botryosphaeriaceae* are thought to be important pathogens in Venezuela and their correct identification is desired.

The aim of this study was to characterise species in the *Botryosphaeriaceae* and their anamorphs found on important forest plantation trees in Venezuela. *Botryosphaeriaceae* were isolated from *Acacia mangium* Willd., *Eucalyptus* spp. and *Pinus caribaea* Morelet var. *hondurensis* (Sénécl.) W.H.G. Barrett & 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*

*Botryosphaeriaceae* 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 the mountain ranges (Cordillera of Los Andes), Mérida state, and thirty two isolates were from *Pinus caribaea* var. *hondurensis* in a seedling orchard in Falcon state and one isolate made 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, die-back and from entirely dead trees. Plant tissue was surface disinfected in 70% ethanol for 30 seconds, after which it was rinsed in sterile water for 1 minute. Pieces of tissue were cut from the specimens and placed on 2% malt extract agar (MEA) (2% DIFCO, Becton Dickinson, MD, USA) at 25°C and stored on this medium at 4°C. Isolates were characterized 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

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

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

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 *Botryosphaeriaceae* were made 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 and amplification***

A modified phenol-chloroform protocol based on Reader and Broda (1985) was used to isolate DNA from all the fungi as described in Slippers *et al.* (2004b). The dissolved samples were treated with 5 µl RNase (1mg/ml) and left at 37°C overnight to degrade all RNA. Nucleic acids were quantified using a spectrophotometer with an absorbance at 260 nm and 280 nm (OD<sub>260</sub>: OD<sub>280</sub>).

The extracted DNA was used as template to amplify a part of the nuclear rRNA operon in PCR reactions using the primers ITS1 and ITS4 (White *et al.*, 1990). The amplified fragments included the 3' end of the small subunit (SSU) rRNA gene, the internal transcribed spacer ITS (ITS1), the complete 5.8S rRNA gene, the second region ITS2 and the 5' end of the large subunit (LSU) rRNA gene. PCR reactions and conditions were as described before (Mohali *et al.*, 2005). PCR amplicons were separated on 1.5% (w/v) agarose gels, stained

with ethidium bromide and visualized under UV light. 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 *Botryosphaeriaceae*. 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, Alameda, 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 and Huelsenbeck, 1992) were determined.

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

| Culture No <sup>1</sup> | Other No <sup>1</sup> | Identity                          | Host                                    | Location                    | Collector                 | GenBank <sup>2</sup> |
|-------------------------|-----------------------|-----------------------------------|---|-----------------------------|---------------------------|----------------------|
| CMW10126                | BOT16                 | <i>Neofusicoccum eucalyptorum</i> | <i>Eucalyptus grandis</i>               | Mpumalanga, South Africa    | H. Smith                  | AF283687             |
| CMW10125                | BOT24                 | <i>N. eucalyptorum</i>            | <i>E. grandis</i>                       | Mpumalanga, South Africa    | H. Smith                  | AF283686             |
| CMW992                  |                       | <i>N. luteum</i>                  | <i>Actinidia deliciosa</i>              | New Zealand                 | G.J. Samuels              | AF027745             |
| CMW1112                 |                       | <i>N. australe</i>                | <i>Widdringtonia nodiflora</i>          | South Africa                | W. Swart                  | AY615167             |
| CMW9073                 |                       | <i>N. australe</i>                | <i>Acacia mearnsii</i>                  | Australia                   | J. Roux                   | AY339261             |
| CMW13355                | CBS117915             | <i>N. parvum</i>                  | <i>E. urophylla</i>                     | Portuguesa state, Venezuela | S. Mohali                 | EF118035             |
| CMW9079                 | ICMP7933              | <i>N. parvum</i>                  | <i>A. deliciosa</i>                     | New Zealand                 | S.R. Pennycook            | AY236941             |
| CMW13350                | CBS117923             | <i>N. parvum</i>                  | <i>Psidium guajava</i>                  | Zulia state, Venezuela      | L. Cedeño                 | EF118036             |
| CMW9080                 | ICMP8002              | <i>N. parvum</i>                  | <i>Populus nigra</i>                    | New Zealand                 | G.J. Samuels              | AY236942             |
| CMW9081                 | ICMP8003              | <i>N. parvum</i>                  | <i>P. nigra</i>                         | New Zealand                 | G.J. Samuels              | AY236943             |
| CMW13360                | CBS117916             | <i>N. ribis</i>                   | <i>E. urophylla</i>                     | Portuguesa state, Venezuela | S. Mohali                 | EF118037             |
| CMW13410                | CBS117443             | <i>N. ribis</i>                   | <i>E. urophylla</i>                     | Portuguesa state, Venezuela | S. Mohali                 | EF118038             |
| CMW7054                 | CBS121                | <i>N. ribis</i>                   | <i>Ribes rubrum</i>                     | New York, USA               | N.E. Stevens              | AF241177             |
| CMW7772                 |                       | <i>N. ribis</i>                   | <i>Ribes sp.</i>                        | New York, USA               | B. Slippers/<br>G. Hudler | AY236935             |
| CMW7773                 |                       | <i>N. ribis</i>                   | <i>Ribes sp.</i>                        | New York, USA               | B. Slippers/<br>G. Hudler | AY236936             |
| CMW7801                 | BRIP23396             | <i>N. mangiferum</i>              | <i>Mangiferae indica</i>                | Australia                   | G.I. Johnson              | AY615187             |
| CMW7024                 | BRIP24101             | <i>N. mangiferum</i>              | <i>M. indica</i>                        | Australia                   | G.I. Johnson              | AY615185             |
| CMW13444                | CBS 117451            | <i>N. andinum</i>                 | <i>Eucalyptus sp.</i>                   | Mérida state, Venezuela     | S. Mohali                 | EF118039             |
| CMW13446                | CBS 117452            | <i>N. andinum</i>                 | <i>Eucalyptus sp.</i>                   | Mérida state, Venezuela     | S. Mohali                 | DQ306263             |
| CMW13455                | CBS 117453            | <i>N. andinum</i>                 | <i>Eucalyptus sp.</i>                   | Mérida state, Venezuela     | S. Mohali                 | AY693976             |
| CMW13458                | CBS117921             | <i>N. andinum</i>                 | <i>Eucalyptus sp.</i>                   | Mérida state, Venezuela     | S. Mohali                 | EF118040             |
| CMW13381                | CBS117918             | <i>B. dothidea</i>                | <i>E. urophylla</i> x <i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali                 | EF118043             |
| CMW13390                | CBS117919             | <i>B. dothidea</i>                | <i>E. urophylla</i> x <i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali                 | EF118044             |

**Table 2 continued.** Isolates considered in the phylogenetic study in *Botryosphaeriaceae*.

| Culture No <sup>1</sup> | Other No <sup>1</sup>    | Identity   | Host                                       | Location                    | Collector        | GenBank <sup>2</sup> |
|-------------------------|--------------------------|--|--|-----------------------------|------------------|----------------------|
| CMW13400                | CBS<br>117442            | <i>B. dothidea</i>                                       | <i>E. urophylla</i> x <i>E. grandis</i>    | Portuguesa state, Venezuela | S. Mohali        | EF118045             |
| CMW7999                 |                          | <i>B. dothidea</i>                                       | <i>Ostrya</i> sp.                          | Crocifisso, Switzerland     | B. Slippers      | AY236948             |
| CMW13425                | CBS<br>117445            | <i>B. mamane</i>   | <i>Acacia mangium</i>                      | Portuguesa state, Venezuela | S. Mohali        | EF118046             |
| CMW13437                | CBS<br>117450            | <i>B. mamane</i>   | <i>A. mangium</i>                          | Cojedes state, Venezuela    | S. Mohali        | EF118047             |
| CMW13429                | CBS<br>117446            | <i>B. mamane</i>   | <i>Eucalyptus</i> -<br><i>hybrid</i>       | Cojedes state, Venezuela    | S. Mohali        | EF118048             |
| CMW13433                | CBS<br>117447            | <i>B. mamane</i>   | <i>Eucalyptus</i> -<br><i>hybrid</i>       | Cojedes state, Venezuela    | S. Mohali        | EF118049             |
| CMW13416                | CBS<br>117444            | <i>B. mamane</i>   | <i>E. urophylla</i> x<br><i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali        | EF118050             |
| CMW13370                | CBS<br>117917            | <i>B. mamane</i>   | <i>E. urophylla</i> x<br><i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali        | EF118051             |
| CMW13363                | CBS<br>118624<br>ZS97-59 | <i>B. mamane</i>   | <i>E. urophylla</i> x<br><i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali        | EF118052             |
|                         |                          | <i>B. mamane</i>   | <i>Sophora</i><br><i>chrysophylla</i>      | Hawaii                      | D. Gardner       | AF246930             |
| CMW13487                |                          | <i>L. theobromae</i>                                     | <i>E. urophylla</i> x<br><i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali        | EF118053             |
| CMW13489                | CBS<br>117922            | <i>L. theobromae</i>                                     | <i>E. urophylla</i> x<br><i>E. grandis</i> | Portuguesa state, Venezuela | S. Mohali        | DQ103525             |
| CMW13426                | PREM<br>58513            | <i>Pseudofusicoc</i><br><i>cum</i><br><i>stromaticum</i> | <i>A. mangium</i>                          | Portuguesa state, Venezuela | S. Mohali        | EF118041             |
| CMW13434                | CBS<br>117448            | <i>P.</i><br><i>stromaticum</i>                          | <i>Eucalyptus</i> -<br><i>hybrid</i>       | Cojedes state, Venezuela    | S. Mohali        | AY693974             |
| CMW13435                | CBS<br>117449            | <i>P.</i><br><i>stromaticum</i>                          | <i>Eucalyptus</i> -<br><i>hybrid</i>       | Cojedes state, Venezuela    | S. Mohali        | DQ436935             |
| CMW7063                 |                          | <i>Bionectria</i> sp.                                    | Unknown                                    | Netherlands                 | H. A. van der Aa | AY236956             |

<sup>1</sup>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.

<sup>2</sup> Sequences obtained from GenBank.

### **Restriction analysis**

An 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 showed that the enzyme could separate all the species of *Botryosphaeriaceae* from *Eucalyptus* and *Acacia* in

Venezuela, except *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips.

Restriction analysis of the amplified ITS regions was consequently done using the RE *CfoI* (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 N. ribis-N. parvum complex***

Restriction patterns for an unidentified DNA Locus *BotF15*-(Slippers *et al.*, 2004a) were used to distinguish cryptic species residing in *N. ribis-N. parvum* 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 *CfoI* 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 pycnidia or conidiomata on sterilized pine needles on WA after two weeks. The *Botryosphaeriaceae* 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 *Botryosphaeria mamane* Gardner (Figs 1-5), *Lasiodiplodia theobromae*, the *Neofusicoccum ribis-N. parvum* complex (Slippers *et al.*, 2004b) (Figs 6-10), *Botryosphaeria dothidea* (Moug. ex Fries) Ces. & de Not. (Figs 11-15),



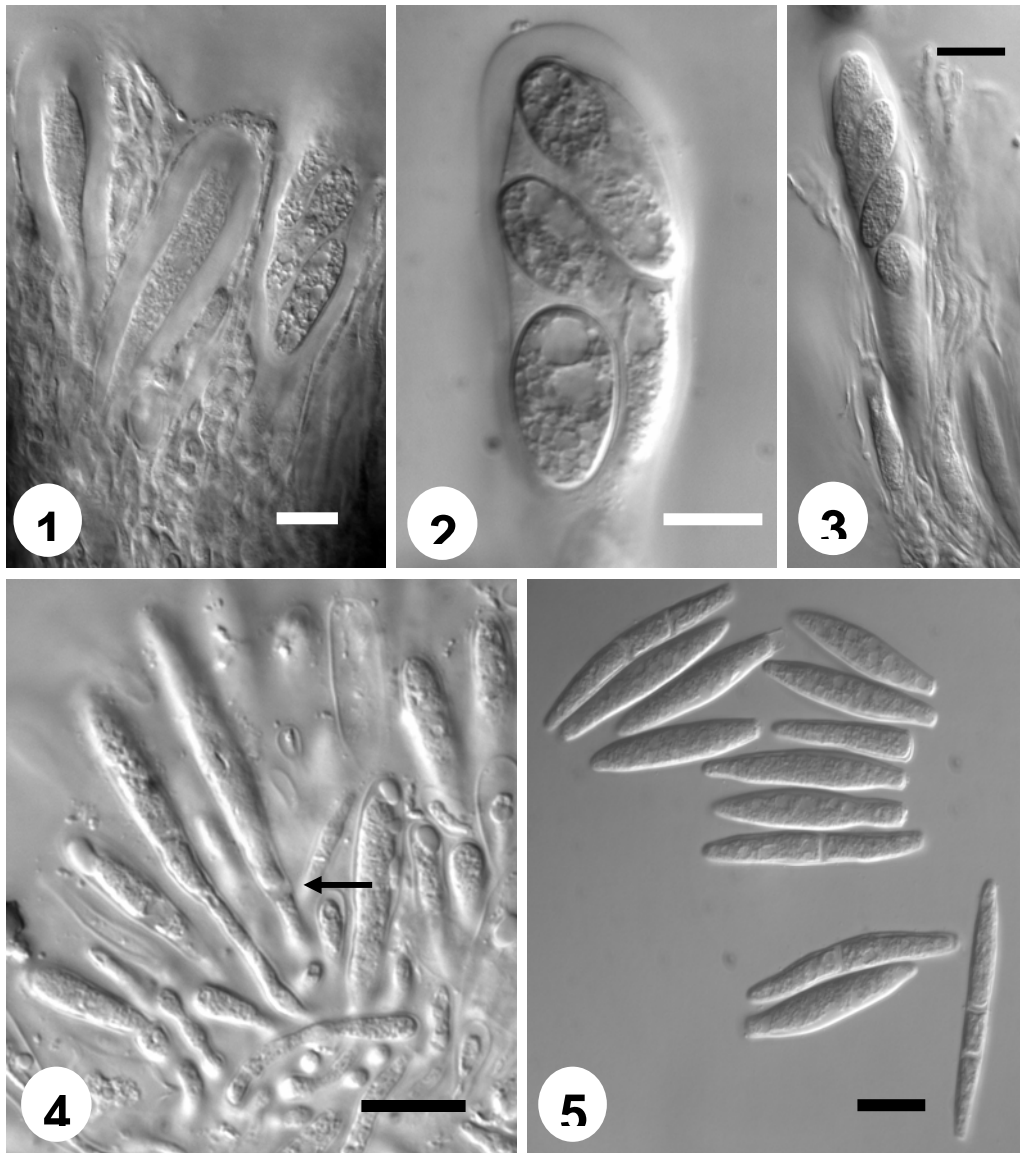
**Table 3.** Conidia (*in vitro*) measurements and descriptions for species of *Botryosphaeriaceae* from Venezuela.

|             | <i>B. mamane</i>  | <i>B. dothidea</i>  | <i>N. ribis</i>  | <i>N. parvum</i>  | <i>L. theobromae</i>  | <i>N. andinum</i>   | <i>P. stromaticum</i>  |
|-------------|---|---|--|---|---|---|--|
| Size        | (21-) 28-43<br>(-52) × (4-)<br>5-7 (-8)                                       | (18-) 21-26<br>(-32) × (3-)<br>4-6  | (12-) 15-20<br>(-22) × 5-6<br>(-7)   | (16-) 17-20<br>(-22) × 5-6<br>(-7)  | (20-) 23-27<br>(-30) × (10-)<br>12-15   | (19-) 23-<br>31 (-40) ×<br>(4-) 5-6 (-<br>8)                                    | (19-) 20-23<br>(-24) × (4-)<br>5-6   |
| Average     | 35.5 × 6.1  | 23.4 × 4.9  | 17.2 × 5.6   | 18.6 × 5.8  | 24.5 × 12.8   | 27.1 × 5.6  | 21.7 × 5.4   |
| L/W         | 5.8   | 4.7   | 3.7  | 3.2   | 1.9   | 4.84  | 4.01   |
| Shape       | Fusiform,<br>straight or<br>slightly<br>curved,<br>with<br>truncated<br>base. | Narrowly<br>fusiform<br>with<br>subobtuse<br>apex, base<br>subtruncate<br>or round<br>base. | Fusiform to<br>ellipsoidal<br>with apex<br>round and<br>base<br>subtruncate. | Fusiform to<br>ellipsoidal<br>with apex<br>round and<br>base<br>subtruncate | Ellipsoid or<br>oblong,<br>straight.  | Clavate to<br>slightly<br>navicular,<br>apex<br>obtuse and<br>base<br>truncate. | Bacilliform,<br>straight to<br>slightly<br>curved, apex<br>and base<br>both bluntly<br>rounded or<br>blunt.                                    |
| Description | 0-2 septa<br>and hyaline  | 0-2 septa,<br>hyaline,<br>smooth<br>with<br>granular<br>contents                            | Unicellular,<br>hyaline,<br>smooth<br>with<br>granular<br>contents           | Unicellular,<br>hyaline,<br>smooth<br>with<br>granular<br>contents          | Hyaline,<br>aseptate,<br>becoming<br>dark brown<br>and one-<br>septate with<br>irregular<br>longitudinal<br>striations,<br>broadly<br>rounded at<br>apex,<br>truncate at<br>base. | 0-1 septa,<br>hyaline<br>and<br>granular<br>contents.                           | Aseptate,<br>hyaline, thin<br>to slightly<br>thickened<br>walled,<br>contained by<br>a persistent<br>mucous<br>layer,<br>granular<br>contents. |

*Neofusicoccum andinum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf., and *Pseudofusicoccum stromaticum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf. (Mohali *et al.*, 2005).

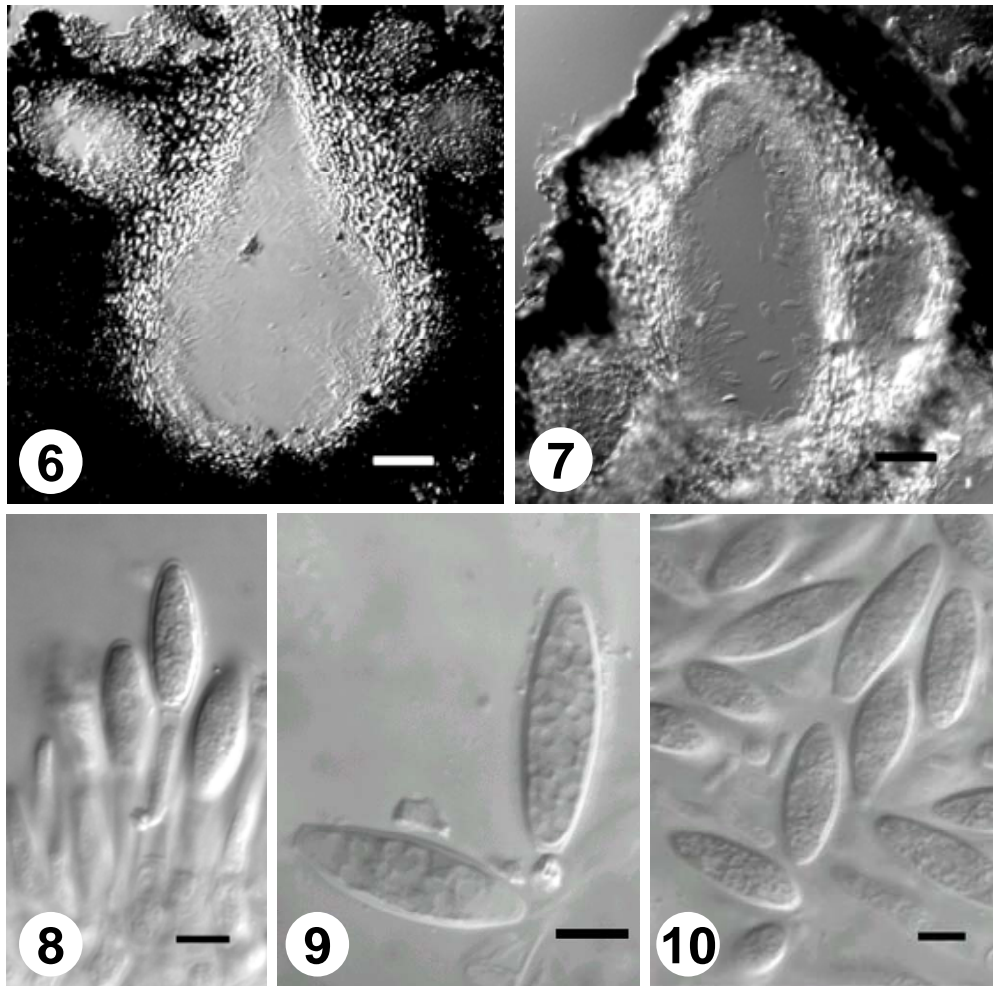
### *Phylogenetic analyses*

ITS sequence data (after alignment 562 characters) were obtained from 39 isolates (Table 2). Of the total data set, 255 characters were constant, and of the variable characters 166 were parsimony informative. Heuristic search analysis of the sequence data resulted in 119 trees of 505 steps [Consistency Index (CI) = 0.848; Retention Index (RI) = 0.919; Homoplasy Index (HI) =



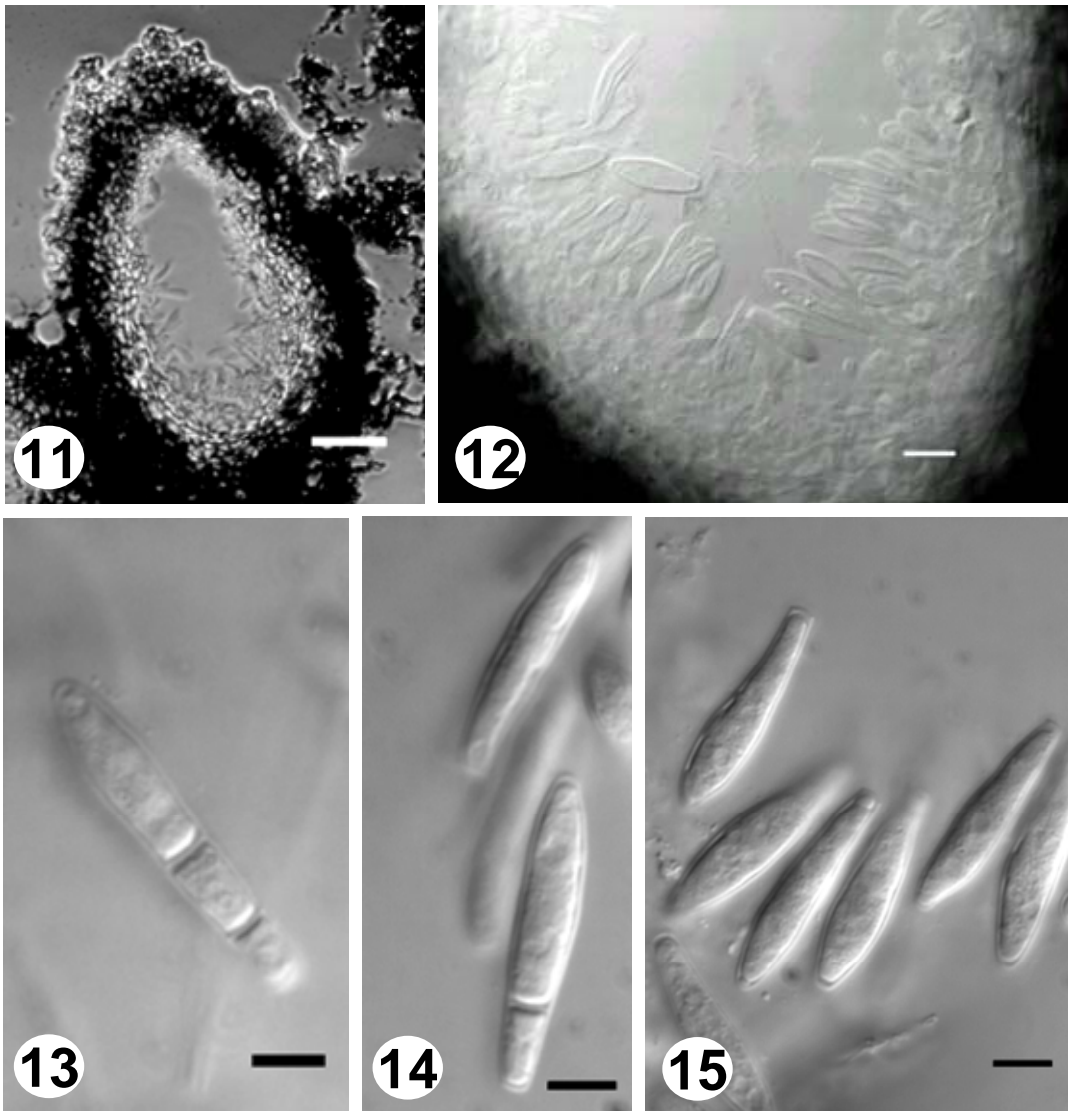
**Figs 1-5.** Microscopic characteristics of *Botryosphaeria mamane*. **1.** Immature and mature asci. **2-3.** Mature ascus and ascospores with granular, textured contents. **4.** Conidiogenous cells (arrow) with macroconidium. **5.** Macroconidia with 0-2 septa. Bars = 10  $\mu$ m.

0.152] (Fig. 16). Nine principal clades (I to IX) were obtained by comparing isolates from Venezuela with sequences from the GenBank. Venezuelan isolates grouped in six clades (III, IV, VI, VII, VIII, IX), which correspond to the morphological groups noted above. The ITS rDNA sequence data analysis



**Figs 6-10.** Micrographs of structures for isolates in the *Neofusicoccum ribis-N. parvum* complex. **6.** Pyriform pycnidium with a short and acute papilla. **7.** Globose pycnidium. Bars = 50  $\mu$ m. **8-9.** Conidiogenous cells and macroconidia produced in culture on MEA (2%) with pine needles. **10.** Macroconidia. Bars = 5  $\mu$ m.

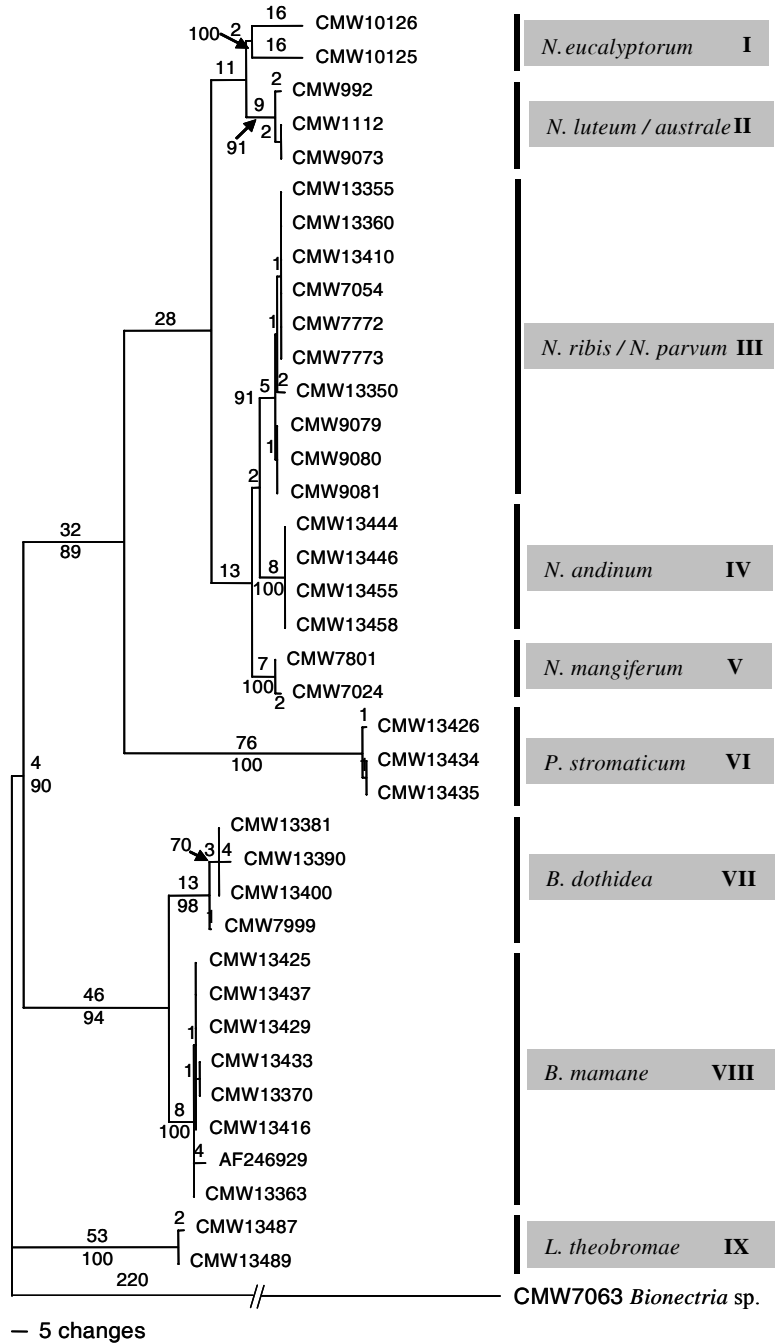
could not distinguish between isolates belonging to the *N. ribis* / *N. parvum* complex, which grouped in clade III. *Botryosphaeria dothidea*, *B. mamane* and *Lasiodiplodia theobromae* grouped in clades supported with bootstrap value  $\geq$  90%. *Neofusicoccum andinum* (Clade IV) and *Pseudofusicoccum stromaticum* (Clade VI) recently described from Venezuela (Mohali *et al.*, 2005) were also strongly supported by bootstrap values of 100% (Fig. 16).



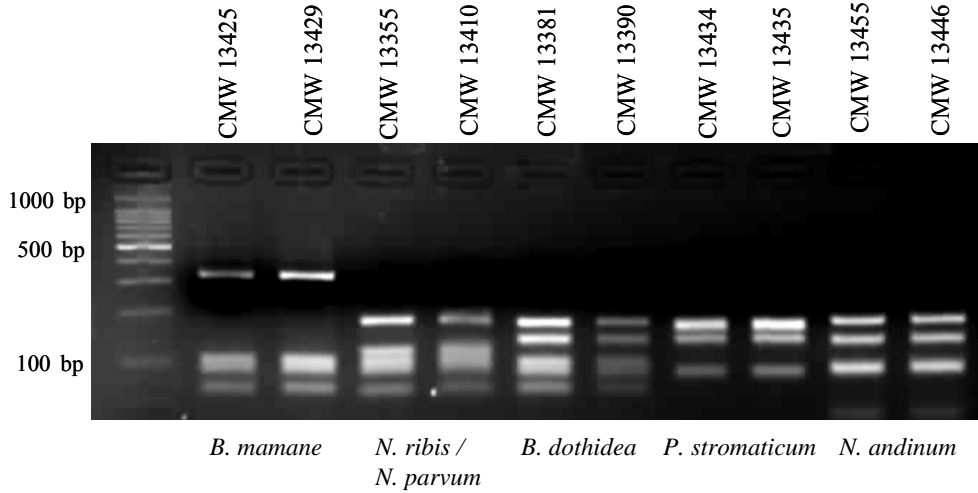
**Figs 11-15.** Micrographs of structures of *Botryosphaeria dothidea* in Venezuela. **11.** Globose pycnidium. Bar = 50 µm. **12.** Section through pycnidium with conidia. Bar = 10 µm. **13-15.** Conidia with 0-2 septa produced in culture on MEA (2 %) with pine needles. Bars = 5 µm.

#### ***PCR-RFLP analysis***

All the *Botryosphaeriaceae*, except *Neofusicoccum ribis* and *N. parvum*, 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

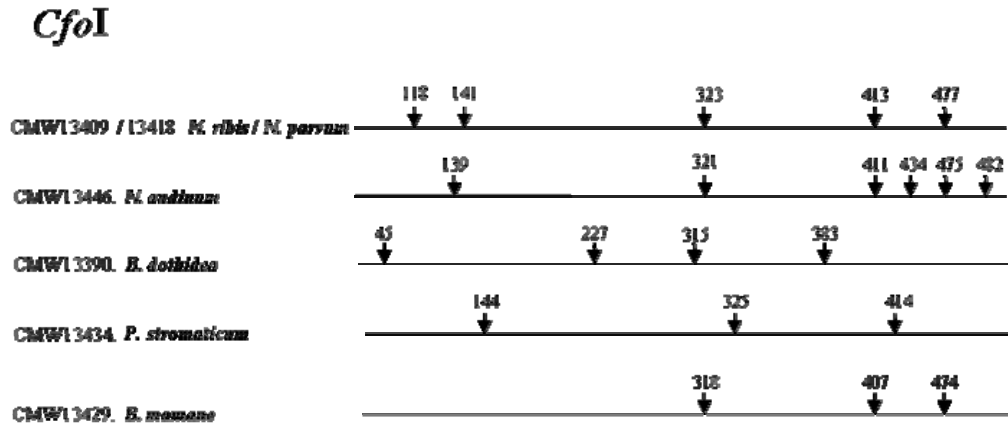


**Fig. 16.** Phylogenetic relationships amongst *Botryosphaeriaceae* from Venezuela and elsewhere (GenBank data) based on maximum parsimony analysis of ITS-1 and ITS-2 and 5.8S rDNA sequence data. The phylogram is rooted to the outgroup *Bionectria* sp. Bootstrap values greater than 50 % from 1000 replications 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.

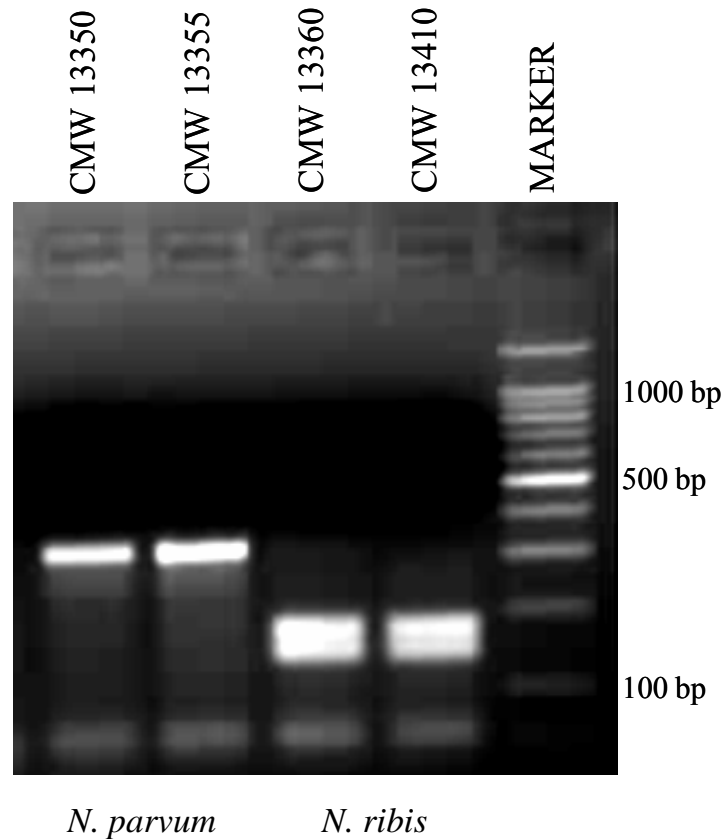


**Fig. 17.** Restriction fragments for the restriction enzyme *CfoI* of the ITS-PCR products of different species of *Botryosphaeriaceae* from Venezuela on a 3% agarose gel stained with ethidium bromide. Lane one contains a 100 bp size marker.

using the RE *CfoI* showed different restriction patterns for the isolates of *Botryosphaeria mamane* (CMW 13432 / 13429), *Neofusicoccum ribis* / *N. parvum* (CMW 13409 / 13418) as a complex, *Botryosphaeria dothidea* (CMW 13373 / 13390), *Pseudofusicoccum stromaticum* (CMW 13434 / 13435) and *Neofusicoccum andinum* (CMW 13446 / 13455).



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



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

#### ***RFLP analysis of N. ribis-N. parvum complex***

The DNA locus *BotF15*, amplified with the primers BOT 15 and BOT 16 for the isolates grouping in the *N. ribis-N. parvum* complex (Clade III), was polymorphic, with a restriction site for the enzyme *CfoI* in the isolates of *N. ribis*, but not in the *N. parvum* 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 species of *Botryosphaeriaceae* 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 *Botryosphaeriaceae* 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 *Botryosphaeria 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, *Botryosphaeria 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 *Botryosphaeriaceae* (Swart and Wingfield, 1991; Smith *et al.*, 1996a, b; 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 *Botryosphaeria mamane* and its anamorph, *Fusicoccum mamane* (Figs 1-5), differs somewhat from the description of the fungus from Hawaii (Gardner, 1997). The macro-conidia of the Venezuelan specimens sometimes have septa, but these were not reported previously. The asci and ascospores 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 *Neofusicoccum ribis* and *N. parvum* (Figs 6-10) are well-known pathogens of forest tree species, including *Eucalyptus* spp. (Frezzi, 1952; Davison and 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 and 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 these fungi in Venezuela will be needed to resolve this question.

Considerable problems have been experienced in distinguishing between *Neofusicoccum ribis* and *N. parvum* based on morphology (Zhou and 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 III) 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  $\beta$ -tubulin and translation elongation factor (EF) 1-  $\alpha$ ) were used to distinguish between isolates of these species.

We used an RFLP method to distinguish between *Neofusicoccum ribis* and *N. parvum*. This method, using primers that amplify a microsatellite-containing region in both species (Slippers *et al.*, 2004a), has a unique restriction site for *N. ribis*. We consider our isolates distinguished in this way as representing *N. ribis sensu lato* and *N. parvum 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 *Fusicoccum aesculi* (Figs 11-15) is one of the most commonly reported species in *Botryosphaeriaceae*. '*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 *Botryosphaeriaceae*, it has been associated with diseases on a wide range of hosts (Arx von, 1987; Slippers *et al.*, 2004b), but the identification of the species prior to the use of DNA comparisons is uncertain.

*Neofusicoccum andinum* and *P. stromaticum* were isolated and characterized originally from Venezuela (Mohali *et al.*, 2005). These fungi originate from branches and stems on *Eucalyptus*-hybrid, *E. urophylla* x *grandis* hybrids, *Eucalyptus* sp. 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 seems 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).

*Lasiodiplodia theobromae* 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* (Sénécl.) W.H.G., *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 and Palacios-Pru, 1992; Cedeño *et al.*, 1995, 1996; Mohali, 1993; Mohali and 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 and Petiffor, 1939; Findlay, 1959). Furthermore, *L. theobromae* produces cankers and kino exudation on *E. 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, *L. theobromae* 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 *Botryosphaeriaceae*, 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.

*Lasiodiplodia theobromae* was the most abundant species identified during this study, isolated from all three hosts. The next most common species isolated, in order of decreasing abundance were, *Botryosphaeria mamane*, *B. dothidea*, *Neofusicoccum ribis*, *N. parvum* and *Pseudofusicoccum stromaticum*, *Neofusicoccum andinum* was isolated from all the sampled *Eucalyptus* trees in the mountainous areas of Venezuela, but was absent from all other areas (Table 4).

The species composition of *Botryosphaeriaceae* on *Eucalyptus* in Venezuela differs from those reported in other recent studies on this host from South America, South Africa and Australia, which used similar techniques. In Colombia, only *Neofusicoccum ribis* and *Botryosphaeria dothidea* were found to cause *Eucalyptus* diseases (Rodas, 2003). In Chile, South Africa and Australia, *Neofusicoccum parvum*, *N. australis*, *N. eucalyptorum* and *N. eucalypticola* are the dominant species associated with *Botryosphaeria* canker and die-back diseases of *Eucalyptus* (Ahumada 2003, Slippers *et al.*, 2004b, c; Burgess *et al.*, 2005). Thus, *Eucalyptus* in Venezuela share some pathogens

**Table 4.** Identification of species of the *Botryosphaeriaceae* 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                                |
|------------------------------|--|----------------|---|
| <i>Botryosphaeria mamane</i> | <i>E. urophylla</i> x <i>E. grandis</i> ,<br><i>Eucalyptus</i> -hybrid, <i>Acacia mangium</i> , <i>Dipteri</i> x <i>punctata</i> . | 41             | Portuguesa, Barinas and Cojedes states. |
| <i>B. dothidea</i>           | <i>E. urophylla</i> x <i>E. grandis</i> ,<br><i>Eucalyptus</i> -hybrid   | 24             | Portuguesa and Cojedes states.          |
| <i>N. andinum</i>            | <i>Eucalyptus</i> sp.  | 20             | Mountain range, Mérida state            |
| <i>N. parvum</i>             | <i>E. urophylla</i> x <i>E. grandis</i> ,<br><i>Psidium guajava</i>  | 07             | Portuguesa and Zulia states             |
| <i>N. ribis</i>              | <i>E. urophylla</i> x <i>E. grandis</i>  | 16             | Portuguesa state                        |
| <i>L. theobromae</i>         | <i>Pinus caribaea</i> var.<br><i>hondurensis</i> , <i>E. urophylla</i> x <i>E. grandis</i> , <i>A. mangium</i> .                   | 89             | Falcon, Portuguesa and Cojedes state    |
| <i>P. stromaticum</i>        | <i>E. urophylla</i> x <i>E. grandis</i> ,<br><i>Eucalyptus</i> -hybrids, <i>A. mangium</i> .                                       | 07             | Portuguesa and Cojedes state            |

with other *Eucalyptus*-growing countries (e.g. the *Neofusicoccum parvum* - *N. ribis* complex with all areas, and *Botryosphaeria dothidea* with Colombia), but lacks some *Botryosphaeriaceae* from native *Eucalyptus* (e.g. *N. eucalyptorum* and *N. eucalypticola*), and is affected by unique species, possibly native to the country (e.g. *Botryosphaeria mamane*, *Pseudofusicoccum stromaticum* and *Neofusicoccum andinum*). These results confirm the importance of detailed identifications of *Botryosphaeriaceae* involved in causing specific disease symptoms in different areas and at different times. It also shows that *Eucalyptus* plantations in many different parts of 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 a large number of species of *Botryosphaeriaceae* are 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 *Botryosphaeriaceae* considerably and they will be used as a foundation to re-evaluate the importance of diseases associated with these fungi in Venezuela.

## Key to *Botryosphaeriaceae* from Venezuela occurring on *Eucalyptus*, *Acacia* and *Pinus*

All *Botryosphaeriaceae* 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* Fr. Apud Mont. (= teleomorph: *B. stevensii* Shoemaker) is included because it was isolated in Venezuela and might resemble some of the species treated here. Data for *D. mutila* 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 × 10-15 µm (average 24.5 × 12.8) ..... ***L. theobromae***
2. Conidia smooth, cylindrical with broadly rounded ends, some with a large central guttule, with a thick glassy wall, 28-32 × 13-15 µm (average 25.3 × 13.2) ..... ***D. mutila***
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 × 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 × 4-8 µm (average 27.1 x 5.6), l/w 4.84 ..... ***N. 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, aseptate to two septate, 18-32 × 3-6 µm (average 23.4 × 4.9), l/w 4.7 ..... ***B. dothidea***
6. Conidia mainly bacilliform, covered in a persistent mucous, apex and base both bluntly rounded or just blunt, 19-24 × 4-6 µm (average 21.7 × 5.4), l/w 4 ..... ***P. stromaticum***
7. Conidia 12-22 × 5-7 µm (average 17.2 × 5.6), l/w 3.7 ..... ***N. ribis***
7. Conidia 16-22 × 5-7 µm (average 18.6 × 5.8), l/w 3.2 ..... ***N. parvum***

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