Taxonomy and ecology of *Botryosphaeria* species and their anamorphs from Venezuela

by

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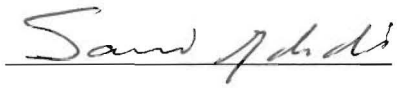
Declaration

I, the undersigned hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria, contains my own independent work, and has hitherto not been submitted for any degree at any other University.



Sari Ramón Mohali Castillo

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Dedicated to my beloved wife, Enid, my sons Jesus and Andres, my parents Sari and Gladys and to my brothers and sister.

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PREFACE

The fungal genus *Botryosphaeria* including its anamorphs has a cosmopolitan distribution and occurs on a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts, including woody twigs and branches, herbaceous leaves, stems of grasses, and even lichen thalli. These fungi give rise to a wide variety of symptoms such as shoot blights, stem cankers, fruit rots, die-back and gummosis. They are also known as saprophytes on dead or dying stems, branches or leaves of plants. In Venezuela, the following species have been reported: *Lasiodiplodia theobromae* (the anamorph of *Botryosphaeria rhodina*), *Diplodia pinea*, *D. mutila* and *Dothiorella* spp. However, their characterization has been based only on morphological descriptions. The most common and well characterised species, *L. theobromae*, is associated with pines and other hosts.

The focus of the studies presented in this thesis as to survey *Eucalyptus* and *Acacia* plantations in Venezuela for the presence and influence of *Botryosphaeria* spp., and to characterise these fungi using morphological characteristics and DNA sequence data. I also evaluated the pathogenicity and population biology of *Botryosphaeria* species present in the plantations. It was anticipated that the various studies would characterize a relatively large number of *Botryosphaeria* species, potentially recognise new species and provide some perspective of their relative importance to the *Eucalyptus* growing industry.

The thesis has been divided into chapters that reflect discrete units prepared for future publication. The first chapter presents a review of the relevant literature. Two chapters treat the taxonomy of the *Botryosphaeria* spp. collected in this study. An additional two chapters consider the population biology of the most commonly

encountered species and the remaining chapter deals with the pathogenicity of these fungi to *Eucalyptus*.

In the literature review I treat the taxonomy of *Botryosphaeria*, which has been the subject of much uncertainty for many years. I also briefly review recent findings related to their molecular characterization. A focus is also placed on the pathogenicity of *Botryosphaeria* species on various host plants and their known importance in Venezuela. The focus concerns mainly *Eucalyptus* spp.

Surveys that formed part of this study logically gave rise to a large collection of *Botryosphaeria* isolates. An important component of this thesis was to characterise these fungi. This was done based on morphological characteristics and also comparisons of DNA sequence data for various gene regions known to be informative for these fungi.

Lasiodiplodia theobromae (anamorph of *B. rhodina*), *B. ribis* and *B. parva* were studied from a population biology perspective. These fungi were chosen for study because they were the most common species encountered on *Eucalyptus* in Venezuela. To study populations, I made use of simple sequence repeat markers (SSR). Three populations of each of *L. theobromae* (Venezuela, Mexico and South Africa) and *B. ribis-B. parva* complex (Venezuela, Colombia and Hawaii) were analysed and the data considered in terms of population differentiation, gene flow, mode of reproduction, gene and genotype diversity.

In the final chapter of this thesis, I evaluated the pathogenicity of the seven *Botryosphaeria* species identified from Venezuela. These seven species were thus inoculated on *Eucalyptus urophylla* x *E. grandis* hybrid clones. A second series of inoculations was then carried out with the two most pathogenic fungi to determine the relative tolerance of the most important clones to infection.

This thesis was conducted over a period of four years. Work was undertaken both in Venezuela and South Africa and this necessitated long periods of time away from my home University and family. Surveys were conducted in many parts of Venezuela to collect the *Botryosphaeria* spp. of interest for latter study in South Africa. The research chapters have been completed systematically and they evolved over time. Each represents a discrete unit implying that there is some overlap, at least in the references, between them. I would like to believe that the thesis will provide a firm foundation for further studies of *Botryosphaeria* spp. and prove valuable to the small but important *Eucalyptus* growing industry in Venezuela.

SUMMARY

Species in the fungal genus *Botryosphaeria* are generally regarded as weak pathogens that attack stressed or wounded plants. Such stresses include drought, hail, wind and frost damage or insect infestation. It is interesting in this regard that these fungi have, in recent years, also been shown to exist endophytically in asymptomatic tissue as latent pathogens on *Eucalyptus*.

The taxonomy of *Botryosphaeria* spp. has been problematic from more than 100 years. The identification of species in this genus has been traditionally based on morphological descriptions, which tend to be interpreted subjectively. In recent years, molecular techniques and particularly analyses of DNA sequences have been used to identify closely related *Botryosphaeria* species. Thus, in the first chapter of this thesis, a combination of morphological characteristics and analyses of multiple gene sequences were successfully used to identify cryptic *Botryosphaeria* species occurring in Venezuela.

Studies in this thesis have treated the taxonomy, phylogenetic relationships, population structure and pathogenicity of *Botryosphaeria* species. The species were isolated in various areas of Venezuela and from various woody hosts, such as *Acacia*, *Eucalyptus* and *Pinus*. The DNA based comparisons used to identify *Botryosphaeria* spp. were based on sequence data for the ITS regions of the ribosomal DNA operon. These sequences were from fragments including the 3' end of the small subunit (SSU) rRNA gene, the internal transcribed spacer ITS (ITS1), the complete 5.8S rRNA gene, the ITS2 and the 5' end of the large subunit (LSU) rRNA gene. In addition, part of the EF 1-α was amplified using the primers EF1-728F and EF1-986R. In total, seven *Botryosphaeria* spp. were characterised from Venezuela. Two new *Botryosphaeria*

anamorphs, *Fusicoccum andinum prov. nom.* and *F. stromaticum prov. nom.* (chapter 2), were identified using the combination of the two-gene regions (ITS and EF1-α). An additional five *Botryosphaeria* species, *Botryosphaeria mamane*, *B. dothidea*, *B. rhodina*, *B. parva* and *B. ribis*, were characterized using the ITS region (chapter 3).

The population biology for *L. theobromae* and the *B. ribis-B. parva* complex (Chapter 4 and 5) was considered using SSR markers previously developed for these fungi. Three populations of *L. theobromae* (Venezuela, Mexico and South Africa) and *B. ribis-B. parva* complex (Venezuela, Colombia and Hawaii) were used in each of the comparisons. *Lasiodiplodia theobromae* showed no evidence for host specificity, and there was very high gene flow between populations of isolates from different hosts. Reproduction was predominantly clonal with some genotypes widely distributed within a region. The isolates representing the *B. ribis* - *B. parva* complex were separated into two distinct groups, confirming the species barrier within this species complex that has been reported previously based on combined gene genealogies, and SSR and PCR-RFLP data from other populations. The *B. ribis* populations had a low degree of isolation and an asexual (clonal) mode of reproduction. Populations of *B. parva* in Colombia and Hawaii showed recombination within each population, and a high degree of isolation between Colombia and Hawaii.

Pathogenicity tests were undertaken on *Eucalyptus urophylla* x *E. grandis* hybrid clones in the field using the seven *Botryosphaeria* species identified in an earlier study (Chapter 3). These tests showed that not all *Botryosphaeria* species collected from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela are pathogenic on the selected *Eucalyptus*-hybrid. *Botryosphaeria parva* and *B. ribis* were the most pathogenic on *Eucalyptus*, but of the five clones inoculated, one had obviously greater tolerance to infection by these species. The remainder of the clones inoculated were less tolerant to infection.

Botryosphaeria parva gave rise to longer lesions than did isolates of *B. ribis* and the former species appears to be most pathogenic.

This study represents the first comprehensive treatment of *Botryosphaeria* spp. in Venezuela. The focus has been on species from forestry crops, but it is clear that these fungi are well represented in the country and I believe that many new species remain to be discovered. It is my hope that studies presented in this thesis will not only provide a foundation for future work on *Botryopshaeria* spp., but that they will also stimulate an elevated interest in the field of forest pathology.

OPSOMMING

Spesies in die fungus genus *Botryosphaeria* word as swak patogene beskou wat slegs plante onder stres of gewonde plante aanval. Sodanige stres op plante word veroorsaak deur droogte, hael, wind- en rypskade of insekinfestasie. Dit is ook onlangs getoon dat die swamme endofities as latente patogene in gesonde plantweefsel kan leef.

Die taksonomie van *Botryosphaeria* spp. is al vir meer as 'n 100 jaar 'n probleem. Die identifikasie van spesies in die genus is tradisioneel op morfologiese beskrywings gebaseer, wat dikwels ruimte laat vir subjektiewe interpretasies. Molekulêre tegnieke, in besonder analises van DNA basisopeenvolging, is onlangs gebruik om nabyverwante *Botryosphaeria* spp. te onderskei. Vir hierdie rede word 'n kombinasie van morfologiese karakters en DNA basisopeenvolging data van verskillende gene in Hoofstuk 1 gebruik om kriptiese *Botryosphaeria* spp. in Venezuela te identifiseer.

Die verskillende studies in hierdie tesis ondersoek die taksonomie, filogenetiese verwantskappe, populasie struktuur en patogenisiteit van *Botryosphaeria* spp. in verskillende areas van Venezuela en van verskillende gashere soos *Acacia*, *Eucalyptus* en *Pinus*. Die DNA gebaseerde vergelykings tussen verskillende *Botryosphaeria* spp. is eerstens geskoei op DNA basisopeenvolging van die ITS gedeelte van die ribosomale DNA operon, insluitende die 3' kant van die SSU rRNA, die hele ITS1, 5.8S geen en ITS2, asook die 5' kant van die LSU rRNA geen. Hierbenewens is gedeeltes van die EF1-α geen geamplifiseer met behulp van die primers EF1-728F en EF1-986R. Sewe *Botryosphaeria* spp. is met behulp van die data in Venezuela geïdentifiseer. Twee nuwe *Botryosphaeria* anamorwe, *Fusicoccum andinum prov. nom.* en *F. stromaticum prov. nom.* (Hoofstuk 2) is geïdentifiseer met behulp van die twee geen areas (ITS en EF1- α).

'n Addisionele vyf *Botryosphaeria* spesies, *B. mamane*, *B. dothidea*, *B. rhodina*, *B. parva* en *B. ribis* is geidentifiseer met behulp van ITS data en morfologie (Hoofstuk 3).

Die populasie biologie van *Lasiodiplodia theobromae* en die *B. ribis-B. parva* kompleks (Hoofstukke 4 en 5) was ondersoek met behulp van SSR merkers wat vroeër vir die swamme ontwikkel is. Drie populasies elk van *L. theobromae* (Venezuela, Mexico en Suid-Afrika) en die *B. ribis – B parva* kompleks (Venezuela, Colombia en Hawaii) is ondersoek. Daar was geen aanduiding van gasheer seleksie in populasies van *L. theobromae*, met hoë vlakke van geenbeweging tussen die populasies van verskillende gashere. Reproduksie was hoofsaaklik klonaal, met sommige genotipes wydverspreid in die areas. Die isolate wat die *B. ribis* - *B. parva* kompleks verteenwoordig het, is in twee groepe verdeel gebaseer op die SSR data. Hierdie data bevestig die hipotese van 'n spesiegrens tussen *B. parva* en *B. ribis* soos vroeër gevind uit 'n studie van multigeen DNA basisopeenvolging, en SSR en PCR-RFLP data van ander populasies. Die *B. ribis* populasies was swak onderskei (hoë geenvloei) en hoofsaaklik aseksuele reproduksie. Populasies van *B. parva* vanaf Hawaii and Colombia was egter afgeskei (lae geenvloei) en toon beide tekens van seksuele reproduksie.

Patogenisiteitsproewe is gedoen met die sewe *Botryosphaeria* spp., wat in Hoofstuk 3 geïdentifiseer is, op *Eucalyptus urophylla* x *E. grandis* hibried klone in die veld. Hierdie proewe het gewys dat nie al die *Botryosphaeria* spp. patogenies is op die *Eucalyptus* hybried nie. *Botryosphaeria parva* en *B. ribis* was die mees patogeniese spesies op *Eucalyptus*, maar van die vyg klone wat getoets was het ten minste een beduidende weerstand teen die patogene gehad. Die oorblywende klone was minder bestand teen infeksie. *Botryosphaeria parva* het langer letsels veroorsaak as *B. ribis*, en die spesie blyk dus die mees patogeniese te wees.

Hierdie studie verteenwoordig die eerste oorhoofse ondersoek na *Botryosphaeria* spp. in Venezuela. Die fokus was op spesies geassosieer met bosboubome, maar dit is duidelik dat die swamme goed verteenwoordig is in die land en daar is waarskynlik meer onontdekte spesies. Dit is my hoop dat die studies wat hier verteenwoordig word nie net die fondasie sal vorm van toekomstige werk op *Botryosphaeria* nie, maar dat dit ook navorsing in die veld van bosboupatologie as geheel sal stimuleer.

Chapter 1

The taxonomy and pathology of *Botryosphaeria* spp., with special reference to their relevance in *Eucalyptus* plantations of Venezuela

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INTRODUCTION

Fungi in the genus *Botryosphaeria* (Loculoascomycetes, Dothideales, Botryosphaeriaceae) (Barr, 1987; Hawksworth *et al.*, 2001) have a cosmopolitan distribution. They occur on a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts, on woody twigs and branches, herbaceous leaves, stems of grasses, and in lichen thalli (Barr, 1987). *Botryosphaeria* spp. result in many different symptoms such as shoot blights, stem cankers, fruit rots, die-back and gummosis (Ciesla *et al.*, 1996; Old, 2000; Old & Davison, 2000).

Very little is known regarding the *Botryosphaeria* spp. in Venezuela. The anamorphs of *Botryosphaeria* spp., have been reported to 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, have been based on conidial morphology. In recent years there have, however, been significant advances in the identification of *Botryosphaeria* spp. through combination of morphological and DNA-based techniques (Jacobs & Rehner,

1998; Denman *et al.*, 2000; Smith *et al.*, 2001a; Smith & Stanosz, 2001; Zhou & Stanosz, 2001a; Slippers *et al.* 2004b). These studies are given rise to a relatively robust taxonomy for *Botryosphaeria* and this is rapidly leading to a deeper understanding of host pathogen relationships and geographic distribution of species. This knew knowledge has not been applied to *Botryosphaeria* in Venezuela.

Botryosphaeria species from Australian woody plants such as *Acacia* spp., *Eucalyptus* spp. and Proteaceae, have been relatively well studied because the plants have been used in commercial plantations and orchards world-wide (Ciesla *et al.*, 1996; Old, 2000; Old & Davison, 2000; Wingfield, *et al.*, 2001a, b; Denman *et al.*, 2003, Slippers *et al.* 2004c, d). These studies have shown that the establishment of such exotic plantations is followed by the introduction of pathogens such as *Botryosphaeria* spp. on infected planting stock and seeds (Ciesla *et al.*, 1996; Old, 2000; Old & Davison, 2000; Wingfield, *et al.*, 2001a, b; Denman *et al.*, 2003, Slippers *et al.* 2004c, d). These exotic plantations are also affected by pathogens that are native to the regions where these trees are planted. Both natural and introduced pathogens are thus a potential threat to the productivity of these plantations, as well as the native plants that surround them. This is very likely also true for Venezuela, but has not been studied to date.

Large-scale clonal plantations of *Eucalyptus* have been established in Venezuela for the production of wood and fiber. The *Eucalyptus* clones are selected for fast growth and desireable wood properties. The selection and need for uniformity in plantations results in lowered genetic variation. Such low genetic diversity in the host increases the risk of potential pathogens, such as *Botryosphaeria* spp., especially when the eucalypt trees are growing under stress.

Botryosphaeria spp. occur in Venezuela on various hosts on which they are thought to be important pathogens. Basic knowledge regarding many of these fungi is,

however, lacking in many cases, as illustrated above. This review, therefore, aims to provide a framework and foundation for the characterisation, risk assessment and attempts at control of *Botryosphaeria* spp. in Venezuela.

HIGHER CLASSIFICATION AND TELEOMORPH TAXONOMY

The genus of *Botryosphaeria* was established in 1863 by Cesati and De Notaris to accommodate bitunicate Ascomycetes with eight, aseptate, hyaline ascospores. Before this time, these taxa were placed under the group name *Sphaeria* (Fries 1823). Cesati and De Notaris (1863) identified 12 species at the time, including *B. dothidea*. More species were soon added, starting with four species, including *B. berengeriana* De Not added by De Notaris (1863). Today, more than 200 species have been described in *Botryosphaeria* (Index Fungorum; http://www.indexfungorum.org).

The type species for *Botryosphaeria* was not selected by Cesati and De Notaris (1863). Barr (1972) rejected propositions that either *B. quercuum* (Schwein.) Sacc. or *B. berengeriana* be designated as lectotype species for the genus because these species were not part of the original description. Therefore, Barr (1972) designated *B. dothidea* (Moug.:Fr.) Ces. & De Not. (= *Sphaeria dothidea* Moug.:Fr.), one of the original species included by Cesati and De Notaris (1863), as the lectotype of the genus.

The higher classification of *Botryosphaeria* and the related genus *Guignardia* has been controversial. Arx and Müller (1954) regarded both *Botryosphaeria* and *Guignardia* as genera of the family Botryosphaeriaceae. Petrak (1957) studied *Guignardia* and concluded that it should be merged with *Botryosphaeria*. Barr (1970), however, argued that *Botryosphaeria* and *Guignardia* are amerosporous representatives of the families Dothioraceae and Dothideaceae, respectively. Luttrell (1973) classified

Botryosphaeria in the order Pleosporales and *Guignardia* in the order Dothideales. Soon afterwards, however, Arx and Müller (1975) placed the two genera in the order Dothideales, family Botryosphaeriaceae. Sivanesan (1984) followed the same classification, but placed *Botryosphaeria* in the family Dothideaceae. Barr (1987) again placed *Botryosphaeria* in the order Pleosporales. The currently accepted classification, however, considers *Botryosphaeria* as member of the family Botryosphaeriaceae in the order Dothideales and *Guignardia* in the family Mycosphaerellaceae in the order Mycosphaerellales (Hawksworth *et al*., 2001).

Despite the fact that the genus has been known for more than 140 years, the taxonomy of *Botryosphaeria* species is still problematic for several reasons. Teleomorphs are uncommonly encountered in nature and are difficult to induce in culture (Laundon, 1973; Jacobs & Rehner, 1998). Furthermore, there is often not sufficient diversity of teleomorph morphology to allow differentiation at the species level. There are also differences between young developing ascospores and older released ascospores, which often complicate comparisons between collections (Laundon, 1973). Despite recent clarifications of the taxonomy of key species, the majority of species in the genus are in need of revision using modern taxonomic methods.

ANAMORPH TAXONOMY

Botryosphaeria species have coelomycetous anamorphs (Sutton, 1980; Barr, 1987). Identification of *Botryosphaeria* spp. is often dependent on its anamorphs. These forms are more commonly encountered in nature and in culture and the morphological features are also more variable and useful for species identification (Sutton, 1980; Pennycook &

Samuels, 1985; Hanlin, 1990; Jacobs & Rehner, 1998). The anamorph characters most frequently used are conidial size, colour, septation, wall thickness and texture, as well as the presence of microconidia, and mode of conidiogenesis (Sutton, 1980; Sivanesan, 1984; Pennycook & Samuels, 1985; Denman *et al.*, 2000; Smith *et al.*, 2001b; Zhou and Stanosz, 2001a). The use of the conidial characters can also be problematic because the conidial size varies within species and on different hosts, or may overlap between species and change with age (Butin, 1993; Jacobs & Rehner, 1998; Sutton, 1980; Sivanesan, 1984; Pennycook & Samuels, 1985; Slippers *et al.*, 2004b).

Sutton (1980) used variations in the pycnidia and conidiogenesis to separate *Botryosphaeria* anamorph genera. Denman *et al.* (2000) studied the taxonomy of the most common *Botryosphaeria* anamorphs identified at the time, which amounted to 22 (even more have since been recognised). Of these, the most commonly used were *Fusicoccum* Corda, *Diplodia* Fr., *Macrophoma* (Sacc.) Berl & Vogl., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Sphaeropsis* Sacc. and *Botryodiplodia* Sacc. Denman *et al.* (2000) separated these *Botrypsphaeria* anamorphs into two groups based on conidial colour, shape and wall texture. One group has hyaline, fusoid and thin-walled conidia that might become translucent brown and septate prior to germination. These taxa are typical of *Fusicoccum*. The other group has hyaline to dark-conidia, most often ellipsoidal, 0-1 euseptate and opaque brown when mature They can have prominent melanin deposits on the insides of the conidial walls giving the impression of striations, or the conidial walls can be smooth and thick (sometimes glassy). These taxa are considered representative of *Diplodia* (including *Lasiodiplodia*).

Zhou and Stanosz (2001a) studied the relationships of 52 *Botryosphaeria* isolates and their associated anamorphic fungi based on conidial morphology. They too identified two sections amongst the anamorphs. The first group, section *Hyala*, included

B. corticis, *B. dothidea*, *B. mamane*, *B. parva*, and *B. ribis* (all with known *Fusicoccum* anamorphs), and anamorph species *Fusicoccum luteum*. The conidia in this group were considered to usually be hyaline, but to become light brown when old or before germination. Widths of the conidia usually less than 10 µm. This section corresponds to the *Fusicoccum* group of Denman *et al.* (2000). The second group was named as section *Brunnea*, and included *B. obtusa*, *B. quercuum*, *B. rhodina*, *B. stevensii*, and *B. tsugae* (having *Diplodia*, *Lasiodiplodia* or *Sphaeropsis* anamorphs), *Diplodia pinea* f. sp. *cupressi*, and both the A and B groups of *Diplodia pinea*. The mature conidia were described as usually light to dark brown, but could be hyaline when young, and widths of conidia were usually greater than 10 µm. This section corresponds to the *Diplodia* group of Denman *et al.* (2000).

Alves *et al.* (2004) highlighted the fact that some *Botryosphaeria* species accommodated in *Diplodia*-anamorphs have mostly hyaline conidia such as: *B. corticola*, *B. stevensii* and *B. quercuum*. Therefore, they considered the distinction between the two sections developed by Zhou and Stanosz (2001a) on the basis of conidium coloration as tenuous. The name *Diplodia* should thus not be restricted to anamorphs with brown conidia, but also apply to some species with hyaline conidia, which also was reflected by Denman *et al.* (2000). Alves *et al.* (2004) pointed out that there was a clearer distinction between *Fusicoccum* and *Diplodia* in the widths of the conidia and cell wall thickness. *Fusicoccum* is generally <10 µm wide and thin walled, while *Diplodia* is generally >10 µm wide and thicker walled.

Today there are three genera validly recognized and used for anamorphs of *Botryosphaeria*. These are *Fusicoccum* with hyaline and narrow conidia; *Diplodia* and *Lasiodiplodia* with dark and broad conidia, becoming longitudinally striate in the latter

genus (Denman *et al.*, 2000; Zhou & Stanosz, 2001a; Phillips *et al.*, 2002; Alves, *et al.*, 2004).

***Fusicoccum* Corda.**

Petrak (1922) placed the type species of *Fusicoccum*, called *F. aesculi* Corda by Saccardo (1880, 1884, 1886), in the genus *Dothiorella*. Sutton (1980), however, considered the material described by Saccardo (*F. aesculi*) as best accommodated in the genus *Fusicoccum*, including the anamorphs of ascomycetes such as *B. dothidea* and *B. ribis*. Sutton (1980) described *Fusicoccum* as a Coelomycetes group that has hyaline, aseptate, straight conidia, with obtuse apices and truncate bases (fusiform). Pennycook and Samuels (1985) described two new *Fusicoccum* species, *F. parvum* and *F. luteum*, and they distinguished these two species from *F. aesculi* based on conidial size and pigmentation, as well as culture morphology. They thereby showed that *Fusicoccum* conidia can also be pigmented. Crous and Palm (1999) and Denman *et al.* (2000) supported the observations of Sutton (1980) and Pennycook and Samuels (1985). They, however, added other characteristics which could be used to identify the species in this genus such as the mode of proliferation of the conidiogenous cell for example, proliferation at the same level resulting in periclinal thickening, or percurrently resulting in annellations.

The 180 *Fusicoccum* species listed in Index fungorum might be misleading. Denman *et al.* (2000) and Slippers *et al.* (2005) suggested that many species previous described under *Dothiorella* are likely to be *Fusicoccum* species. There are more than 350 species of *Dothiorella* (Index fungorum: http: www.indexfungorum.org). Similarly, a number of species of *Phyllosticta* have recently been transferred to

Fusicoccum (Van der Aa & Vanev, 2002). Furthermore, recent studies have shown that some well recognized *Fusicoccum* species might contain cryptic species (Slippers *et al.*, 2004b, c, d). There are thus likely to be many more *Fusicoccum* spp. than those currently described and this genus, as well as the allied genera, are clearly in need of revision.

***Diplodia* Fr. (= *Sphaeropsis* Sacc.)**

According to Sutton (1980), the original generic description of *Diplodia* was compiled by Fries in 1834, and identified as *Diplodia mutila* Fr. The teleomorph of *D. mutila* was discovered by Stevens (1936) who cited it as *Physalospora mutila* (Fr.) N. E. Stevens. Shoemaker (1964) renamed *P. mutila* as *Botryosphaeria stevensii* Shoem. Sutton (1980) described the genus as having pycnidial, unilocular conidiomata, with a central ostiole; conidiophores are absent; proliferation is percurrent in conidiogenous cells; conidia are oblong to clavate, straight, aseptate (but developing eusepta prior to germination). The apexes obtuse and tapered to a truncate base.

Recent treatments have shown that the name *Diplodia* should preside over names such as *Sphaeropsis* (Denman *et al.*, 2000; De Wet *et al.*, 2003). Denman *et al.* (2000) and De Wet *et al.* (2003) considered the argument of percurrent proliferation and time of septation of *S. sapinea* as insufficient to separate it from the genus *Diplodia*. They suggested, therefore, that the older name *Diplodia pinea* be used for *Sphaeropsis sapinea* and also described a new cryptic species from isolates of this latter taxon as *D. scrobiculata* De Wet, Slippers & Wingfield (De Wet *et al.*, 2003).

Diplodia is the largest of the anamorph genera of *Botryosphaeria*, with more than 1200 species listed in Index Fungorum (http://www.indexfungorum.org). Many of

these species are, however, likely to be synonyms, while others will refer to species complexes. Like the other *Botryosphaeria* anamorph genera, *Diplodia* is in need of revision.

***Lasiodiplodia* Ellis & Everh.**

Lasiodiplodia theobromae, is a common plant pathogen in the tropics where it is often reported as *Botryodiplodia theobromae*. *Lasiodiplodia*, however, produces solitary pycnidia, which differentiates it from *Botryodiplodia*, which produces pycnidia in a valsoid stroma (Crous & Palm, 1999). The genus was first described by Ellis and Everhart in 1896 with *Lasiodiplodia tubericola* Ellis & Everh., as the type species (Taubenhaus, 1915). *Lasiodiplodia* spp. are characterized as having eustromatic, uni or multilocular conidiomata; conidiophores are absent; conidia are hyaline when young, later dark-brown and euseptate, and thick walled. Bases are truncate with longitudinal striations from apex to base (Sutton, 1980). Uduebo (1975) showed that the conidial wall ornamentation is made up by deposits of melanin on the inside of the wall, creating an illusion of striations on surfaces of conidia.

Lasiodiplodia theobromae (= *Botryodiplodia theobromae* Pat.) is the most commonly reported species (Punithalingam, 1976, 1980; Arx von, 1987). *Lasiodiplodia theobromae* has previously been reported as the anamorph of *Physalospora rhodina* Berk. & Curt. Apud Cooke (Punithalingam, 1980; Sutton, 1980), but now considered as the anamorph of *Botryosphaeria rhodina* (Cooke) Von Arx (Arx von, 1987).

There are 10 *Lasiodiplodia* species recorded in Index Fungorum (http://www.indexfungorum.org/). Some of these species are likely synonyms of *L. theobromae*. However, the recent description of a new species, *L. gonubiensis* Pavlic,

Slippers & M. J. Wingf. (Pavlic *et al.*, 2004), also shows that there might be more species to be identified on previously unstudied hosts and environments.

DNA-BASED CHARACTERIZATION

The identification of *Botryosphaeria* spp. and their anamorphs based on morphological characteristics requires an experienced researcher and large numbers of samples to compensate for variation within and between species. DNA based characters have been successful in resolving species level taxonomic questions in different groups of fungi (Berbee & Taylor, 1993). Many researchers have thus attempted to develop non-subjective molecular techniques to identify *Botryosphaeria* spp. RAPDs and isozymes have been used in *Botryosphaeria* taxonomy since the 1990's, especially with regard to *D. pinea* (= *S. sapinea*) morphotypes (Smith & Stanosz, 1995; Stanosz, *et al.*, 1999). Jacobs and Rehner (1998) were the first authors to combine DNA sequence data and morphological characters to consider interspecific relationships in *Botryosphaeria*. Since then, numerous other studies have combined morphological characters and different forms of DNA based data to characterize and study the phylogeny of *Botryosphaeria* (Jacobs & Rehner, 1998; Burgess *et al.* 2001b; Smith *et al.*, 2001a; Zhou & Stanosz, 2001a; Phillips *et al.*, 2002; Denman *et al.*, 2003; Alves *et al.*, 2004; Slippers *et al.*, 2004b and others).

Isozymes and Randomly Amplified Polymorphic DNA (RAPD) markers

Isozyme patterns and Randomly Amplified Polymorphic DNA (RAPD) markers have been useful tools to study the A and B morphotypes of *D. pinea* from different hosts and

localities (Smith & Stanosz, 1995; Stanosz *et al.*, 1996, 1999). With this technique the relationship between the *Botryosphaeria* spp. is determined using cluster analyses of presence or absence data for amplification fragments produced by RAPD markers and proteins extracted from different cultures (Smith & Stanosz, 1995; Stanosz *et al.*, 1999). The advantage of this technique is that it gives a genome-wide perspective.

Data from isozymes and RAPD's did not provide sufficient support to separate some closely related *Botryosphaeria* species, such as those in the *B. ribis-B. parva* and *B. lutea-B. australis* species complexes (Smith & Stanosz, 2001). In other cases these markers can also over-estimate diversity, such as in the four distinct groups in *D. pinea*, which were separated using RAPD's (Stanosz, *et al.*, 1996). The four groups were later shown to be only three *Botryosphaeria* species (*D. pinea*, *D. scrobiculata* and *B. obtusa*) (De Wet *et al.*, 2000).

***Internal Simple Sequence Repeat* (ISSR)**

Zhou *et al.* (2001) worked with inter simple or short sequence repeats (ISSR) to differentiate *Botryosphaeria* species with very similar or identical ITS sequences and morphologies. The cluster analysis of the ISSR fingerprints among species of the light and narrow conidial-group (*Hyala*) indicated a close relationships between a group composed of *F. luteum*, *B. ribis* and *B. parva*; and another group composed of *B. mamane*, *B. corticis* and *B. dothidea*, respectively. However, fingerprint analysis also indicated that all these species are distinct. The results supported earlier work that separated *B. dothidea* and *B. ribis* (Zhou *et al.*, 2001), however, it also suggested the differentiation of *B. parva* and *B. ribis*, for which ITS sequences and RAPD marker analyses previously suggested were the same species (Zhou *et al.*, 2001). These results

have subsequently been confirmed with other techniques (see below). Thus, there is value in using ISSRs for species delimitation in *Botryosphaeria*, given that sufficient isolates are available for comparison.

Polymerase Chain Reaction - Restriction Fragment Length Polymorphism *(PCR-RFLPs)* analysis

A method that has been commonly used in recent years to distinguish *Botryosphaeria* spp. is that based on RFLP profiles. This technique provides a quick and effective way to distinguish species and study their geographic distribution and population compositions (Slippers, 2003).

Jacobs (2002) developed an identification system for *Botryosphaeria* species from mango using sequence data of the ITS region of sequenced isolates. Slippers (2003) and Slippers *et al.* (2004c) also used RFLPs of the ITS region to distinguish *Botryosphaeria* spp. from fruit trees and *Eucalyptus* respectively. Pavlic (2004) used this technique to distinguish *Botryosphaeria* spp. from *Syzigium* trees in South Africa. The results of these studies allowed a quick and easy identification of some *Botryosphaeria* species, but could not distinguish between the cryptic species group's *B. parva*, *B. ribis* and *B. australis* (Jacobs, 2002; Slippers, 2003).

Slippers *et al.* (2004a) developed primers that amplified a microsatellite-containing region in both species *B. ribis* and *B. parva*. Restrictions patterns for one of these unidentified DNA regions (Locus *BotF15*) have a unique restriction site for *B. ribis* and were thus used to distinguish *B. ribis* and *B. parva* (Slippers *et al.* 2004b). The isolates distinguished in this way are considered to represent *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).

Alves, *et al.* (2005) extended the ITS RFLP based techniques for distinguishing *Botryosphaeria* species, by also including the D1 and D2 regions of the LSU rDNA in the initial amplification. The method is termed amplification of the ribosomal DNA and restriction analysis (ARDRA), and is also used for taxonomic studies of other fungi (Guarro *et al.*, 1999). The technique was used to separate 35 strains into the respective 10 *Botryosphaeria* species. The results obtained demonstrated that the ARDRA technique is a useful tool for the identification of the major species in the genus *Botryosphaeria*, including some cryptic species such as *B. ribis* / *B. parva* and *B. sarmentorum* / *B. iberica* (Alves *et al.*, 2005; Phillips, *et al.*, 2005). The differentiation of these cryptic species was impossible by ARDRA analysis of the ITS region alone (Slippers *et al.*, 2004c).

Internal Transcribed Spacers* (ITS) *sequence data

Jacobs & Rehner (1998) considered nuclear rDNA ITS sequence analysis together with conidial characters and cultural studies to distinguish *Botryosphaeria* species. For 22 isolates, they found inconsistencies between the ITS clustering and traditional identifications. For example *B. dothidea* grouped in two clades, of which one was shared with *B. ribis*. This study showed the usefulness of DNA sequence data in identifying such problems and laid the foundation for future taxonomic studies.

Smith and Stanosz (2001) and Smith *et al.* (2001b) supported the separation of *B. ribis* from *B. dothidea* with data of ITS and 5.8 rDNA sequences. These studies indicated that the two species are distinct monophyletic groups, with *B. ribis* more

closely related to *B. parva* and *F. luteum*, and *B. dothidea* more closely related to *B. corticis* and *B. mamane*. This affirmation was supported with the addition of ITS sequences and conidial characterization of many more isolates of these taxa (Smith & Stanosz, 2001; Slippers *et al.*, 2004b), allowing clear separation of *B. ribis* and *B. dothidea*.

The use of internal transcribed spacers (ITS1 and ITS2) has been successfully employed to separate *Botryosphaeria* isolates on several hosts. Examples of different hosts include *Vitis* spp., *Eucalyptus* spp., *Quercus* sp., *Pinus* spp., *Pistacia vera* and fruit trees (Ogata *et al.*, 2000; Smith *et al.*, 2001a; Phillips *et al.*, 2002; Alves *et al.*, 2004). PCR primers have been developed based on ITS sequences for identification of *Fusicoccum* sp. from pistachio and other host plants in California (Ma & Michailides, 2002).

Despite the fact that ITS sequence data supported the separation of some *Botryosphaeria* species, this single DNA locus can not resolve all species uncertainties. Like RAPD markers, closely related species such as *B. ribis* and *B. parva* can not be separated with confidence based on ITS data alone (Smith & Stanosz, 2001; Zhou & Stanosz, 2001a; Slippers *et al.*, 2004b).

Mitochondrial Small Subunit Ribosomal RNA (mt SSU rDNA)

Zhou and Stanosz (2001b), worked with mitochondrial small subunit ribosomal RNA (mt SSU rDNA) to differentiate *Botryosphaeria* species with very similar or identical ITS sequences and morphologies. This phylogenetic analysis of mt SSU rDNA sequences did not support the separation of the genera *Fusicoccum* and *Diplodia*, as was

Botryosphaeria spp. can also be involved in causing a specific canker disease on a host. For example, the fungal gummosis or gummosis cankers, characterized by numerous gum deposits on the trunk, limbs, and twigs of peach (*Prunus persica* (L.) Batsch) trees (Reilly & Okie, 1982), is caused by three *Botryosphaeria* species, namely *B. obtusa*, *B. dothidea* and *B. rhodina*. These species are indistinguishable on the basis of symptoms. They, however, differ in frequency with *B. obtusa* isolated more frequently than *B. dothidea* and *B. rhodina* (Britton & Hendrix, 1982, 1986; Britton *et al.*, 1990).

Apart from *Prunus* mentioned above, *Botryosphaeria* spp. are important for causing canker diseases in various other agricultural crop trees or woody shrubs. *Botryosphaeria dothidea* and *B. obtusa* cause major diseases of apple in Georgia (Britton & Hendrix, 1986). Both *B. dothidea* and *B. obtusa* produce cankers on apple that can result in decline of entire trees. In Chile, cankers on the trunks of Red King Oregon apple trees are caused by *B. dothidea* (Latorre & Toledo, 1984). *Lasiodiplodia theobromae* is associated with tan lesions of the inner wood, copious gumming, and a consistent association with freeze-damage tissue, which was observed on young citrus trees (Davis *et al.*, 1987; Sangchote, 1991). *Botryosphaeria corticis* causes cankers on different varieties of blueberry (Clayton & Fox, 1963; Milholland & Galletta, 1969; Milholland, 1984).

Botryosphaeria spp. are important canker pathogens of forestry trees, such as *Eucalyptus*. The most common symptoms on *Eucalyptus* are the presence of cankers on stems and branches and resultant dieback (death of tree tops). The cankers are characterized by swelling of the stems or lateral branches. Usually the bark cracks around the lesions and copious amounts of black reddish resin (excretion of polyphenols) are produced (Ciesla, *et al.*, 1996; Old, 2000; Old & Davison, 2000).

Other symptoms

Apart from cankers, *Botryosphaeria* spp. also produces various other symptoms such as sapwood (blue stain), fruits, leaves, stems, twigs and roots of woody plants. Some of these are as follows:

Fruits - *Botryosphaeria obtusa* causes white and black rot of apple fruit, while *B. dothidea* causes ring rot on immature and mature fruits (Brown & Britton, 1986; Brown-Rytlewski & McManus, 2000; Ogata *et al.*, 2000). *Lasiodiplodia theobromae* is associated with stem end rot disease of mango (Davis *et al.*, 1987; Sangchote, 1991). Ripe fruit rot of *Actinidia deliciosa* (kiwifruit) is caused by *F. aesculi, F. parvum* and *F. luteum* (Pennycook & Samuels, 1985). *Dothiorella aromatica* (which possibly represents *F. aesculi* or *F. luteum*) is associated with symptoms of fruit rot on avocado (Darvas & Kotze, 1987).

Die-backs and other diseases on twigs, branches and stems - A complex of *B. ribis*, *F. luteum* and other fungi with *Fusicoccum* anamorphs cause panicle and shoot blight on California pistachio (Smith *et al.*, 2001b; Ntahimpera *et al.*, 2002; Ma & Michailides, 2002). Plants of high-bush blueberry with stem blight in North Carolina was reportedly caused by *B. dothidea* (Witcher & Clayton, 1963). *Botryosphaeria populi* (synonym of *B. dothidea*) was found on dead branches of *Populus nigra* L. (Phillips, 2000). Dieback on branches of macadamia (*Macadamia integrifolia* and *M. tetraphylla*) is induced by *B. ribis* (Herbert & Grech, 1985).

Leaves - *Botryosphaeria pipturi* causes leaf spot on the endemic understory species *Pipturus hawaiiensis* Levl. (Gardner & Hodges, 1998). Leaf blight on coconut palms in Brazil is attributed to *B. cocogena* (Subileau *et al.*, 1994). In South Africa, *B. ribis* occurs in lesions on *Eucalyptus camaldulensis*, *E. cladocalyx*, *E. grandis*, *E. globulus* and *E. nitens* leaves (Crous *et al.*, 1989). Likewise, *Botryosphaeria ribis* has been reported from leaf spots on eucalypts in Spain (Ruperez & Munoz, 1980).

Uncommonly reported diseases - *Botryosphaeria ribis* has been reported causing seed capsule abortion and twig dieback on *E. camaldulensis* in Florida (Barnard *et al.*, 1987; Webb, 1983). Root rot on *Eucalyptus* sp. is reportedly caused by *B. ribis* in Argentina (Frezzi, 1952). Uncommon branch contortions, swellings, witches-broom, and eventual death of tissue are associated with *B. mamane* on the leguminous forest species *Sophora chrysophylla* in Hawaii (Gardner, 1997).

BOTRYOSPHAERIA AS AN ENDOPHYTIC FUNGUS

Endophytic fungi are able to colonise healthy plant tissue without exhibiting virulence, thus not causing obvious damage at the time of infection (Carroll, 1990; McCutcheon *et al.*, 1993). Latent pathogens also share an endophytic relationship with their hosts, causing quiescent infections for long periods of time and symptoms appear only when the physiological or ecological conditions favour virulence (Tokuna & Ohira, 1973; Pusey, 1989; Bettucci & Saravay, 1993; Smith *et al.*, 1996a, b).

Species of *Botryosphaeria* can be endophytic in *Eucalyptus* tissue (Fisher *et al.*, 1993; Smith *et al.*, 1996a), as well as pathogenic in stressed trees (Pusey, 1989; Old *et al.*, 1990). In South Africa, potentially pathogenic *Botryosphaeria* spp. (e.g. *B. dothidea*

and *B. parva*) have been shown to occur as symptomless endophytic infections on leaves and in xylem of *Eucalyptus* (Smith *et al.*, 1996b). The presence of *Fusicoccum eucalypti* in healthy (endophytic fungus) and symptomatic tissue of twigs of *E. grandis* was reported from Uruguay (Bettucci & Alonso, 1997; Bettucci *et al.*, 1999).

OCCURRENCE OF *BOTRYOSPHAERIA* ON *EUCALYPTUS*

Various *Botryosphaeria* spp. have been associated with *Eucalyptus* cankers in Australia where these trees are native. In species selection trials of *E. radiata*, *B. ribis* was reported to cause dark brown discoloration of the phloem with extensive kino veins often formed ahead of phloem necrosis and light purplish brown discoloration of the xylem (Davison & Tay, 1983; Shearer *et al.*, 1987). *Botryosphaeria dothidea* and *B. ribis* were isolated from the bark of trees and stumps of *E. marginata*, causing basal canker and die-back in Australia (Davison & Tay, 1983). Slippers *et al.* (2004c) identified five *Botryosphaeria* spp. (*B. parva*, *B. dothidea*, *B. eucalyptorum*, *B. australis* and *B. eucalypticola*) from *Eucalyptus* in native forest and plantations in eastern Australia. *Botryosphaeria eucalyptorum* was the most abundant, and represented almost 50% of isolates in this last study.

Different *Botryosphaeria* spp. have been associated with *Eucalyptus* in the different countries where these trees have been introduced. A survey of the most important forestry areas of South Africa showed several eucalypt species and clones with die-back and canker associated with extreme environmental conditions, and where *B. dothidea* was consistently isolated from the trees (Smith *et al.*, 1994). *Botryosphaeria ribis* was also reported from leaves of eucalypts in South Africa (Crous *et al.*, 1989). Later studies by Smith *et al.* (2001a) and Slippers *et al.* (2004c), however,

showed that *B. parva, B. eucalyptorum* and *B. eucalypticola* are the dominant species on *Eucalyptus* in this country. In other regions of Africa, such as the Congo and Uganda, *Lasiodiplodia theobromae* (teleomorph = *B. rhodina*) has been reported as an important threat to the eucalypt plantations, causing stem cankers with copious exudation of resin and xylem discoloration (Roux *et al.*, 2000, 2001).

In South America several *Botryosphaeria* spp. are known to occur on *Eucalyptus*. For example, *B. ribis* is reported to cause symptoms such as stick rot and canker on *E. grandis* and *E. citriodora* in Brazil (de Arruda Silveira, 2001) and cankers on stems and branches in Argentina (Frezzi, 1952). In Colombia, both *B. ribis* and *B. dothidea* were found to cause *Eucalyptus* diseases (Rodas, 2003). In Chile, however, *B. parva, B. eucalyptorum* and *B. eucalypticola* are the most dominant species associated with *Botryosphaeria* canker and die-back diseases on *Eucalyptus* (Ahumada, 2003).

Despite the fact that *Botryosphaeria* spp. are commonly associated with disease of *Eucalyptus*, these fungi are considered to be weak pathogens of this host. They cause disease mostly on wounded or stressed plants, following drought, hot or cold winds, nutritional imbalance, water logging, hail wounds, insect damage and damage by other pathogens (Smith *et al.*, 1994, 1996b). In Australia, the pathogenicity (based on their ability to cause stem lesions) of several fungal species was tested on 12-month-old seedlings of *E. nitens* and *E. globulus* (Yuan & Mohammed, 1999). The *Botryosphaeria* isolates used in that study were shown to be intermediately or weakly pathogenic.

OCCURRENCE OF *BOTRYOSPHAERIA* ON *PINUS*

Botryosphaeria spp. cause damage on differents *Pinus* species. The *Botryosphaeria* species most commonlhy reported on *Pinus* spp. is *Diplodia pinea* (=*Sphaeropsis*

sapinea). This pathogen causes extensive losses in commercial plantation forestry, especially where susceptible *Pinus* spp. are intensively propagated (Zwolinski *et al.*, 1990).

In Brasil, *Diplodia pinea* has been reported on *Pinus radiata* and *P. pinaster* from 3-4 year-old trees causing die-back, blue-stain and mortality (Ferreira, 1989). In South Africa the fungus is considered the most important pathogen of pines, causing serious annual losses due to dieback after hail on *Pinus radiata* and *P. patula* (Swart *et al.*, 1985, 1987; Zwolinski *et al.*, 1990). In addition, *P. radiata* is susceptible to drought, and are vulnerable to damage by *D. pinea* in New Zealand (Thomson, 1969) and Australia (Marks & Minko, 1969; Davison *et al.*, 1991)

D. pinea has been shown to be present as latent endophytic infections in cones, shoots, needles and from stems of pines seedlings (Smith *et al.*, 1996a; Stanosz *et al.*, 1997; Burgess *et al.*, 2001a), and it has aslso been found on various *Pinus* spp. producing various disease symptoms, of which die-back, cankers, root disease, crown wilt and a saprophytic inhabitant of sapwood (blue stain) are most common (Mohali, 1997; Swart *et al.*, 1985, 1987; De Wet *et al.*, 2000).

Botryosphaeria dothidea has been reported causing wilt and death of *Pinus taeda* and *P. elliottii* var. *elliottii* in Hawaii (Hodges, 1983). *Lasiodiplodia theobromae* has been associated with discolouration from seeds of *Pinus elliottii* in South Africa, which reduced the germination (Carneiro, 1986; Rees, 1988; Fraedrich & Miller, 1989). Tip die-back of *Pinus taeda* and *P. elliottii* seedlings and blue stain of *P. massoniana* has been attributed to *L. theobromae* (Rowan, 1982; Fu *et al.*, 1988).

BOTRYOSPHAERIA IN VENEZUELA

One of the most serious problems caused by a *Botryosphaeria* spp. in Venezuela is blue stain of Caribbean pine (*Pinus caribaea* var. *hondurensis*). There are approximately 400.000 ha of this pine species planted in the country. Blue stain of the Caribbean pine timber is a problem for sawmills, especially in the eastern part of Venezuela. The blue stain produces changes in the natural colour of the wood, making it aesthetically undesirable for the carpentry and paper production industry. This alteration in wood colour also results in a reduction in the wood price of up to 50%, representing large economical losses to the forestry industry (Mohali, 1993).

Blue stain of Caribbean pine is caused by different species of *Botryosphaeria*, of which *L. theobromae* (teleomorph *B. rhodina*) is the most important. Recently it has been shown that *D. mutila* also causes blue stain on Caribbean pine logs (Mohali & Encinas, 2001). Furthermore, Mohali (1997) reported *D. pinea* (= *S. sapinea*) as the causal agent of chlorosis and needle fall, as well as blue stain of the stems on Caribbean pine, in Yaracuy state, Venezuela.

Botryosphaeria anamorphs have been reported on agricultural hosts in Venezuela. Brown rot disease was observed on harvested peach fruits and is consistently associated with *Dothiorella dothidea* (an invalid name, which might refer to a number of different *Fusicoccum* species) (Cedeño *et al.*, 1994). *Lasiodiplodia theobromae* was reported as the causal agent of the dieback on passion fruit vines, which caused a significant reduction in the production (Cedeño *et al.*, 1995).

OCCURRENCE OF *BOTRYOSPHAERIA* ON *ACACIA*

Very severe cankers have been reported by Pongpanich (1997) on *Acacia auriculiformis* associated with infection by *Botryosphaeria* spp. in a trial in Western Thailand, with 80 % mortality in some seed. A trial the same provenances (*A. auriculiformis*) from Thailand planted in South Kalimantan, Indonesia, was severely affected by a canker disease caused by *Lasiodiplodia theobromae* (Hadi & Nuhamara, 1997).

A plantation of 2 ha of single seed source of *Acacia aulacocarpa* at Sakaerat in eastern Thailand suffered about 40% mortality through combined attack by borers and infection by *Botryosphaeria* spp. (Pongpanich, 1997). In southern India, severe basal cankers in a small planting of *Acacia crassicarpa* in two locations were associated with infection by *L. theobromae* (Sharma & Florence, 1997).

During a 2-year period, a survey of diseases, pathogenicity tests on *Acacia mearnsii* were conducted in South Africa (Roux & Wingfield, 1997). *Diplodia* and *Botryosphaeria* species were frequently isolated from diseased tissue, besides producing noticeable lesions in inoculation tests (Roux & Wingfield, 1997).

Botryosphaeria rhodina and *B. dothidea* have both been reported from native Australian *Acacia* spp., where these trees are planted as exotics in South Africa (Roux, 1998). In contrast, isolations from the same species as natives Australia have yielded *Botryosphaeria australis*, as new specie which appears to be native to the Southern Hemisphere (Slippers *et al.*, 2004).

CONCLUSIONS

Botryosphaeria spp. are known as important pathogens of various crops in Venezuela. Identifications thus far have, however, only been based on morphological data. Those identifications are outdated with respect to modern taxonomic treatments of the group. The correct identification of such *Botryosphaeria* spp., including previously unrecognized species and cryptic species, must be the first step for future studies and eventual efforts to control them. Based on such information, questions regarding the role of different pathogens, distribution and movement of pathogens and host resistance to key pathogens, can be addressed. This is especially true for *Botryosphaeria* spp. that occur on *Eucalyptus*. The growing importance of this crop in Venezuela and the dangers of common pathogens such as *Botryosphaeria* to clonal forestry makes this important and urgent.

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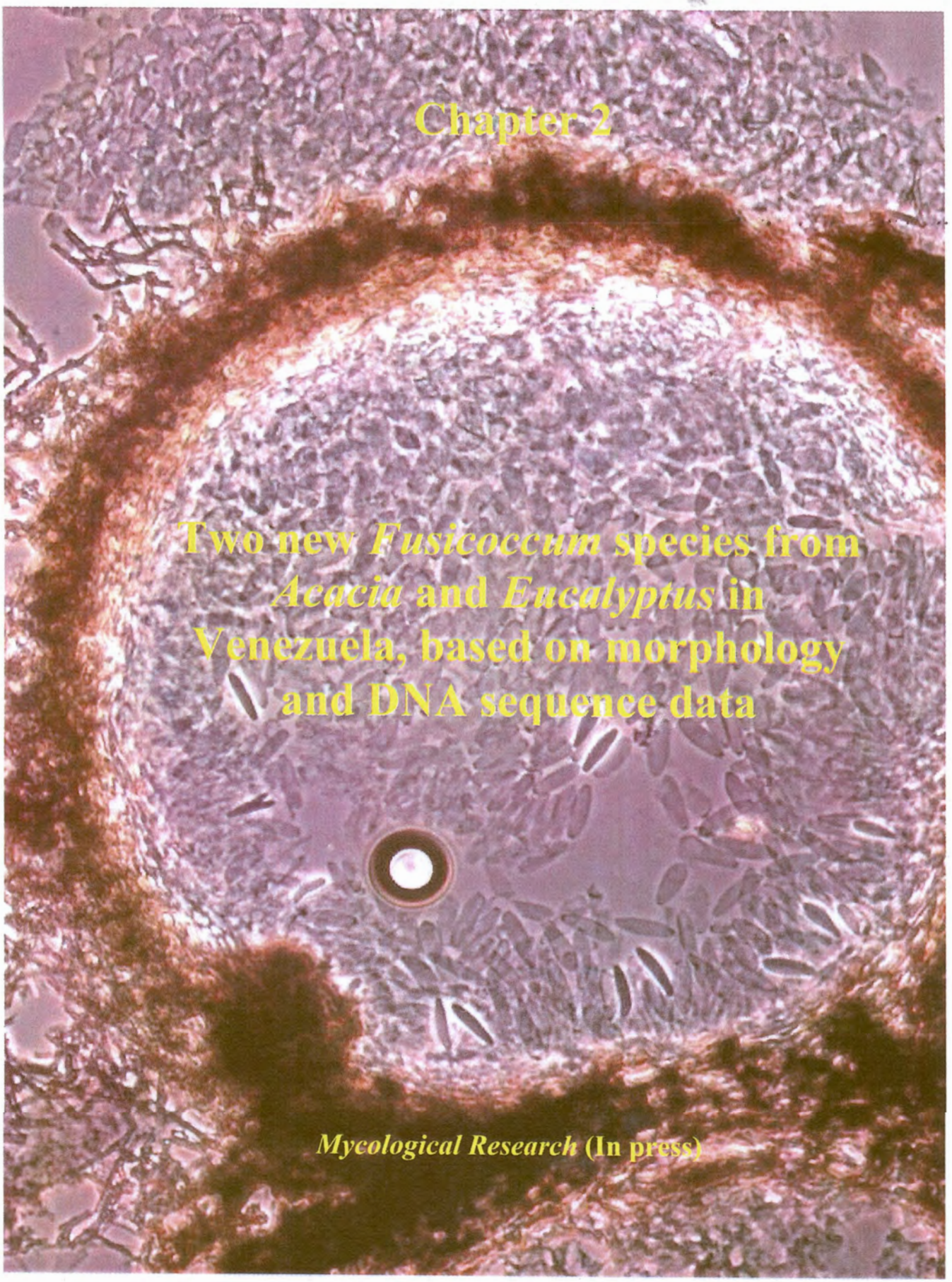

Chapter 2

Two new *Fusicoccum* species from *Acacia* and *Eucalyptus* in Venezuela, based on morphology and DNA sequence data

Mycological Research (In press)

Two new *Fusicoccum* species from *Acacia* and *Eucalyptus* in Venezuela, based on morphology and DNA sequence data

Botryosphaeria spp. are common endophytes of woody plants and they also include some serious pathogens of *Eucalyptus* and *Acacia* species. In this study, we characterise two new *Botryosphaeria* anamorphs, isolated from *Eucalyptus* and *Acacia* trees in Venezuela. These fungi were characterised based on morphological features in culture and comparisons of DNA sequence data. The two taxa, which have been provided the names *Fusicoccum andinum* and *Fusicoccum stromaticum*, resided in two well-supported clades (bootstrap values = 100 %) based on a combined data set of the internal transcribed spacers (ITS) of the rDNA operon and translation elongation factor 1-α (EF1- α) gene sequences. The conidia of *F. andinum* are unusually large amongst *Botryosphaeria* anamorphs and peripherally resemble those of *B. mamane* and *B. melanops*. *Fusicoccum stromaticum* is characterized by large conidiomata in culture, growth at 35 °C and slightly thickened conidial walls, characteristics different from most other *Fusicoccum* spp. No teleomorph states were observed for these fungi, but DNA sequence data show that they are anamorphs of *Botryosphaeria*.

INTRODUCTION

Species of *Botryosphaeria* Ces. & De Not have a cosmopolitan distribution and occur on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts. *Botryosphaeria* spp. infect the stems, branches, twigs and leaves of many woody plants and they have also been found in the stems of grasses and thalli of lichens (Barr, 1987). These fungi include opportunistic pathogens that give rise to symptoms such as shoot blights, stem cankers, fruit rots, die-back and gummosis (von Arx, 1987; Old, 2000; Old & Davison, 2000).

The taxonomy of *Botryosphaeria* has been confused for many years. This is mainly due to the fact that the morphology of the teleomorphs is very similar and these states are rarely encountered either in nature or under laboratory conditions (Jacobs & Rehner, 1998; Slippers *et al.*, 2004a). Host association has been used to assign names to species but this has led to confusion because some species are host specific, while others are generalists (Jacobs & Rehner, 1998; Smith *et al.*, 2001; Smith & Stanosz, 2001; Crous & Palm, 1999; Slippers *et al.*, 2004a).

The anamorphs of *Botryosphaeria* species are generally encountered in culture or on diseased plant parts. For this reason, identification of *Botryosphaeria* spp. has commonly been based on conidial morphology of the anamorphs (Jacobs & Rehner, 1998; Smith & Stanosz, 2001; Smith *et al.*, 2001; Phillips *et al.*, 2002; Slippers *et al.*, 2004a, d).

Conidial characters considered to be useful for the taxonomic delimitation of *Botryosphaeria* anamorphs are size, color, septation, wall thickness and texture, as well as the presence of microconidia and mode of conidiogenesis (Sutton, 1980; Sivanesan, 1984; Pennycook & Samuels, 1985). These characters, however, require careful

interpretation, as there is substantial overlap between the characters of many species. Thus conidial size represents a continuous character and it is also variable between isolates and may change with age or on substrates and hosts (Pennycook & Samuels, 1985; Butin, 1993; Crous & Palm, 1999; Slippers *et al.*, 2004a).

In recent years, analyses of DNA sequence data have contributed substantially towards resolving taxonomic questions in *Botryosphaeria*. Nucleotide sequences of the internal transcribed spacers (ITS) have in particular been used to resolve phylogenetic relationships between species and these have aligned with morphological characters (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Zhou & Stanosz, 2001; Phillips *et al.*, 2002; Alves *et al.*, 2004; Slippers *et al.*, 2004a). Another approach to characterise *Botryosphaeria* spp. is to use comparisons of multiple gene sequences and restriction fragment length polymorphism (RFLP) of anonymous simple sequence repeat (SSR) loci to distinguish closely related species such as *Botryosphaeria parva* Pennycook & Samuels and *B. ribis* Grossenb. & Duggar (Slippers, 2003; Slippers *et al.*, 2004a).

Botryosphaeria spp. are known to occur on various forestry and agricultural crops in Venezuela, but very little attention has been given to their identity (Cedeño *et al.* 1994, 1996; Mohali, 1997; Mohali & Encinas, 2001; Mohali, Encinas & Mora, 2002). *Lasiodiplodia theobromae* (Pat.) Griffon & Maublanc., *Diplodia pinea* (Desm.) Kickx (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton), *D. mutila* (Fries) Mont., and a species of *Dothiorella* Sacc. have been identified as the disease causing agents (Cedeño *et al.*, 1994, 1996; Mohali, 1997; Mohali & Encinas, 2001; Mohali *et al.*, 2002; De Wet *et al.*, 2003).

The aim of this study was to characterise two *Fusicoccum* spp. commonly isolated from *Eucalyptus* and *Acacia* trees in Venezuela, and which appeared to be undescribed. These fungi were thus studied based on morphology and a comparison of

DNA sequence data for the ITS rDNA (ITS 1 and ITS 2) and translation elongation factor 1-α (EF1- α).

MATERIALS AND METHODS

Isolates and morphological characterization

A survey was conducted in plantations of *Eucalyptus urophylla* S.T. Blake, an unidentified *Eucalyptus* sp., a *Eucalyptus*-hybrid and *Acacia mangium* Willd., during 2003. Isolations were made from twigs, stems and branches displaying symptoms of blue stain or dieback, and from dead trees. Single conidial isolates were obtained after cultures were induced to sporulate on water agar to which sterile pine needles had been added.

For isolations, plant tissues were surface disinfested with 70 % ethanol for 30 s and thereafter rinsed in sterile water for 1 min. Small tissue pieces (4-5 mm) were cut from the plant tissue and placed on 2 % malt extract agar (MEA; DIFCO, Detroit, MI, USA) and incubated at 25 °C. Cultures resembling *Botryosphaeria* spp. were transferred to water agar (WA) (2 % Biolab agar, Midrand, South Africa) with sterilized pine needles placed on the agar surface. These were incubated for 3-6 weeks at 25 °C under a combination of near-ultraviolet and cool-white fluorescent light to induce sporulation. All isolates used in this study are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

Conidial 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 with an American Optical Freezing Microtome. Length, width; shape and color of the conidia were recorded after mounting in clear lactophenol. At least 50 conidia of each isolate of two different *Fusicoccum* spp. were measured.

The growth of selected isolates was determined by placing mycelial discs (5 mm diam) at the centres of MEA plates, with three replicate plates for each of three isolates for each of the two morphologically different *Fusicoccum* spp. Plates were incubated at temperatures ranging from 15-40 °C at 5 °C intervals. Two diameter measurements were taken perpendicular to each other after 4 d for each colony, and averages computed. Colony colors were determined using the color charts of Rayner (1970).

DNA isolation and amplification

DNA was extracted from isolates of unknown identity (Table 1) using the technique described by Slippers *et al.* (2004a). The quantification of nucleic acids was made using a spectrophotometer with a radio of absorbance at 260 nm and 280 nm.

The DNA extraction was used as template to amplify 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. A part of the EF1-α was amplified using the primers EF1-728F and EF1-986R (Carbone *et al.*, 1999). The PCR reaction mixture contained 0.02 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), 1X PCR buffer containing $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA), 0.4 mM of each dNTPs, 0.2 µM of each primer and 20-25 ng/µl of DNA template and made up to a final volume of 25 µl with

Sabax water. Standard PCR reaction cycles were followed with primer annealing at 58 °C. Due to difficulties in amplifying the EF 1-α region for some isolates it was necessary to vary the PCR annealing temperature between 52 to 60 °C for this region. PCR amplicons were separated on 1.5 % (w/v) agarose gels, stained with ethidium bromide and visualized under UV light. The sizes of the PCR amplicons were estimated using DNA molecular weight marker XIV (100 bp ladder) (Roche Molecular Biochemicals, Mannheim, Germany).

Sequence analysis

A total of twenty-seven isolates were used in the phylogenetic analysis (Table 1). All the sequences used are from isolates maintained in the CMW and CBS culture collection. BLAST searches were done to determine whether any related sequences are present in GenBank, but none were found that were more closely related to the test isolates than those chosen for comparison here. The trees were rooted to sequence data of an isolate of a *Bionectria* sp., which was included as an outgroup taxon in the analysis of 30 ingroup taxa.

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, ITS4 and EF1-728F, EF1-986R. 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 automated sequencer (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. Gaps were treated as a fifth character and all characters were given equal weight. Phylogenetic analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford, 1999). Heuristic searches were done using random stepwise addition tree bisection and reconstruction (TBR) as branch swapping algorithm for the construction of maximum parsimonious trees. 1000 bootstrapping replicates (Felsenstein, 1985) were run to determine the confidence intervals of branching 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.

RESULTS

Morphological characters

The two unknown *Fusicoccum* spp. from *Acacia* and *Eucalyptus* in Venezuela which residing in distinct clades in the phylogenetic trees, produced conidiomata on sterilized pine needles at 25 °C on WA after 3 weeks.

The colonies of *Fusicoccum* sp., isolated from *Eucalyptus* and *Acacia* in the states of Portuguesa and Cojedes (Table 1), grew rapidly and covered the surface of the Petri dishes at 30 °C in four days, but produced little or no growth at extremes of 15 °C and 40 °C. This fungus produced few, but large conidiomata, on MEA. The conidia were hyaline, with thin to slightly thickened walls, aseptate, bacilliform, straight to slightly curved, and their apices and bases were both blunt or bluntly rounded, 21.7 x 5.4 µm, l/w 4.01 (average of 50 conidia) (Figs. 2-8).

Colonies of the *Fusicoccum* sp. isolated from *Eucalyptus* spp. growing on the mountains in Merida state (Table 1), grew at 15 °C with an optimum growth temperature of 20 to 30 °C. Abundant pycnidia were produced on MEA at 25 °C with conidia oozing from these structures (Figs 9-12). Conidia were clavate to slightly navicular and large when compared with other *Fusicoccum* anamorphs, reaching 40 µm in length, 27.1 x 5.6 µm, l/w 4.84 (average of 50 conidia) (Table 2, Figs 13-16).

Phylogenetic analyses

The partition homogeneity test indicated that the ITS-rDNA (547 characters) and EF1-α (340 characters) sequence partitions were congruent and that the data sets could be combined (P value = 0.440). This resulted in a final data set of 887 characters after alignment of which 289 characters were constant, 163 variable characters were parsimony-uninformative and 435 were parsimony informative. Heuristic search analysis in PAUP of the sequence data resulted in one tree [Consistency Index (CI) = 0.763; Retention Index (RI) = 0.865; Homoplasy Index (HI) = 0.237].

The isolates in the tree obtained from the combined data sets resided in thirteen principal clades (I to XIII)(Fig. 1). Isolates in clades I to X all have hyaline and thin-walled conidia and are thus *Fusicoccum*-like anamorphs. In contrast, isolates residing in clades XI to XIII all have pigmented and thick-walled conidia that can be referred to as *Diplodia*-like anamorphs (Denman *et al.*, 2000; Slippers *et al.*, 2004a). Isolates from Venezuela (VZLA) resided in clade I and clade IX and were distinct from all other clades that included known *Botryosphaeria* spp. (Fig. 1). The *Fusicoccum* sp. from Venezuela residing in clade I and *B. dothidea* (Fr.:Mough.) Ces. & De Not. (Slippers *et al.*, 2004a) in clade X were each strongly supported (100% bootstrap) and distinct from other *Botryosphaeria* spp with *Fusicoccum*-like anamorphs. The *Fusicoccum* sp. from

Venezuela residing in clade IX (100 % bootstrap support) was related to all the following species: *B. parva* and *B. ribis* (clade II, III), *B. eucalyptorum* Crous, H. Smith et M. J. Wingf. (clade IV), *B. eucalypticola* Slippers, Crous & M. J. Wingf. (clade V), *B. australis* Slippers, Crous & M. J. Wingf. (clade VI), *B. lutea* A. J. L. Phillips (clade VII) and *Fusicoccum mangiferum* (Syd. & P. Syd.) Johnson, Slippers & M. J. Wingf. (clade VIII) (Phillips *et al.*, 2002; Slippers *et al.*, 2004b, c, d), *Botryosphaeria obtusa* (Schwein.) Shoemaker, *B. stevensii* Shoemaker and *B. rhodina* (Berk. & Curt.) Arx (clades XI, XII, XIII), all with *Diplodia*-like anamorphs, formed a well defined group (Alves *et al.*, 2004; Punithalingam, 1976).

TAXONOMY

Based on conidial morphology, cultural characteristics and DNA sequence phylogeny we conclude that the two *Fusicoccum* spp. from *Eucalyptus* and *Acacia* in Venezuela represent undescribed taxa. We thus provide the following descriptions for them here.

Fusicoccum stromaticum. Mohali, Slippers & M. J. Wingf., **sp. nov.** (Figs 2-8)

Etym.: The name refers to the very large conidiomata on MEA at 25 °C.

Culturae laxe lanuginosae, superficie viride-olivaceae, infra post 15 dies in MEA ad 25 °C olivaceae. Coloniae ad 70-75 mm diametro post 4 dies in tenebris ad 25 °C. Aegre vel non creverunt ad 15 °C, non ad 40 °C, optime inter 30 °C et 35 °C creverunt. *Conidiomata* magna in superficie MEA, multilocularia, eustromatica, cum hyphis tecta; *loculus* omnino inclusus sine ostiolis, parietibus e textura angulari atrobrunnea compositis, ad aream conidiogenam tenuescens hyalinescensque. *Cellulae conidiogenae* hyalinae, holoblasticae, laeves, cylindricae, conidium unicum apicale efferentes, primo

reaching 80 mm diam on MEA after 4 d in the dark at 25 °C. Cardinal temperatures for growth were min 15 °C (reaching an average 24 mm in diam.), max 35°C (no growth), optimum 20 to 30 °C. *Pycnidia* superficial, produced abundantly on MEA surface at 25 °C (Fig. 9), oozing conida after 30 d at 25 °C on MEA (Fig. 10), solitary or botryose on the colonies, stromatic, globose (Figs 11-12), (331-) 374-597 (-740) x (302-) 339-557 (-671) µm (average of 50 pycnidia 486 x 448 µm, l/w 1.08); pycnidial wall, composed of brown *textura angularis*, 6-8 cell layers thick. *Conidiogenous cells* hyaline, holoblastic, smooth, cylindrical, producing a single apical conidium, the first conidium holoblastic and subsequent conidia enteroblastic (Fig. 13), (8-) 11-17 (-23) x (1.5-) 2 -2.5 (-3.0) µm (average of 50 conidiogenous cells 13.9 x 2.1 µm, l/w 6.62). *Conidia* hyaline, granular, clavate to slightly navicular, apex obtuse and base truncate, 0-1 septa (Figs 14-16), (19-) 23-31 (-40) x (4-) 5-6 (-8) µm (average of 50 conidia 27.1 x 5.6 µm, l/w 4.84).

Teleomorph. Not observed, but expected to be a *Botryosphaeria* sp. based on phylogenetic analyses.

Additional specimens examined.- **VENEZUELA**. *Merida State*: Merida, Mucuchies (3140 m) Cordillera of Los Andes, on branches of *Eucalyptus* sp., Feb. 2003; S. Mohali (PREM 58518, 58519, 58520, 58521, 58522, 58523, 58524, 58525, 58526, 58527, 58528, 58529, 58530, 58531, 58532).

DISCUSSION

Two new *Fusicoccum* spp. collected in Venezuela have been characterised in this study, based on both morphology and their unique DNA sequences. One of these fungi, now known as *Fusicoccum andinum*, was isolated exclusively from *Eucalyptus* spp. at high altitude sites, whereas *F. stromaticum* was from both *Eucalyptus* spp and *Acacia* spp. at

lower altitude sites in Venezuela. To the best of our knowledge, these are the first two species of *Fusicoccum* to be described from Venezuela.

Fusicoccum andinum was collected from *Eucalyptus* spp. growing in the Cordillera Los Andes mountains of Venezuela at an altitude of approximately 3000 m. The daily mean temperature of this region is 10 °C and these extreme environmental conditions most probably explains the lower optimum growth temperature of *F. andinum* in culture. *Fusicoccum andinum* grew at 15 °C, had an optimum at 20 to 30 °C and showed no growth above 35 °C. This is a low optimum temperature for growth when compared with other *Botryosphaeria* species such as *B. dothidea*, *B. parva*, *B. ribis*, *B. mamane*, *B. corticola* A. J. L. Phillips, Alves et Luque, *B. lutea*, *B. eucalyptorum*, *B. eucalypticola*, *B. australis* and *B. protearum* Denman & Crous (Morgan-Jones & White, 1987; Smith *et al.*, 2001; Gardner, 1997; Denman *et al.*, 1999; Alves *et al.*, 2004; Slippers *et al.*, 2004a, c, d).

Fusicoccum andinum was isolated from old *Eucalyptus* trees mainly from asymptomatic branches without causing apparent damage to trees. We thus assume that the fungus is an endophyte and that it is not pathogenic. This would be consistent with many *Botryosphaeria* spp. that are known to reside as endophytes in asymptomatic or healthy plant tissues on a non-native host (Smith *et al.*, 1996; Fisher *et al.*, 1993). In different areas or under different environmental conditions, such endophytic species have, however, been considered important pathogens (Fisher *et al.*, 1993; Smith *et al.*, 2001).

Isolates of *F. andinum* formed a well-defined group based on analyses of sequence data. They are also morphologically distinct. The conidia of this fungus are large when compared with those of other *Fusicoccum* species. Two other *Botryosphaeria* spp. with comparatively large conidia are *B. mamane* (Gardner, 1997)

and *B. melanops* (Tul.) Wint. (Shear & Davidson, 1936), although these are larger than those of *F. andinum*. Other than the relatively large conidia found in *F. andinum*, this species can also be distinguished by its clavate to slightly navicular conidia. These are different to those of *B. mamane* and *B. melanops* that have fusiform conidia. Thus, the combination of relatively large and clavate to slightly navicular conidia, makes *F. andinum* easy to recognise.

Fusicoccum stromaticum was isolated from branches and stems of *Eucalyptus* and *Acacia* trees, with and without symptoms. These trees were growing in the Portuguesa and Cojedes states at an altitude of 150 to 200 m. The annual medium temperatures of these regions ranges between 26 to 30 °C and this is also consistent with the fact that the fungus had a relatively high optimum temperature for growth in culture of between 30-35 °C, compared with many other *Botryosphaeria* spp. (Pennycook & Samuels, 1985; Morgan-Jones & White, 1987; Smith *et al.*, 2001; Gardner, 1997; Denman *et al.*, 1999; Alves *et al.*, 2004).

Isolates of *Fusicoccum stromaticum* resided in a well-defined group with strong bootstrap support. This confirmed that the fungus represents the anamorph of an undescribed *Botryosphaeria* sp. Furthermore, there were three conspicuous morphological characteristics that distinguished this fungus from other *Fusicoccum* spp. *Fusicoccum stromaticum* has unusually large conidiomata, it grows at 35 °C and its conidia have slightly thickened walls.

Fusicoccum stromaticum was isolated from asymptomatic, as well as dead and dying branches and stems of *Eucalyptus* spp. and *A. mangium* trees. The presence of the fungus on asymptomatic tissue suggests that it is an endophyte. In this regard, it is also similar to *F. andinum* described in the present study. Whether *F. stromaticum* is pathogenic is unknown as it may have simply been present on dying tissue as

saprophyte, without necessarily being the cause of the symptoms observed. *Acacia mangium* and *Eucalyptus* species are important plantation trees in Venezuela and pathogenicity tests with this fungus should be conducted to determine its relative importance in tree health.

Isolates of *F. andinum* and *F. stromaticum* originated from trees that are not native to Venezuela. The fact that these fungi have not been found elsewhere in the world, despite the extensive surveys that have been conducted on *Acacia* and *Eucalyptus* spp. (Roux & Wingfield, 1997; Hadi & Nuhamara, 1997; Sharma & Florence, 1997; Old, 2000; Old & Davison, 2000; Slippers *et al.*, 2004b, c), suggests that these newly described species might be native to Venezuela. However, surveys of native woody plants would be necessary to establish this fact.

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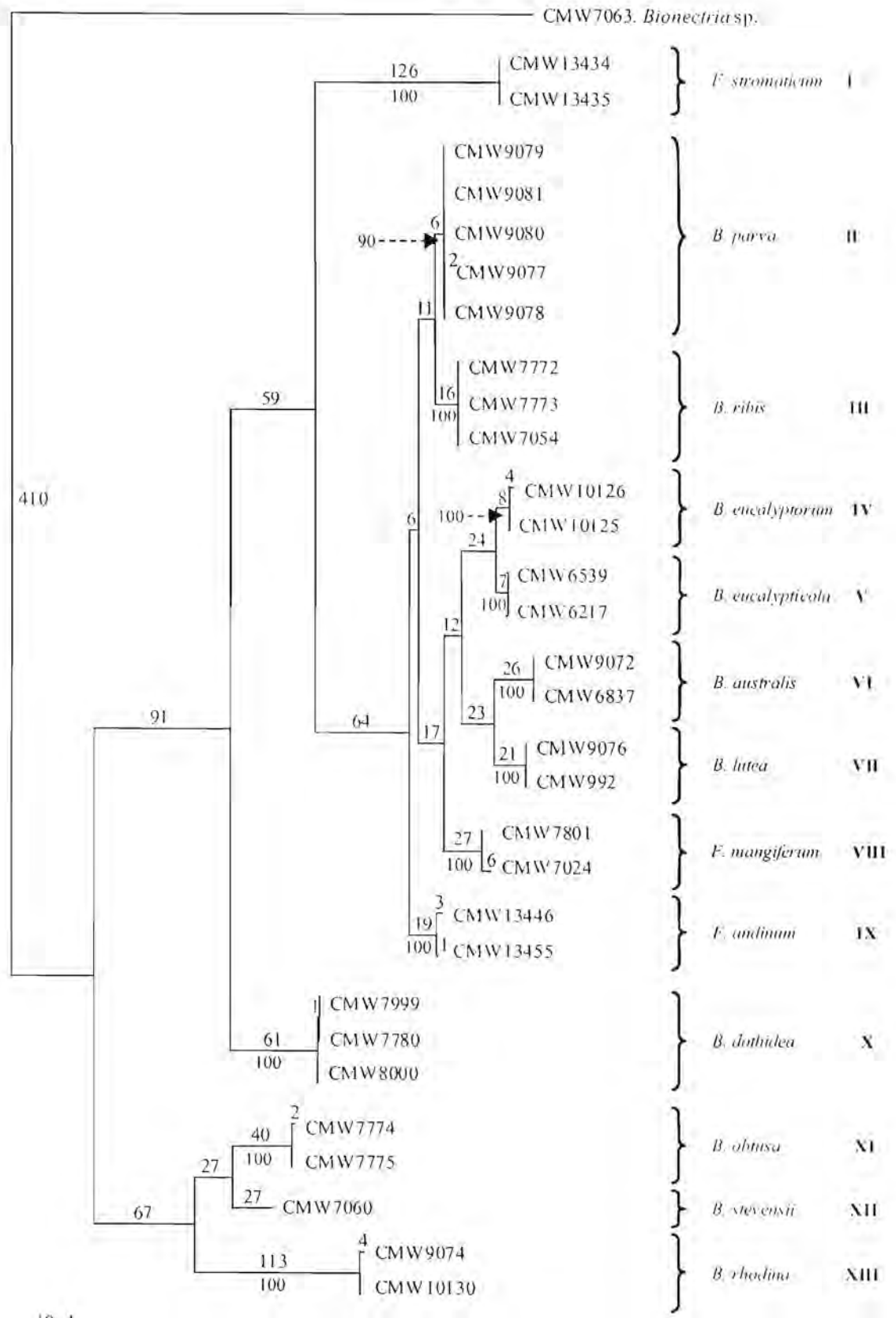
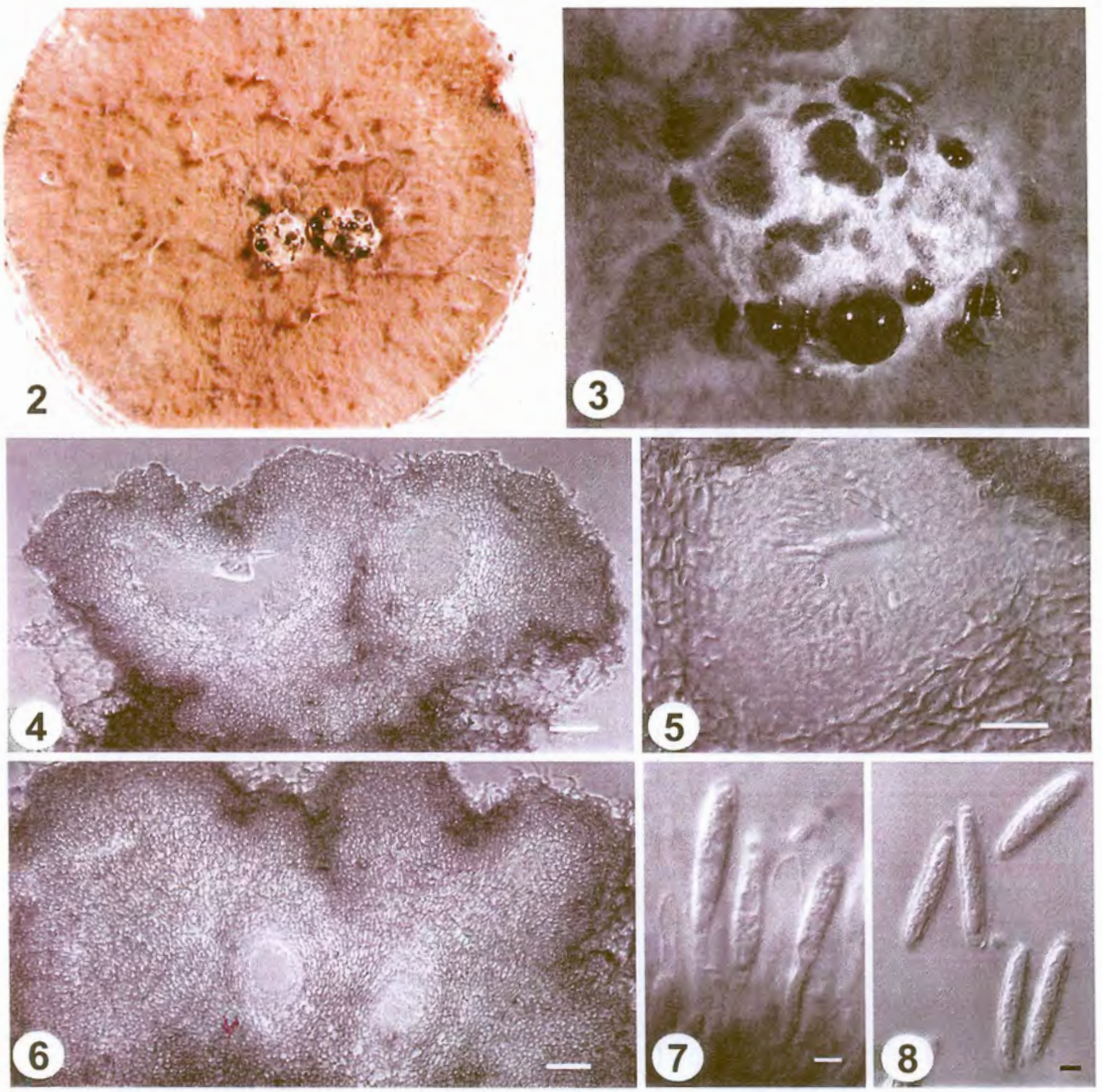
Fig. 1. Phylogenetic relationships amongst *Fusicoccum andinum* and *F. stromaticum* from Venezuela and related species from elsewhere, based on the most parsimonious tree obtained through heuristic searches of the combined dataset of the ITS rDNA and EF1- α regions. The phylogram is rooted with the outgroup *Bionectria* sp. Bootstrap frequencies 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.


CMW7063. Bionectria sp.
CMW13434
CMW13435
F. stromaticum I
CMW9079
CMW9081
CMW9080
CMW9077
CMW9078
B. parva II
CMW7772
CMW7773
CMW7054
B. ribis III
CMW10126
CMW10125
B. eucalyptorum IV
CMW6539
CMW6217
B. eucalypticola V
CMW9072
CMW6837
B. australis VI
CMW9076
CMW992
B. lutea VII
CMW7801
CMW7024
F. mangiferum VIII
CMW13446
CMW13455
F. andinum IX
CMW7999
CMW7780
CMW8000
B. dothidea X
CMW7774
CMW7775
B. obtusa XI
CMW7060
B. stevensii XII
CMW9074
CMW10130
B. rhodina XIII
410
126
100
59
91
64
90
6
2
11
16
100
6
100
4
8
24
7
100
12
26
100
23
17
21
100
27
100
6
3
19
100
1
1
61
100
2
40
100
27
27
67
4
113
100
10 changes

Figs 2-8. *Fusicoccum stromaticum*. **Fig. 2.** Culture with few conidiomata. **Fig. 3.** Big conidioma produced on 2% MEA after 30 d at 25 °C. **Figs 4-6.** Multilocular conidiomata without ostioles and embedded locule. **Fig. 5.** Close-up of a locule. Bars = 50 µm. **Fig. 7.** Conidiogenous cells and conidia. **Fig. 8.** Conidia with thin to slightly thickened walls. Bars = 5 µm.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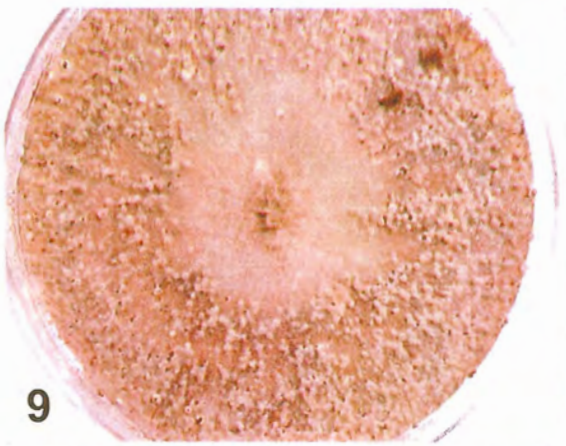
Figs 9-16. *Fusicoccum andinum*. **Fig. 9.** Abundant pycnidia on 2% MEA after 30 d at 25 °C. **Fig. 10.** Pycnidia oozing spore masses. **Fig. 11.** Botryose pycnidia. Bar = 100 µm. **Fig. 12.** Solitary pycnidia. Bar = 50 µm. **Fig. 13.** Conidiogenous cell with conidium. **Fig. 14.** Germinating conidium. **Figs 15-16.** Conidia. Bars = 5 µm.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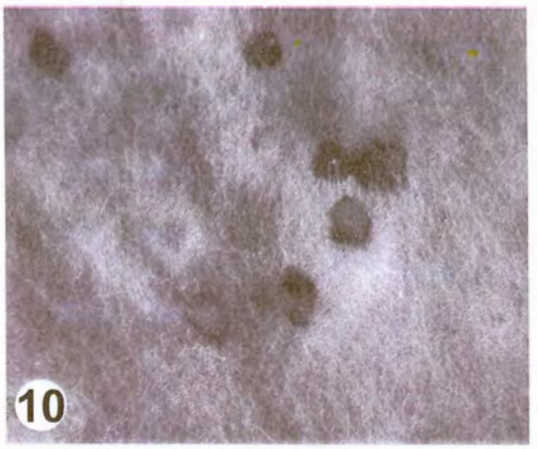
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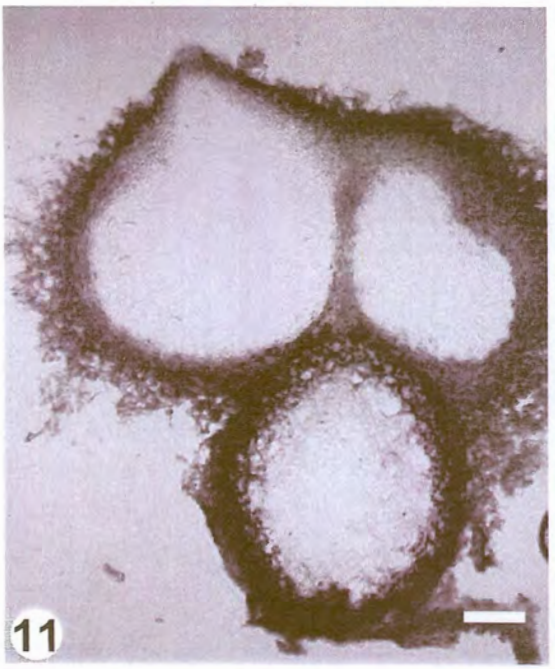
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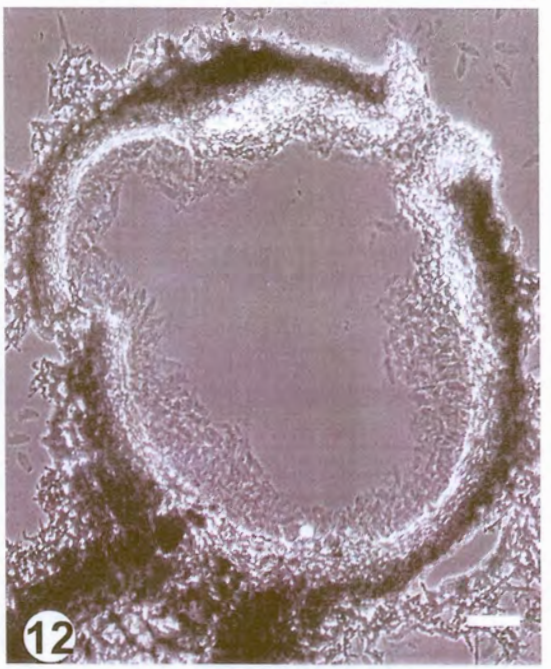
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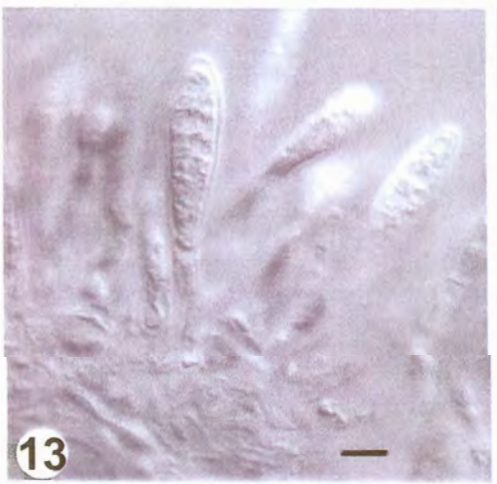
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Table 1. Isolates used in the phylogenetic study.

Culture No.[1]	Other No.[1]	Identity[2]	Host	Location	Isolator	GenBank	
						ITS	EF1-α
CMW7780		*Botryosphaeria dothidea*	*Fraxinus excelsior*	Molinizza, Switzerland	B. Slippers	AY236947	AY236896
CMW7999		*B. dothidea*	*Ostrya* sp.	Crosifisso, Switzerland	B. Slippers	AY236948	AY236897
CMW8000		*B. dothidea*	*Prunus* sp.	Crosifisso, Switzerland	B. Slippers	AY236949	AY236898
CMW9077	ICMP7924	*B. parva*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236939	AY236884
CMW9078	ICMP7925	*B. parva*	*A. deliciosa*	New Zealand	S. R. Pennycook	AY236940	AY236885
CMW9079	ICMP7933	*B. parva*	*A. deliciosa*	New Zealand	S. R. Pennycook	AY236941	AY236886
CMW9080	ICMP8002	*B. parva*	*Populus nigra*	New Zealand	G. J. Samuels	AY236942	AY236887
CMW9081	ICMP8003	*B. parva*	*P. nigra*	New Zealand	G. J. Samuels	AY236943	AY236888
CMW7772		*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	AY236935	AY236877
CMW7773		*B. ribis*	*Ribes*, sp.	New York, USA	B. Slippers/ G. Hudler	AY236936	AY236878
CMW7054	CBS121	*B. ribis*	*Ribes*, sp.	New York, USA	N. E. Stevens	AF241177	AY236879
CMW9076	ICMP7818	*B. lutea*	*Malus x domestica*	New Zealand	S. R. Pennycook	AY236946	AY236893
CMW992	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G. J. Samuels	AF027745	AY236894
CMW7801	BRIP23396	*Fusicoccum mangiferum*	*Mangifera indica*	Australia	G. I. Johnson		
CMW7024	BRIP24101	*F. mangiferum*	*M. indica*	Australia	G. I. Jonnson		
CMW10125		*B. eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, South Africa	H. Smith	AF283686	AY236891
CMW10126		*B. eucalyptorum*	*E. grandis*	Mpumalanga, South Africa	H. Smith	AF283687	AY236892
CMW13446	PREM 58532	*F. andinum*[2]	*Eucalyptus* sp.	Mérida state, Venezuela	S. Mohali		
CMW13455	PREM 58238	*F. andinum*[2]	*Eucalyptus* sp.	Mérida state, Venezuela	S. Mohali	AY693976	AY693977
CMW13434	PREM 58516	*F. stromaticum*[2]	*Eucalyptus*-hybrid	Cojedes state, Venezuela	S. Mohali	AY693974	AY693975

Table 1. Continued.

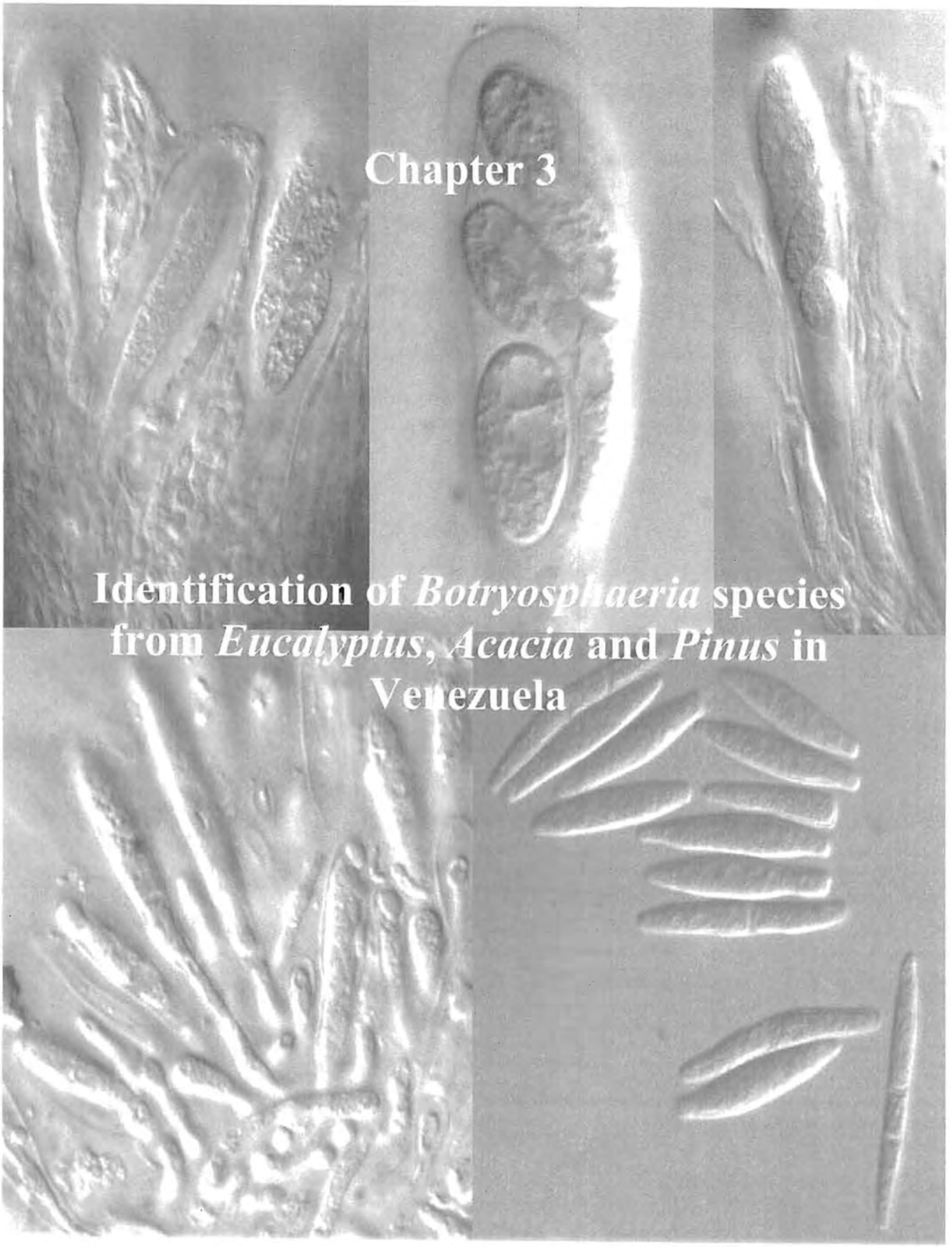
Culture No.[1]	Other No.[1]	Identity[2]	Host	Location	Isolator	GenBank	
						ITS	EF1-α
CMW13435	PREM 58517	*F. stromaticum*	*Eucalyptus*-hybrid	Cojedes state, Venezuela	S. Mohali		
CMW7060	CBS431	*B. stevensii*	*F. excelsior*	Netherlands	H. A. van der Aa	AY236955	AY236904
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	AY236953	AY236902
CMW7775		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	AY236954	AY236903
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901
CMW10130		*B. rhodina*	*Vitex donniana*	Uganda	J. Roux	AY236951	AY236900
CMW7063		*Bionectria* sp.	Unknown	Netherlands	H. A. van der Aa	AY236956	AY236905

[1] Culture collections and isolates abbreviations: CMW = Collection Michael Wingfield, 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; KJ = Jacobs and Rehner (1998); Isolates CMW7999; CMW7772 are ex-type isolates.

[2] Identities determined in this study.

Table 2. Conidial measurement comparisons of the two new *Botryosphaeria* anamorphs with other *Fusicoccum* anamorphs of *Botryosphaeria*.

Identity	Conidial size in vitro (µm)	L/W	Source of data
B. dothidea	(20-) 23-27 (-30) x 4-5 (-6) [Ave. 24.7 x 4.9]	5	Slippers *et al.*2004a
B. parva	(14.7-) 17-21 (-25.5) x 4.5-6 (-7) [Ave. 19 x 5.2]	3.7	Slippers *et al.*2004b
B. ribis	(16-) 19-23 (-24) x 5-6 (-7) [Ave. 20.8 x 5.5]	3.8	Slippers *et al.*2004a
B. lutea	(15-)18-22.5 (-24) x 4.5-6 (-7.5) [Ave. 19.7 x 5.6]	3.6	Phillips *et al.* 2002
B. eucalyptorum	(18-) 20-23 (-25) x 7-8 (-12)	----	Smith *et al.* 2001
B. eucalypticola	(20-) 25-27 (-35) x (5-) 7-9 (-10) [Ave. 26.3 x 7.2]	3.6	Slippers *et al.* 2004c
B. australis	(18-) 23-26 (-30) x 5-6 (-7.5) [Ave. 24.7 x 5.1]	4.8	Slippers *et al.* 2004d
F. mangiferum	(11-) 12-15 (-17.3) x 5-6.6 [Ave. 13.6 x 5.4]	2.5	Slippers *et al.*2004b
B. melanops	(41–) 47–50 (–53) x (9–) 10–10.5 (–11)	----	Shear & Davidson 1936
B. mamane	(19-) 30-44 (-55) x (7-) 8-9 (-10)	----	Gardner 1997
F. andinum	(19-) 23-31 (-40) x (4-) 5-6 (-8) [Ave 27.1 x 5.6]	4.84	This study
F. stromaticum	(19-) 20-23 (-24) x (4-) 5-6 [Ave. 21.7 x 5.4]	4.01	This study



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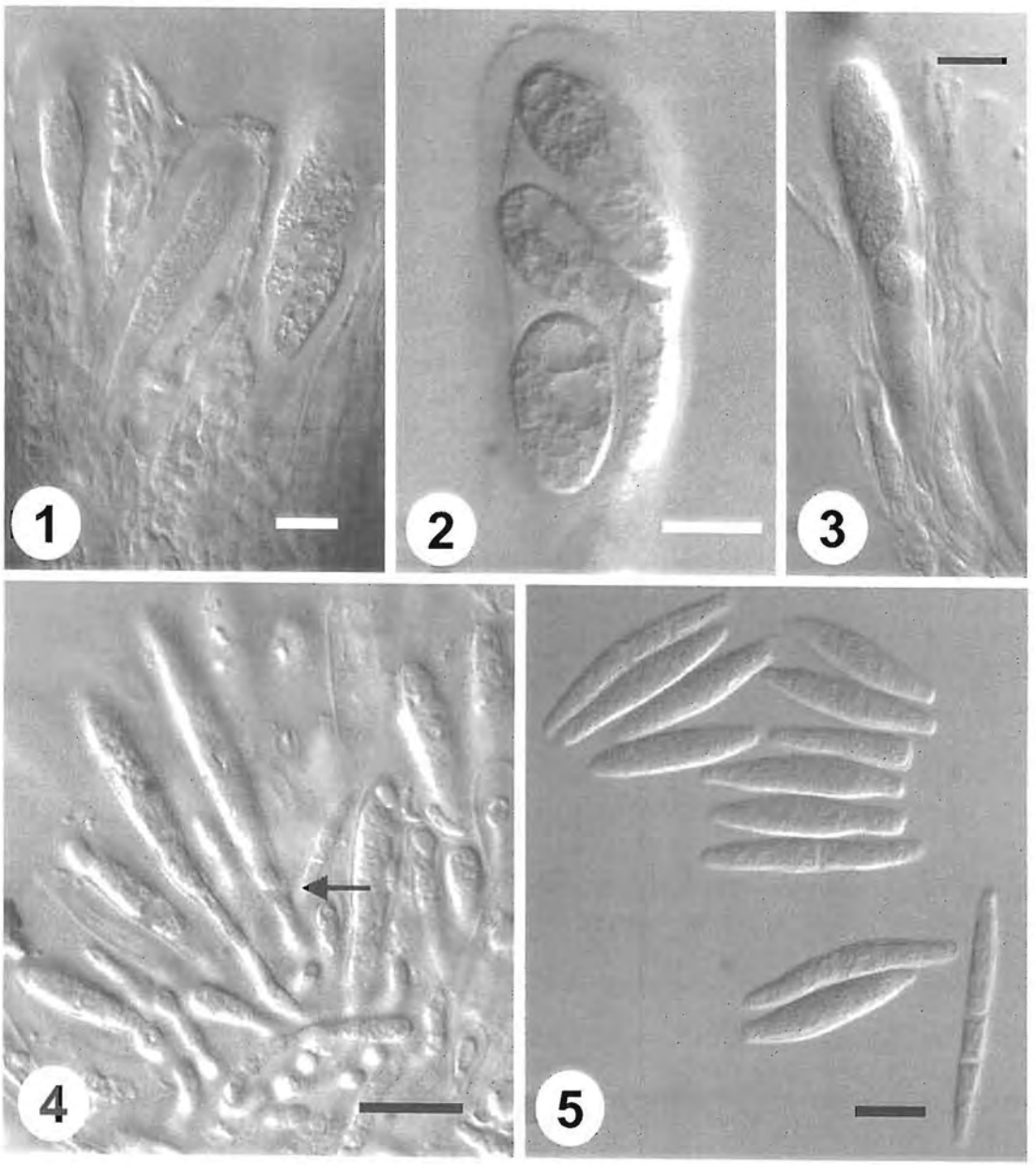
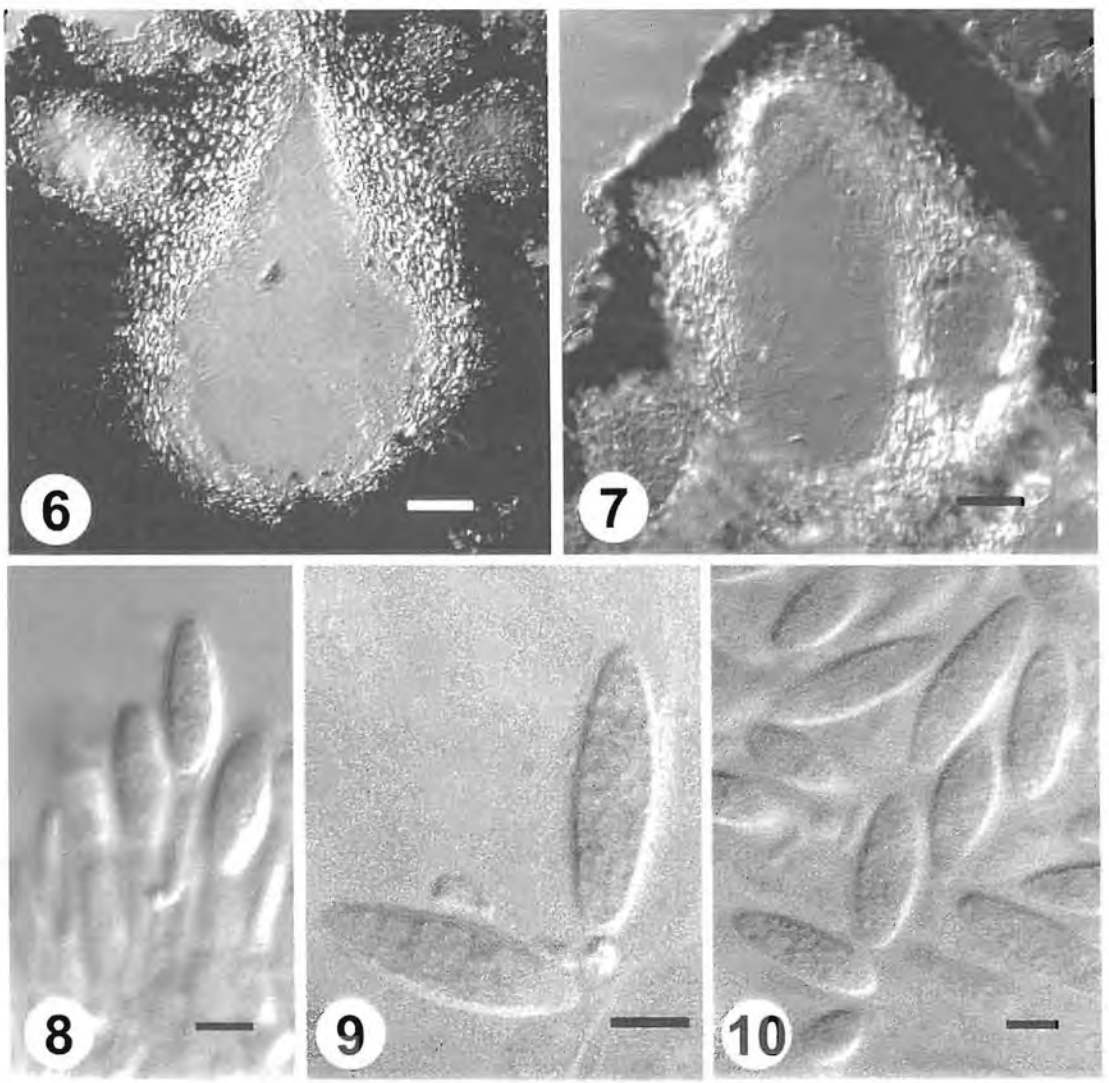
Figs 1-5. Microscopic characteristics of *Botryosphaeria mamane*. **Fig. 1.** Immature and mature asci. **Figs 2-3.** Mature ascus and ascospores with granular, textured contents. **Fig. 4.** Conidiogenous cells (arrow) with macroconidium. **Fig. 5.** Macroconidia with 0-2 septa. Bars = 10 µm.



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.

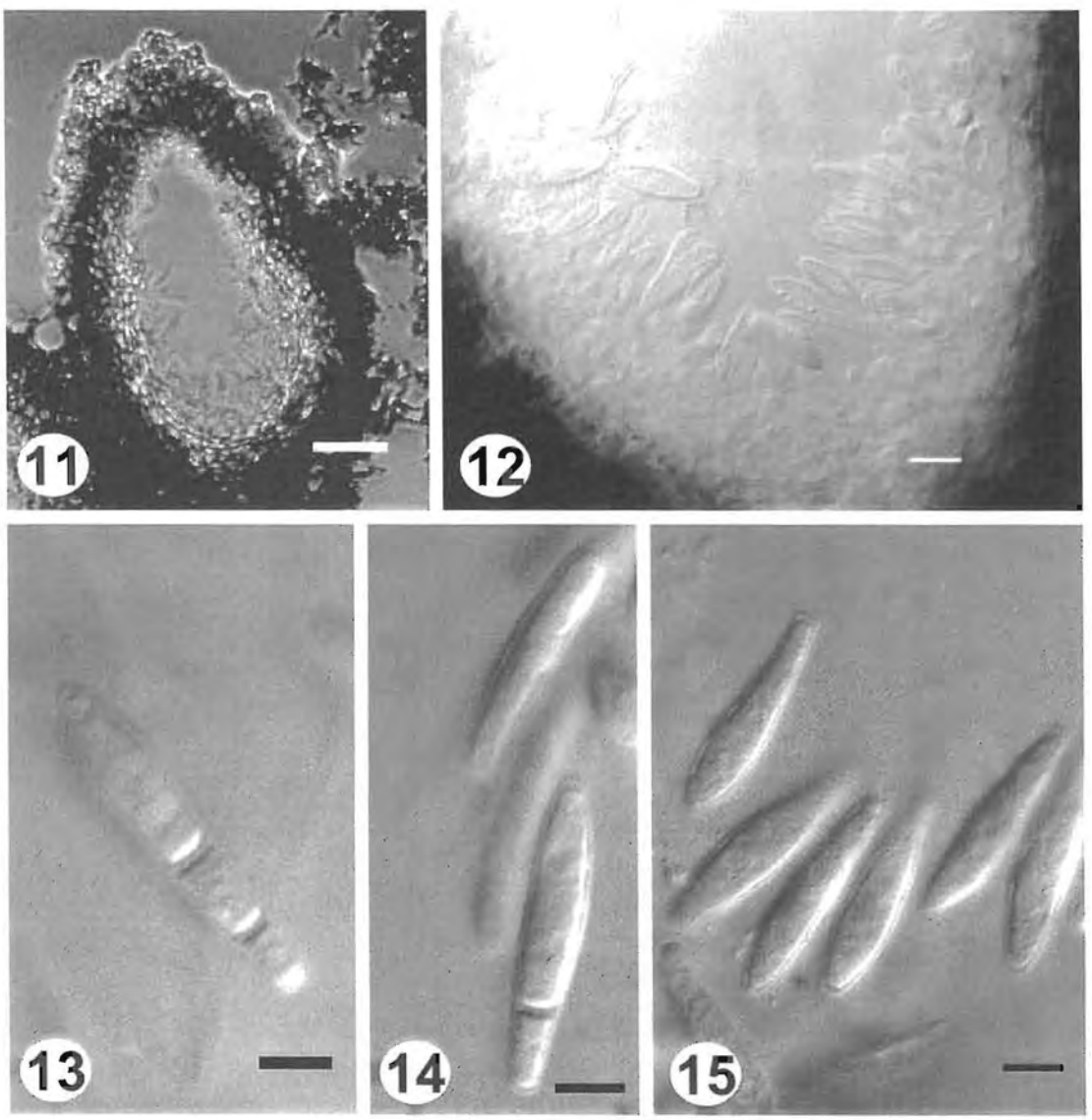


Fig. 16. Phylogenetic relationships among *Botryosphaeria* spp. from Venezuela and elsewhere (GenBank data) based on maximum parsimony analysis of ITS-1 and ITS-2 and 5.8S rDNA sequence data. The phylogram is rooted to the outgroup *Biomectria* sp. Bootstrap values greater than 50 % from 1000 replications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

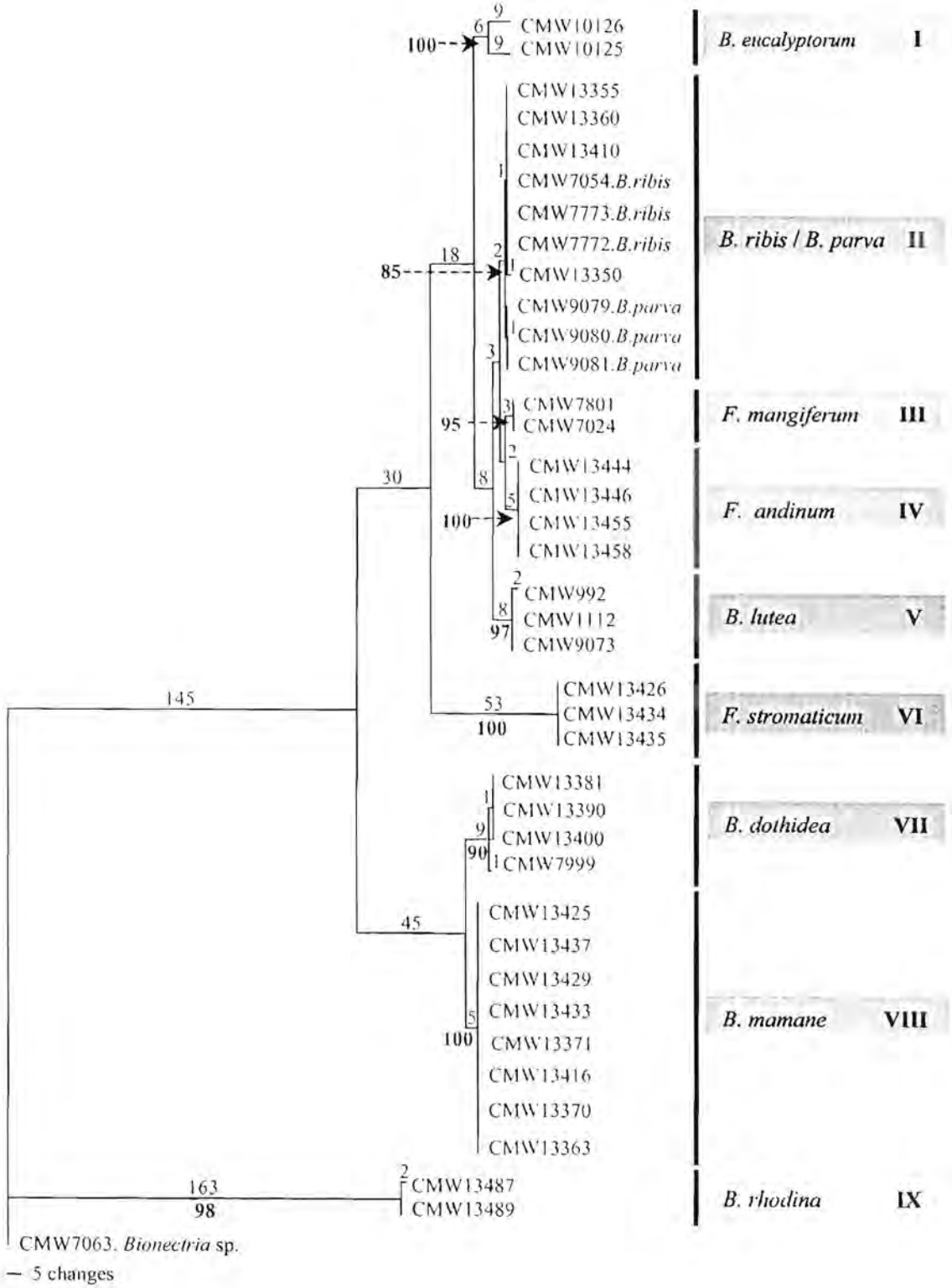


Fig. 17. Restriction fragments for the restriction enzyme *Cfo*I of the ITS-PCR products of different species of *Botryosphaeria* and its anamorphs (*Fusicoccum*) from Venezuela on a 3% agarose gel stained with ethidium bromide. Lane one contains a 100 bp size marker.

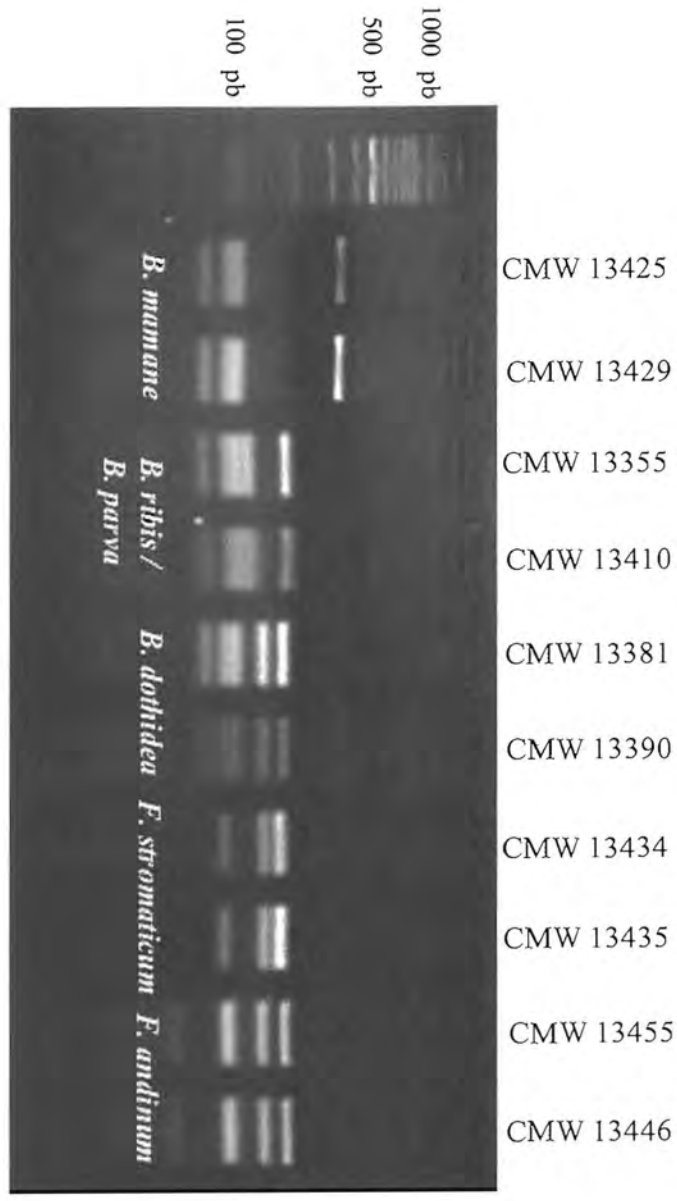


Fig. 18. Restriction maps for the restriction enzyme *Cfo*I to the ITS rDNA regions of different species of *Botryosphaeria* from Venezuela. Fragment sizes (numbers indicate sizes in bp) were inferred from sequence data.

***Cfo*I**

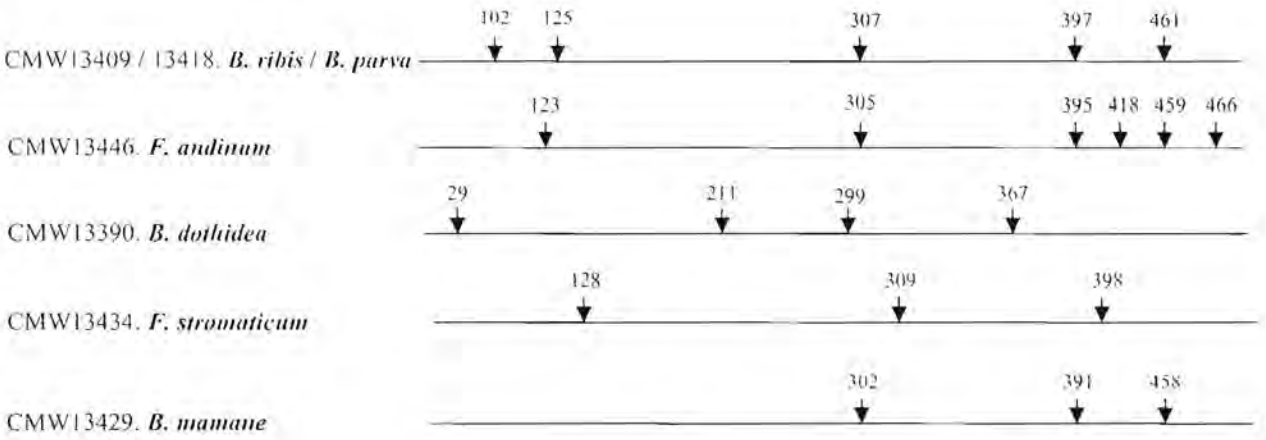


Fig. 19. PCR products (obtained with primers BOT15 & BOT16 (Slippers *et al.* 2004a) for isolates of *B. parva* and *B. ribis* that were digested with the restriction enzyme *Cfo*I, visualized on 2% agarose gel stained with ethidium bromide. The last lane represents a 100 bp size marker.

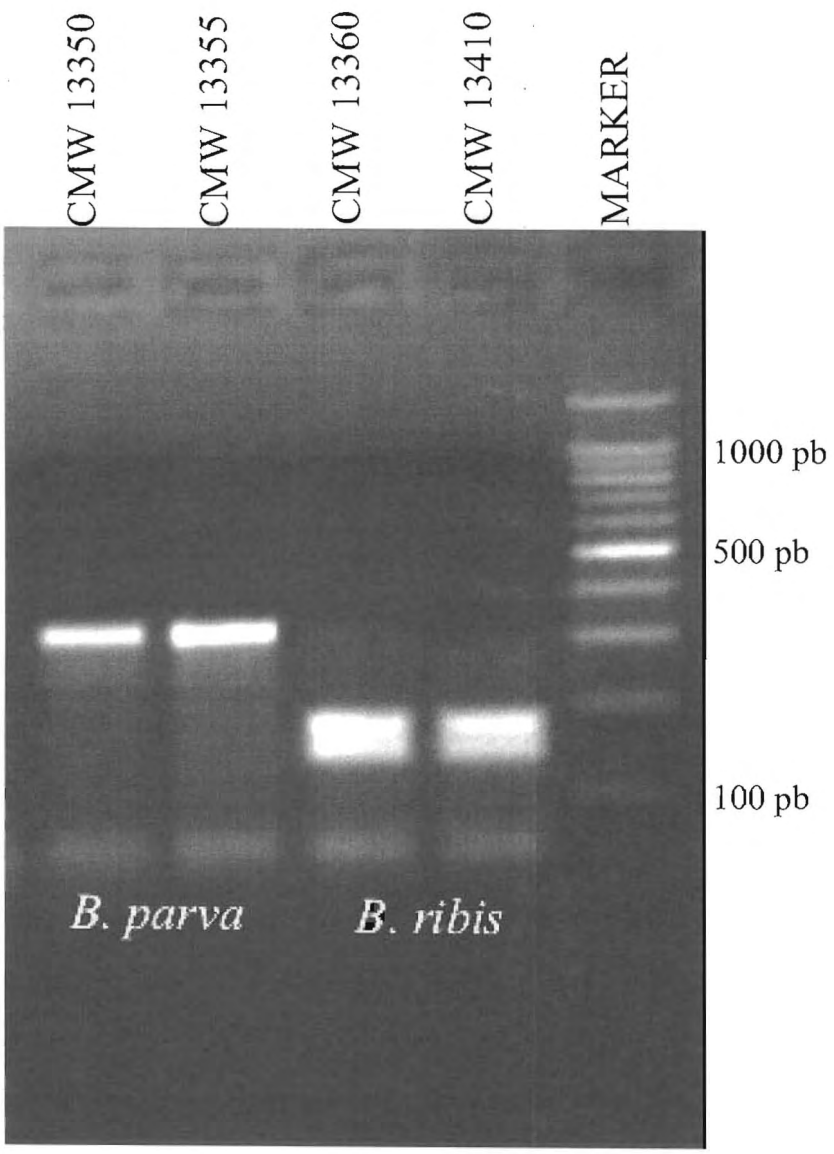

CMW 13350
CMW 13355
CMW 13360
CMW 13410
MARKER
1000 pb
500 pb
100 pb
B. parva
B. ribis

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

Chapter 4

Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobromae* revealed using SSR markers

Forest Pathology 35: 385-396

Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobromae* revealed using SSR markers

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Lasiodiplodia theobromae is a cosmopolitan fungus with a worldwide distribution in the tropics and sub-tropics, where it causes shoot blight and dieback of perennial trees and shrubs and imparts blue stain in timber. In this study, 8 SSR markers were used to evaluate the genetic diversity and gene flow between populations of *L. theobromae*. The relationships between isolates from different host were considered using 3 populations from different tree species in Venezuela and the relationships between isolates from different geographic origins included populations from Venezuela, South Africa and Mexico. A small number of predominant genotypes were encountered in the Venezuela and South African populations and thus genotypic diversity was low. There was no evidence of host specificity for isolates of *L theobromae* and there was very high gene flow between populations from different hosts. Geographic isolation existed between populations of the pathogen from different regions, with unique alleles fixed in the different populations. Gene flow was, however, less restricted between isolates from Mexico and the other populations, consistent with Mexico as a common source of seed in both Venezuela and South Africa. Genetic analysis suggested predominantly clonal reproduction with some genotypes widely distributed within a region. The broad host range of *L. theobromae* and the lack of evidence for host specialization, coupled with its endophytic nature and the common appearance of symptoms only after harvest, is likely to hinder disease management strategies.

INTRODUCTION

The fungal pathogen *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (= *Botryodiplodia theobromae* Pat.) represents the asexual state of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx. It has a worldwide distribution in tropical and subtropical regions and occurs on a very wide range of plants (Punithalingam, 1976). Hosts are mainly woody plants including fruit and tree crops such as mango (Sangchote, 1991), peach (Britton *et al.*, 1990), avocado (Darvas & Kotze, 1987) and *Eucalyptus* spp. (Apetorgbor *et al.*, 2004; Roux *et al.*, 2000, 2001; Sharma *et al.*, 1984). In Venezuela, *L. theobromae* causes shoot blight and dieback of *Pinus caribaea* var. *hondurensis*, *P. oocarpa*, *Azadirachta indica*, *Citrus aurantiifolia*, *C. sinensis*, and *Passiflora edulis* and is also an important agent of blue stain in lumber (Cedeño & Palacios-Pru, 1992; Cedeño *et al.*, 1995, 1996; Mohali, 1993; Mohali *et al.*, 2002). The greatest disease impact is encountered in eastern Venezuela where areas of *P. caribaea* have been established in plantations. *Lasiodiplodia theobromae* is common, causing distension and disruption of the cell walls, weakening the strength and toughness of the Caribbean pine wood, thus reducing its value by up to 50% (Cedeño *et al.*, 1996; Mohali, 1993).

Lasiodiplodia theobromae can colonise healthy plant tissue without exhibiting symptoms. Müllen (1987) for example isolated *L. theobromae* from stem cankers on dogwood (*Cornus florida*). Subsequent pathogenecity tests on dogwood stems with and without drought stress, showed that *L. theobromae* could be isolated from all inoculated plants, but cankers developed only on stressed plants (Müllen, 1987). Thus, *L. theobromae* can be considered as a latent pathogen capable of endophytic infection such as has been reported for the related fungi *Diplodia pinea* (Desm.) Kickx. on *Pinus* spp. (Burgess *et al.*, 2001a; Flowers *et al.*, 2003; Smith *et al.*, 1996) and *B. dothidea* (Fr .:Mough.) Ces. & De Not. on *Eucalyptus* spp. (Smith *et al.*, 1996).

DNA-based markers have been used to recognise and characterise populations, gene flow, and evidence of speciation in many fungal pathogens. SSR markers represent a class of co-dominant molecular markers consisting of tandem repeat loci, rich in polymorphisms with allele size determined by the addition or deletion of one or more repeats (Levinson & Gutman, 1987). SSR markers have recently been used to examine gene and genotype flow, reproductive mode and speciation in a number of fungi, including *Botryosphaeria* spp. and their anamorphs (Barnes *et al.*, 2001; Burgess *et al.*, 2001b, 2003, 2004a, b; Slippers *et al.*, 2004; Zhou *et al.*, 2002).

An earlier study using SSR markers developed for *L. theobromae,* suggested relationships among isolates were more closely linked to host than to geographic origin (Burgess *et al.*, 2003). That study was focussed largely on the development of appropriate markers to study populations of the pathogen and it included only 9 isolates. The aim of the present study was to consider the relationships between host and geographic origin of isolates of *L. theobromae* in greater detail and with a considerably more robust collection of isolates. The study initially emerged from an interest in the fungus in Venezuela, where it causes serious problems on forestry crops. Thus, relatively large populations of *L. theobromae* isolates were available from Venezuela and these could be compared with those available from South Africa and Mexico.

MATERIALS AND METHODS

Fungal isolates

Three *L. theobromae* sub-populations (total 84 isolates) were randomly collected in 2003 from *P. caribaea* var. *hondurensis*, *E. urophylla* and *Acacia mangium* at three locations in Venezuela (Table 1). The isolates were made from asymptomatic plant tissue as well as from

trees exhibiting blue stain, die-back and from entirely dead trees. In addition, two populations of *L. theobromae* were used for comparative purposes. These included 70 isolates randomly collected from blue-stained *P. elliotti* lumber in South Africa and 23 isolates obtained from *P. pseudostrobus* seed cones collected near San Cristobal, Mexico (Table 1). Each of these isolates was selected to originate from a different tree, growing in the same area.

For primary isolations, the plant tissue samples were surface sterilised, rinsed and placed on 2 % malt extract agar (MEA) at 25 °C. To induce sporulation, isolates were transferred onto water agar (WA) supplemented with sterilized pine needles and incubated for 3-6 weeks at 25 °C under near-ultraviolet and cool-white fluorescent light. Isolates were derived from single conidia and maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction and SSR-PCR

Fungal cultures were grown on half strength Potato Dextrose agar (DIFCO, Becton Dickinson, MD, USA) in Petri dishes. Mycelium was scraped from the surface of 7 day-old cultures and freeze-dried. DNA was extracted from the dried mycelium following the protocol of Barnes *et al.* (2001). SSR-PCR was performed on all isolates with 8 fluorescent labelled markers, specifically designed to amplify polymorphic regions in *L. theobromae* as described previously (Burgess *et al.*, 2003).

Labelled SSR PCR products were separated on an ABI Prism 377 DNA sequencer and allele size was estimated by comparing the mobility of the SSR products to that of the TAMRA internal size standard (Applied Biosystems, Perkin Elmer Corp.) as determined by GeneScan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 2 (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility.

Gene and genotypic diversity

For each of the loci, individual alleles were assigned a different letter. For each isolate, a data matrix of 8 multistate characters (one for each locus) was compiled (eg. AABDCGDD). The frequency of each allele at each locus for entire and clone corrected populations was calculated, and allele diversity determined using the program POPGENE (Yeh *et al.*, 1999) and the equation $H = 1-\sum x_k^2$, where x_k is the frequency of the k^{th} allele (Nei, 1973). Chi-square tests for differences in allele frequencies at each locus were performed for clone corrected populations (Chen & McDonald, 1996).

Genotypic diversity (G) was estimated using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the i^{th} phenotype (Stoddart & Taylor, 1988). To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N * 100$ where N is the population size.

Population differentiation

Population differentiation (G_{ST}) as measured by theta (Weir, 1996) was calculated between all pairs of clone corrected populations in Multilocus v. 1.3 (Agapow & Burt, 2001). The statistical significance were determined by comparing the observed G_{ST} value to that of 1000 randomized datasets in which individuals were randomized among populations. The number of migrants (M) that must be exchanged between populations for each generation, to give the observed G_{ST} value, was calculated using the equation M=((1/0)-1)/2 (Cockerham & Weir 1993).

Mode of reproduction

Index of association (I_A) was used to measure multilocus linkage disequilibrium for each clone-corrected population (Maynard Smith *et al.*, 1993). The tests were performed on a data

matrix of 8 multistate characters using the program Multilocus. The distribution under the null hypothesis of recombination was estimated by 1000 randomly recombining data sets and compared with the observed data.

RESULTS

Segregation of SSR alleles

The SSR markers produced 63 alleles across the 8 loci examined (Table 2). There were 47 alleles among the populations from Venezuela, 28 alleles in the Mexican population and 34 alleles in the South African population (Table 2). Of the 63 alleles, 17 (27%) were present in all regions and a further 12 (19%) were present in two of the three populations. Thirty-four alleles (54%) were unique to specific populations of *L. theobromae* (Table 2). There were unique alleles in the Venezuelan population at 7 loci (21 alleles in total), in the Mexican population at 3 loci (3 alleles in total) and in the South African population at 6 loci (10 alleles in total) (Table 2).

Gene and genotype diversity

The mean gene diversity (H) for all 8 loci across all populations of *L. theobromae* was 0.665 for clone corrected populations. The gene diversity among hosts from Venezuela was 0.63 for *Pinus*, 0.67 for *Eucalyptus* and 0.51 for *Acacia* (Table 3). The distribution among geographic regions was 0.70 for Venezuela (VEN), 0.54 for Mexico (MEX) and 0.49 for South Africa (RSA) (Table 5). Values for RSA and MEX were lower than the total mean gene diversity, indicating greater between populations than within population diversity. Diversity for VEN was similar to the total diversity indicating that all observed diversity is reflected in VEN population.

The genotypic diversity for the Venezuelan sub-populations was moderate to low (Table 2) as each of these populations had a single dominant genotype (data not shown). Genotypic diversity for the combined VEN population was also low, again due to the predominance of a single genotype (Table 2). Genotypic diversity in RSA was also low with only 23 genotypes among 70 isolates. Diversity in Mexico was higher because, although there were fewer alleles, a single dominant genotype was not observed (Table 2).

Population differentiation and gene flow

Contingency χ^2 test indicated no significant differences ($P>0.05$) in allele frequencies at any loci for the Venezuelan populations of *L. theobromae* from *Pinus*, *Eucalyptus* and *Acacia* (Table 3). This is reflected in the lack of population differentiation and very high gene flow between the different populations (Table 4). Therefore, all three Venezuelan populations were pooled.

Results of the Chi-square test indicate significant differences ($P<0.05$) in allele frequency between the populations from the three different countries at 6 of the 8 loci (Table 5). Gene flow (number of migrants) between countries was restricted, especially between RSA and VEN (Table 6). Although 0 values indicate significant population differentiation, gene flow was less restricted between MEX and RSA and MEX and VEN, than between RSA and VEN.

Mode of reproduction

The index of association (I_A) of the observed data differed significantly from the values obtained for the recombined data set for all the individual *L. theobromae* populations (Fig. 1A-C).

DISCUSSION

In this study, we have considered for the first time, the population structure of the common, generally tropical pathogen *L. theobromae*. In terms of geographic distribution, this is a relatively poorly understood fungus. Whilst it was first described in South America (Patouillard & De Lagerheim, 1892), its very wide host range and geographic distribution suggests it has been actively moved between countries and its true origin in unknown. Populations of isolates considered in this study were specifically from forest tree crops and the results should be interpreted within the context of the relatively narrow focus of the study.

One of the interesting results of this study was the high gene flow between populations from the three host types considered. These hosts are from three very different families, including confers and hardwood trees and results show clearly that host of origin of isolates plays no role in partitioning of the pathogen genotypes. The study also included isolates from three geographically isolated countries and there was a barrier to gene flow between them.

Many species of *Botryosphaeria*, including *L. theobromae*, are known to have a cosmopolitan distribution with wide host range (Arx, 1987; Barr, 1972; Punithalingam, 1976). Thus, the association of *L. theobromae* with 3 different hosts in Venezuela was not unexpected. However, the lack of host specificity is surprising, with the same genotypes found on all three host species. In the study of Burgess, *et al.* (2003), only 9 isolates of *L. theobromae* were considered, however, those from *Eucalyptus* spp. and *Pinus* spp. grouped separately and host specificity was suggested. All three host species in Venezuela are non-native in that country and the lack of specificity might be associated with this fact. If it is assumed that *L. theobromae* is also non-native in Venezuela, there may have been limited introductions and selection pressure, coupled with the lack of niche competition, could have

forced the same genotypes onto the different hosts. This has been observed for a mycorrhizal fungus, *Pisolithus*, in the non-native environment (Dell *et al.*, 2002). *Pisolithus* spp. exhibit host specificity but, for example, a pine-specific isolate will develop superficial mycorrhizae on *Eucalyptus* spp. in the absence of *Eucalyptus*-specific isolates (Dell *et al.*, 2002). In order to determine whether a similar situation exists with *L. theobromae* in Venezuela, pathogenicity trials using the same fungal genotypes on different host species would be required.

While there appears to be no host specificity for *L. theobromae*, at least on the plants considered in this study, there was a clear restriction to gene flow between geographically isolated regions. The lowest level of gene flow was between populations from Venezuela and South Africa. However, while still somewhat limited, there was evidence of some gene flow between the population from Mexico and those from both Venezuela and South Africa. Across all loci only 3 alleles were unique to Mexico, compared with 21 unique alleles in Venezuela and 10 for South Africa. Mexico is a common source of *Pinus* seed in many sub-tropical countries maintaining plantations of non-native *Pinus* spp. (Burgess & Wingfield, 2001). *Lasiodiplodia theobromae* is well known to occur on *Pinus* seed (Cilliers, 1993) and Mexican isolates used in this study were also from seed collected in a native pine stand. It thus seems likely that this fungus has been distributed with seed to many sub-tropical pine growing regions including South Africa and Venezuela.

This observed linkage of alleles between different loci in all populations, suggests a predominantly clonal mode of reproduction for the fungus. This 'clonal' mode of reproduction can be either due to asexual reproduction or homothallic sexual reproduction (selfing) (Coppin *et al.*, 1997; Turgeon, 1998). However, pseudothecia (sexual structures) of *L. theobromae* are seldom seen in nature. Despite repeated collections, we have failed to connect isolates of *L. theobromae* from *Acacia*, *Pinus* and *Eucalyptus* to sexual structures on

these hosts. On these hosts and at the sites studied, the fungus appears to exist in a predominantly asexual form and we were not surprised to find association of alleles at unlinked loci and a clonal genetic structure. Similarly, Burgess *et al.* (2004b) found no evidence of recombination among populations of the related pine endophyte *Diplodia pinea*, and the same genotypes were found across continents. *Diplodia pinea* is the predominant pine endophyte in temperate regions (Burgess *et al.*, 2001a; Burgess & Wingfield, 2001, 2002) and this niche appears to be replaced by *Lasiodiplodia theobromae* in tropical and sub-tropical regions (Burgess & Wingfield, 2002). *L. theobromae* appears to be similar to *D. pinea* with single genotypes found over large distances.

Generally, fungi undergoing sexual reproduction exhibit greater genotypic diversity than those reproducing asexually (Milgroom, 1996). In our study, low genotypic diversity was observed in populations from Venezuela and South Africa, arising from the predominance of a single genotype. In both cases the area from which the samples were collected was greater than 100 km^2, indicating genotype flow across a region. Although the limited genetic diversity suggests this, the scope of this study was insufficient to be able to say that *L. theobromae* has been introduced into Venezuela and South Africa. The isolates from Mexico originated from native trees in an undisturbed area. The higher genetic diversity among isolates suggests that this population might be native. Confirmation of this fact would require larger numbers of isolates collected in a more structured fashion from a wider diversity of sites.

Lasiodiplodia theobromae is an important pathogen on many tree crops, tempting speculation of host specific groups as is, for example, found with the root pathogen *Fusarium oxysporum* (Gordon & Martyn, 1997). Our study has shown no evidence for host specificity, and demonstrated very high gene flow between populations of isolates from different hosts. Reproduction was predominantly clonal with some genotypes widely distributed with a

region. This was observed for a purported native population (Mexico) and probable introduced populations (South Africa, Venezuela). The broad host range of *L. theobromae* and lack of host specialisation, coupled with its endophytic nature and the appearance of symptoms such a blue stain only after harvest, are likely to hinder efforts to manage this pathogen.

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Table 1. Source of *Lasiodiplodia theobromae* isolates from Venezuela, Mexico and South Africa.

Country	Location	Cultivar	Origin of seed	No. of isolates	Collector
Venezuela	Falcon state	*P. caribaea* var *hondurensis*	Guatemala	30	S. Mohali
	Portuguesa and Cojedes state	*E. urophylla*	Brasil	29	S. Mohali
	Portuguesa and Cojedes state	*A. mangium*	Indonesia	25	S. Mohali
Mexico	San Cristóbal	*Pinus pseudostrobus*	Mexico	23	M. Wingfield
South Africa	Kwa Zulu Natal and Mpumulunga	*Pinus elliotti*	unknown	70	W. de Beer

Table 2. Allele size (bp) and frequency at 8 loci (LAS1-8) for *Lasiodiplodia theobromae* populations collected from Venezuela (VEN), Mexico (MEX) and South Africa (RSA).

Locus	Allele	VEN	MEX	RSA
LAS1	352	0.643	0.391	-
	355	-	-	0.014
	358	-	0.131	-
	360	-	0.217	0.071
	361	0.274	0.217	0.886
	364	0.012	-	-
	367	0.036	-	-
	369	-	-	0.014
	370	0.036	0.044	0.014
LAS2	312	0.060	-	-
	313	0.095	-	-
	314	-	0.044	-
	316	0.560	0.522	0.872
	317	0.274	0.391	0.114
	320	0.012	0.043	0.014
LAS3	326	-	-	0.014
	329	0.012	-	-
	330	0.155	-	-
	334	0.036	-	-
	336	0.262	0.348	0.871
	343	-	-	0.029
	348	-	-	0.029
	352	0.012	0.609	0.043
	354	0.466	-	-
	355	0.036	-	-
	null	-	0.043	0.014
LAS4	248	-	-	0.029
	251	0.012	0.044	0.043
	254	0.095	-	0.014
	255	0.993	0.956	0.900
	258	-	-	0.014
LAS5	383	0.024	0.522	-
	385	0.500	-	-
	387	0.143	0.435	-
	388	0.060	-	0.771
	389	0.202	0.043	0.200
	400	0.071		0.029
LAS6	454	-	-	0.029
	459	0.036	-	-
	463	0.262	0.435	0.828
	465	0.024	0.043	0.029
	468	0.476	0.478	0.100
	488	0.071	-	-
	490	0.048	-	-
	492	0.060	-	-
	496	0.024	-	-
	504	-	0.044	0.014
LAS7	180	0.012	-	-
	182	0.024	-	-
	183	0.643	0.522	-
	192	0.274	0.478	0.986
	195	0.036	-	-
	199	-	-	0.014
	201	0.012	-	-
LAS8	372	-	-	0.029
	376	0.012	0.087	0.428
	377	0.083	-	0.114
	380	0.012	0.261	0.400
	381	0.012	0.043	-
	382	0.190	-	-
	384	0.012	-	-
	385	0.679	0.565	0.029
	392	-	0.044	-
N		**84**	**23**	**70**
No. Alleles		**47**	**28**	**34**
No. Unique Alleles		**21**	**3**	**10**
H		**0.543**	**0.513**	**0.274**
N(g)		**24**	**11**	**23**

G	4.76	5.05	5.09
$\hat{G}$	5.66%	21.94%	7.27%

N = number of isolates
N(g) = number of genotypes
H = gene diversity of the population (Nei 1973)
G = Genotypic diversity (Stoddart and Taylor, 1988)
$\hat{G}$ = G/N% = percent maximum diversity
null = primers failed to amplify a product probably indication a mutation in the primer binding site.

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* from Venezuela collected from *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium*. No χ^2 values were significant.

	Gene diversity (H)				
Locus	*Pinus*	*Eucalyptus*	*Acacia*	χ^2	df
LAS1	0.62	0.58	0.57	10.9	8
LAS2	0.54	0.72	0.49	13.1	8
LAS3	0.80	0.66	0.49	20.4	12
LAS4	0.34	0.42	0.24	3.0	4
LAS5	0.80	0.74	0.69	7.8	10
LAS6	0.78	0.84	0.61	15.5	14
LAS7	0.62	0.74	0.49	12.1	10
LAS8	0.56	0.70	0.49	14.2	12
N (g)	10	10	7		
MEAN	0.63	0.67	0.51		

Table 4. Pairwise comparisons of population differentiation, G_{ST}, (above the diagonal) and number of migrants, M, (below the diagonal) among clone corrected populations of *Lasiodiplodia theobromae* from Venezuela collected from *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium*. There was no significant differentiation between populations.

	Pine	Eucalypt	Acacia
Pine	-	0.020	0.005
Eucalypt	24.5	-	0.065
Acacia	99.5	7.19	-

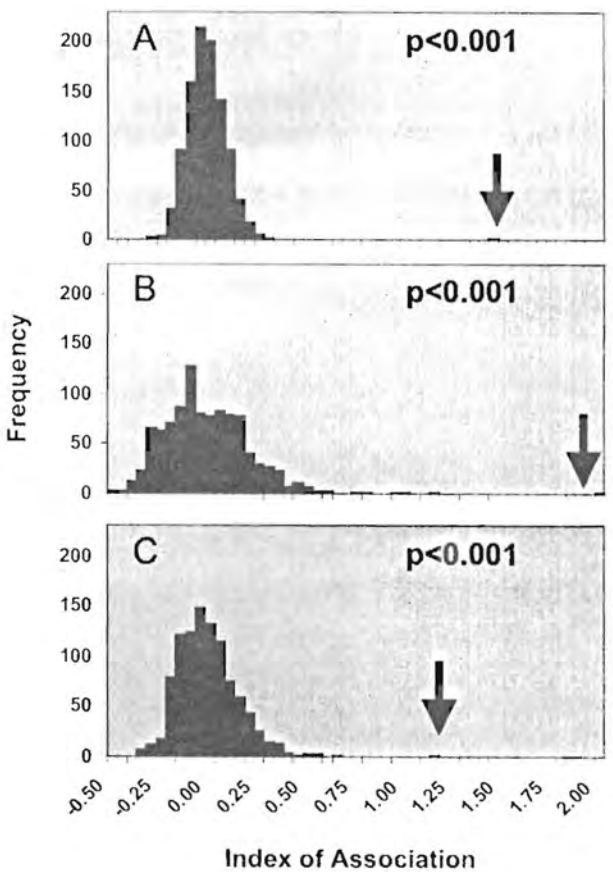
Table 5. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* from Venezuela (VEN) Mexico (MEX) and South Africa (RSA). For χ^2 values stars indicate level of significance (*** P<0.001, **P<0.01, *P<0.05), no stars indicate no significant differentiation between populations.

	Gene diversity (H)				
Locus	VEN	MEX	RSA	χ^2	df
LAS1	0.68	0.76	0.43	45.7***	16
LAS2	0.71	0.61	0.48	19.7*	10
LAS3	0.79	0.58	0.59	43.2**	20
LAS4	0.39	0.16	0.49	10.6	8
LAS5	0.82	0.51	0.57	27.6**	10
LAS6	0.73	0.55	0.58	23.6	18
LAS7	0.69	0.40	0.08	26.3**	12
LAS8	0.67	0.74	0.75	39.0***	16
N (g)	24	11	23		
MEAN	0.70	0.54	0.49		

Table 6. Pairwise comparisons of population differentiation, G_{ST}, (above the diagonal) and number of migrants, M, (below the diagonal) among clone corrected populations of *Lasiodiplodia theobromae* from Venezuela (VEN) Mexico (MEX) and South Africa (RSA). For G_{ST} values, stars represent level of significance (*** P<0.001, **P<0.01, *P<0.05), no stars indicate no significant differentiation between populations.

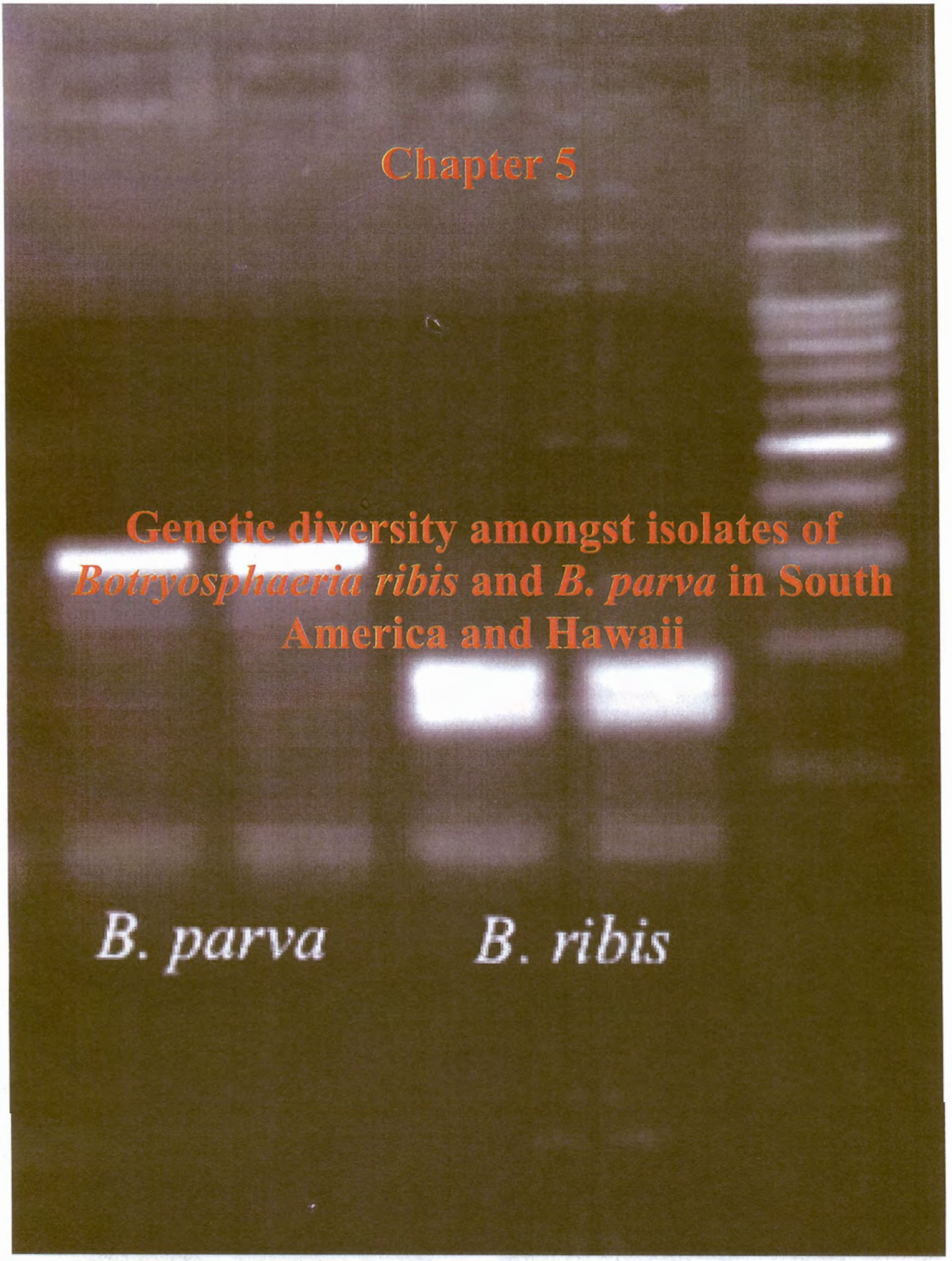
	VEN	MEX	RSA
VEN	-	0.077*	0.152***
MEX	5.99	-	0.087**
RSA	2.79	5.24	-

Fig. 1. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomized datasets. (A) Venezuela, (B) Mexico and (C) South Africa. Results were compared with the observed dataset (arrows).



Chapter 5

Genetic diversity amongst isolates of *Botryosphaeria ribis* and *B. parva* in South America and Hawaii



Genetic diversity amongst isolates of *Botryosphaeria ribis* and *B. parva* in South America and Hawaii

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Botryosphaeria parva and *B. ribis* are common and pathogenic Ascomycete fungi on woody plants including *Eucalyptus* spp., in native forests and plantations around the world. These fungi form part of a complex of cryptic species for which the taxonomic boundaries are unclear. In this study, simple sequence repeat (SSR) markers were used to evaluate the genetic diversity within and between populations of *B. ribis* and *B. parva* from Venezuela, Colombia and Hawaii. Isolates were initially identified based on a PCR RFLP marker, reflecting a fixed single nucleotide polymorphism (SNP) between the species. Distance analyses of the SSR data generally separated these species consistent with separations based on the PCR RFLP marker. For the 59 isolates used (34 multilocus SSR genotypes), there were only three exceptions where isolates identified by PCR RFLP as *B. ribis*, grouped with *B. parva* based on SSR analyses. Whether this result reflects plasticity at the interface of contact and divergence between the two species, or hybridization events, the unfixed status of the SNP used for the initial identification or homoplasy in the SSR data, is not clear. There was no differentiation between the Venezuelan and Colombian populations of *B. ribis*. The data also indicate that these populations mainly reproduce asexually or are homothallic. In contrast, populations of *B. parva* from Colombia and Hawaii were significantly differentiated and alleles were randomly associated, suggesting random (most likely heterothallic) mating.

INTRODUCTION

The genus *Botryosphaeria* Ces. & De Not. occurs worldwide on a large number of mainly woody plants. These fungi are known to occur on dead or dying stems, branches and leaves of plants (saprophytes) or as endophytes within healthy plant tissue (von Arx, 1987; Barr, 1987; Smith *et al.*, 1996). Some species are also known to cause serious canker and dieback diseases when the plants are under stress (von Arx, 1987; Old, 2000; Old & Davison, 2000).

DNA based taxonomic tools have been successfully used to identify some *Botryosphaeria* species and to resolve their phylogenetic relationships, especially when combined with morphologic characters (Jacobs & Rehner, 1998; Crous & Palm, 1999; Denman *et al.*, 2000, 2003; Zhou & Stanosz, 2001; Phillips *et al.*, 2002; Slippers *et al.*, 2004b; Alves *et al.*, 2004). For example, analysis of sequence data for the ITS region of the nuclear rDNA has supported the separation of *B. ribis* Grossenb. & Duggar. from *B. dothidea* (Moug.:Fr.) Ces. & De Not., which was a controversial and important taxonomic problem for many years (Jacobs & Rehner, 1998; Smith & Stanosz, 2001; Smith *et al.*, 2001). Despite the fact that ITS sequence data have supported the separation of some *Botryosphaeria* species, this single DNA locus has not been sufficient to resolve boundaries for all species. Thus, closely related species such as *B. ribis* and *B. parva* Pennycook & Samuels have not been separated with confidence based on ITS, β-tubulin, translation elongation factor 1-α (EF 1-α) or mtSSU rDNA data alone (Smith & Stanosz, 2001; Zhou & Stanosz, 2001; Slippers *et al.*, 2004b).

Internal Simple Sequence Repeat (ISSR) markers have supported the differentiation of *B. parva* and *B. ribis*, but the phylogenetic significance of this finding was uncertain (Zhou *et al.*, 2001). The combination of sequence data from multiple gene regions (the ITS, β-tubulin and EF 1-α), however, confirmed the separation of these species, at least when considering

ex-type isolates (Slippers *et al.*, 2004b). Comparisons of sequence data for these regions has also been used to identify other cryptic *Botryosphaeria* spp. from trees in eastern Australia and South Africa (Slippers *et al.*, 2004c, d).

Botryosphaeria ribis and *B. parva* are well-known pathogens of forest tree species, including *Eucalyptus* spp. (Frezzi, 1952; Davison & Tay, 1983; Shearer *et al.*, 1987; Slippers *et al.*, 2004c). However, in a wide survey of *Botryosphaeria* spp. on *Eucalyptus* spp. growing in plantations and native environments in eastern Australia or as exotics in South Africa, the presence of *B. ribis* could not be confirmed (Slippers *et al.*, 2004b, c). A recent survey in Venezuela and Colombia, however, revealed that both *B. parva* and *B. ribis* occur on *Eucalyptus* in these countries (Chapter 3; Slippers unpublished data). Pathogenicity tests with these fungi on commercially propagated clones of *Eucalyptus* trees, indicated that *B. ribis* and *B. parva* were more pathogenic than other *Botryosphaeria* spp. (Rodas 2003; Chapter 6). Both species produced lesions and cracks in the bark, and black kino exudation from the points of inoculation. Furthermore, *B. parva* was more virulent giving rise to lesions almost double the length of those associated with *B. ribis* (Chapter 6).

SSR markers have been used to determine the gene and genotypic diversity, kinship, gene flow, reproductive mode and differentiation between populations, as well the differentiation between species morphotypes (Queller *et al.*, 1993; Taylor *et al.*, 1999). SSR markers also have recently been used to study populations of some *Botryosphaeria* species, such as *Diplodia pinea* (Desm.) J. Kickx., *D. scrobiculata* De Wet, Slippers & M. J. Wingf., and *Botryosphaeria rhodina* (Cooke) Arx (Burgess *et al.*, 2001a, 2003, 2004a, 2004b; De Wet *et al.*, 2003). These SSR markers that were developed for *Botryosphaeria* spp. with *Diplodia*-like anamorphs did not prove useful for population studies of *Botryosphaeria* spp. with *Fusicoccum*-like anamorphs (Slippers *et al.*, 2004a). Slippers *et al.* (2004a) thus

developed SSR markers that could be used in populations of *B. parva*, and these were also shown to be useful for nine other *Botryosphaeria* species with *Fusicoccum*-like anamorphs.

In this study, the SSR markers developed by Slippers *et al.* (2004a) are applied to isolates representing populations of *B. ribis* from Venezuela as well as *B. ribis* and *B. parva* from Colombia and Hawaii to determine their genetic diversity and distinction, and mode of reproduction. Additionally, the markers were applied to consider the possible spread of the populations or species and between these areas.

MATERIALS AND METHODS

Fungal isolates

Fifty-nine isolates of *Botryophaeria ribis* and *B. parva* from Venezuela, Colombia and Hawaii were choosen for this study. Sixteen isolates from *Eucalyptus urophylla* S.T. Blake x *E. grandis* W. Hill: Maiden hybrids growing in two locations of Venezuela (the states of Portuguesa and Cojedes) were made from asymptomatic plant tissue as well as from trees exhibiting blue stain and die-back, or entirely dead trees. Eighteen isolates originated from *E. grandis* in Hawaii and twenty-five isolates originated from *E. urophylla* in Cauca Valle and Andina zones, Colombia.

The fungi were isolated and single spore isolates were made as described by Slippers *et al.* (2004b). All isolates used in this study are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, RFLP and SSR-PCR analysis

DNA was extracted from mycelium following the protocol of Raeder and Broda (1985) with some modification. These modifications were similar to those described by Slippers *et al.* (2004a). Nucleic acids were quantified using a spectrophotometer with an absorbance at 260 nm and 280 nm.

Restriction enzyme patterns for an unidentified DNA region-Locus *BotF15* (Slippers *et al.*, 2004a) were used to define the isolates belonging to either *B. ribis* or *B. parva* that are considered cryptic species residing in the *B. ribis-B. parva* complex. The DNA fragment was amplified using the primers BOT 15 and BOT 16 and the PCR reaction mixtures were made as described by 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.

SSR-PCR was performed on all isolates with seven fluorescently-labelled markers, specifically designed to amplify polymorphic regions within isolates of the *B. parva-B. ribis* complex. The protocols used were as described previously by Slippers *et al.* (2004a). Fluorescently-labelled SSR-PCR products were diluted and one µl of the dilution was mixed with a LIZ^{TM} internal size standard (Perkin-Elmer Applied Biosystems, Foster City, CA) and formamide mix (1:14). The products were separated on an ABI PRISM 3100 automated sequencer (Perkin-Elmer Applied Biosystems). Allele size was estimated by comparing the mobility of the SSR products as determined by GeneScan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 3.0 (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility.

Gene and genotypic diversity

For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele at each of the 7 polymorphic loci (eg. ABBCDEE). The frequency of each allele at each locus for complete and clone corrected populations was calculated, and allele diversity determined, using the program POPGENE (Yeh *et al.*, 1999) and the equation $H = 1-\sum x_k^2$, where x_k represents the frequency of the k^{th} allele (Nei, 1973).

Each genotype was assigned a number and the genotypic diversity (G) was estimated (Stoddart & Taylor, 1988) using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the i^{th} phenotype and compared to genotypic diversity expected for the null hypothesis of random mating. To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N * 100$ where N is the population size (Chen *et al.*, 1994). Chi-square tests for differences in allele frequencies were calculated for each locus across clone corrected populations (Workman & Niswander, 1970).

Genetic distance

Genetic distance between populations based on microsatellite data was calculated using D_{AD}, a statistic based on absolute distance. Data were represented as total nucleotide length of the locus. The distance between isolates was calculated using the program MICROSAT (Minch *et al.*, 1995) and Neighbor-joining trees constructed in MEGA3 (Kumar *et al.*, 2004).

Mode of reproduction

The index of association (I_A) was used as a measure of the degree of association between loci or multilocus linkage disequilibrium for each population and for all populations combined (Maynard Smith *et al.*, 1993). I_A was calculated for the clone corrected population containing only one representative of each genotype. The tests were performed on a data matrix of seven

multistate characters using the program Multilocus (Agapow & Burt, 2001). The expected data for 1000 randomly recombining data sets was calculated and compared with the observed data. Where the observed data fall within the distribution range of the recombined data, then the hypothesis that the population was undergoing recombination cannot be rejected. If the observed data fall outside the distribution range with a significant P value ($P<0.05$), the population is most likely clonal.

RESULTS

Segregation of SSR alleles

The seven loci examined by the SSR markers produced a total of 44 alleles (Table 1). There were 17 alleles in the Venezuela population, 34 alleles for Colombia (20 alleles for *B. ribis* and 14 for *B. parva*) and 20 alleles for Hawaii isolates. Only one allele, (2.3 %) of the total of all alleles, was shared between populations (Table 1). Within each of these populations unique alleles were present. The Venezuelan population had four unique alleles (9.1 %); for the Colombia population, *B. ribis* had five unique alleles (11.4 %) and *B. parva* had three unique alleles (6.8 %); for the Hawaii population, *B. ribis* had two unique alleles (4.6 %) and *B. parva* 11 unique alleles (25 %) (Table 1).

Gene and genotype diversity

All populations showed a moderate genotypic diversity ($\hat{G}$ %); 32% for *B. ribis*, Venezuela population (eight genotypes), 39 % for Colombia (five genotypes); 36 % for *B. parva*, Colombia (nine genotypes) and 50 % for Hawaii (11 genotypes) (Table 2). There were no genotypes shared among the different populations studied for either *B. ribis* or *B. parva* (Table 2).

The gene diversity (H) for each of the clone corrected populations was 0.35 for Venezuela (*B. ribis* populations), 0.50 for Colombia (for both *B. ribis* and *B. parva* populations) and 0.37 for Hawaii (*B. parva* populations).

Contingency χ^2 test indicated no significant differences ($P<0.05$) in allele frequencies at any loci for the Venezuelan and Colombian populations of *B. ribis*, except the loci *BotF37* (Table 3). This is reflected in the lack of population differentiation, indicating a high gene flow between the different populations.

The Chi-square test indicated significant differences ($P<0.01$ and <0.001) in allele frequency between the Colombia and Hawaii populations of *B. parva* (Table 4). Four loci from seven polymorphic SSR were highly significant (*BotF17, 21, 24, 35*), showing differentiation between *B. parva* populations from Colombia and Hawaii, indicating low levels of gene flow.

Genetic distance

The cluster analysis with the neighbour-joining tree revealed the genetic diversity among 59 *B. ribis* and *B. parva* isolates and two *B. ribis* isolates from New York, (which were used to allow comparison to the type isolates for the species). The dendrogram was constructed based in the seven loci examined by the SSR markers (Fig. 1). The dendrogram separated isolates into two distinct clades, one containing *B. ribis* isolates and the other containing *B. parva* isolates. Two purported *B. ribis* isolates from Venezuela fell into the *B. parva* clade. The *B. parva* clade was further divided into two distinct groups, one for Colombia and one for Hawaii, thus reflecting observations of low gene flow between these populations.

Mode of reproduction

The I_A for the observed data differed significantly from the values obtained for the recombined data set for all the individual *B. ribis* and *B. parva* populations (Fig. 2A, B; Fig.

3). This indicates linkage of alleles between different loci and suggests a predominantly sexual mode of reproduction in *B. parva* from Colombia and Hawaii. This is in contrast to an apparently asexual (clonal) mode of reproduction for *B. ribis* from Venezuela and Colombia.

DISCUSSION

The primers develop by Slippers *et al.* (2004a) successfully amplified SSR containing markers in the populations of *B. parva* and *B. ribis* used in this study. These markers allowed the distinction between these two cryptic species, as well as for significant resolution of population diversity, population separation and reproductive mode in these fungi.

The total number of alleles shared between isolates identified as *B. parva* or *B. ribis* was very low (2.3 %). A neighbour-joining tree reflecting the genetic diversity among the 59 *B. ribis* and *B. parva* isolates from the three populations, separated the isolates into two well defined groups; one group corresponding to *B. parva* and the other one to *B. ribis*. These results support the separation of the taxa as independent species. This separation also has been confirmed in other studies using other techniques such as ISSR markers, combined gene genealogies of the ITS, EF 1-α and β-tubulin loci and PCR-RFLP fingerprints of part of the LSU and ITS regions (Smith & Stanosz, 2001; Slippers *et al.*, 2004b; Alves *et al.*, 2005).

The *B. ribis* and *B. parva* groups identified in the neighbour-joining tree reflect the initial identification of isolates using the PCR RFLP fingerprint of locus *BotF15*, with the exception of three isolates. This microsatellite containing region has a unique restriction site for *B. ribis* based on a single nucleotide polymorphism (Slippers *et al.*, 2004a). The three exceptions were for isolates identified by the PCR RFLP technique as *B. ribis* (one from Hawaii and two from Venezuela), but that grouped as *B. parva* using the multilocus SSR markers. Whether these exceptions reflect plasticity at the interface of contact and divergence

between the two species, hybridisations events across the species boundary, the unfixed status of the SNP used for the initial identification, or homoplasy in the SSR data, is not clear and will be the subject of future investigations. It does, however, suggest that the *Cfo*I restriction profile for locus *BotF15* is relatively reliable for rapidly separating the cryptic species *B. ribis* and *B. parva*, which are otherwise, difficult to distinguish from each other.

The results emerging from the calculation of Index of Association (Taylor *et al.*, 1999) gave the appearance of sexual reproduction for *B. parva* populations (alleles randomly associated) and clonal reproduction for *B. ribis* populations (alleles non-randomly associated). No teleomorphs structures were observed for either *B. ribis* and *B. parva* collections used in this study. However, these fungi are known to exhibit both sexual and asexual reproduction (Grossenbacher & Duggar, 1911; Pennycook & Samuels, 1985; Slippers *et al* 2004a). This result could imply that *B. parva* is heterothallic and *B. ribis* homothallic although this seems unlikely for such closely related. Alternatively, is possible that *B. ribis* relies more strongly on asexual spores for dispersal and infection than *B. parva* on *Eucalyptus* in these environments. This could arise from the absence of one mating type if the fungus is heterothallic or from environmental factors that are not conducive to sexual reproduction. It is also possible that both fungi are homothallic, but that *B. parva* has also retained the ability to outcross. *Botryosphaeria parva* populations from Colombia and Hawaii had a higher genotypic diversity than *B. ribis* populations from Venezuela, Colombia or Hawaii. Both populations of *B. parva* from Colombia and Hawaii had a moderate level of diversity and sexual reproduction reflecting the behaviour of an outcrossing fungus. Slippers (2003) hypothesized that this fungus is homothallic, based on observations in cultures of *B. eucalyptorum* and a low level of diversity in populations of *B. parva* from South Africa despite the presence of sexual fruiting structures. If this fungus is homothallic, then it has retained the ability to occasionally outcross which would be similar to the situation in for

example *Cryphonectria parasitica* (Milgroom *et al.*, 1993; Marra & Milgroom, 2001). Alternatively, this could be a heterothallic fungus, of which only a limited number of genotypes occur on *Eucalyptus*, coupled to preferential reproduction by asexual spores on this host. These results reflect previous studies where a combination of clonal and sexual reproduction has been shown to significantly influence fungal population structure (Taylor *et al.*, 1999).

The *B. parva* populations showing recombination within each population and a high degree of differentiation, suggests that they have been separated geographically for some time (Burgess *et al.*, 2004a; Barnes, *et al.*, 2005). These populations might thus be native to each of the regions where they have been collected and that they have moved onto *Eucalyptus*. Alternatively, multiple introductions of *B. parva* into Hawaii and Colombia from different sources could also explain the result. Irrespective of the reason, the diversity and apparent sexual reproduction in these countries might aid the fungus in overcoming resistance in *Eucalyptus* clones. This adds to the importance of *B. parva* as one of the primary targets for disease screening, given its high virulence (see Chapter 6).

The *B. ribis* populations appeared to be predominantly clonal with a very similar structure in both Venezuela and Colombia. There was only one *B. ribis* isolate from Hawaii, so population statistics could not be done for that region. We found no geographic barriers separating populations from Venezuela and Colombia and the lack of allelic diversity in these populations could thus be due to natural gene flow between these countries (Burgess, *et al.*, 2001b; 2004b). The population differentiation is frequently related to the distance between populations. Thus, when two populations occur in areas closer to each other it is likely that they will have similar allelic frequencies (Slatkin, 1993; Linde *et al.*, 2002). However, the overlap could also be due to movement of *Eucalyptus* germplasm between Colombia and Venezuela. These results emphasize a ready exchange of pathogens between these

neighbouring countries of which forestry companies and researchers should be aware as more damaging diseases could also be exchanged.

Slippers (2003) observed some shared polymorphism between isolates of *B. ribis* and *B. parva* based on DNA sequence and SSR marker data, despite overall distinction between the species based on these data. This is similar to the results of the present study. Such shared polymorphism has been interpreted as: a) speciation is not completed and that some isolates retain the ability to interbreed, or b) not enough time has elapsed since speciation to allow full resolution of polymorphisms, or c) hybridisation between the distinct species (Taylor *et al.*, 2000; Slippers, 2003). This last possibility is important to consider. Hybridization is increased when closely related, but geographically isolated, pathogen populations come into contact (Brasier, 2000, 2001). This is possibly the case for various *Botryosphaeria* spp. (Slippers, 2003). Theoretically, this process presents an opportunity for rapid emergence of new or modified pathogens via gene flow, although it appears to be rare in nature (Brasier, 1995, 2001; Brasier *et al.*, 1998, 1999; Newcombe *et al.*, 2000; Garbelotto *et al.*, 2004). It is thus important to further test these hypotheses for *B. parva* and *B. ribis* as it will reflect on the importance of future quarantine measures to prevent pathogen movement. It might also help predict future emergence of new strains that could potentially be more pathogenic or infect new hosts.

This study was somewhat impaired by a relatively low number of isolates available. However, despite the somewhat limited number of isolates, results showed that *B. ribis* and *B. parva* appear to co-exist in South America and perhaps have different modes of reproduction. Their pathogenicity differs, but whether and how their roles in the ecosystem/plantations differ remains unclear. It will be important to continue these studies because this information can promote an improved understanding of the movement of pathogens between *Eucalyptus* growing regions, both within South America and between this

and other continents. Such knowledge could be important to help prevent pathogen movement, which might have unforeseen consequences. Furthermore it should also promote an understanding of and planning for breeding programs attempting to deal with these pathogens.

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Table 1. Allele size (bp) and frequency at 7 loci for *Botryosphaeria ribis* (R) and *B. parva* (P) isolates collected from Venezuela (VEN), Colombia (COL) and Hawaii (HAW).

Locus	Allele	COL(R)	VEN(R)	HAW(R)	COL(P)	HAW(P)
BOT11	421	-	-	-	-	0.14
	428	0.11	0.06	-	0.75	0.72
	430	0.44	-	-	0.25	0.14
	431	0.33	0.88	-	-	-
	434	0.11	0.06	-	-	-
	438	-	-	1.00	-	-
BOT15	377	0.56	1.00	1.00	1.00	1.00
	389	0.44	-	-	-	-
BOT17	228	0.89	0.88	-	-	-
	230	-	0.06	-	-	-
	232	-	0.06	-	0.87	-
	234	-	-	1.00	-	-
	240	-	-	-	-	0.57
	242	-	-	-	-	0.07
	248	-	-	-	-	0.21
	252	-	-	-	-	0.14
	null	0.11	-	-	0.13	-
BOT21	196	-	-	1.00	-	0.15
	207	0.11	0.25	-	1.00	0.21
	208	0.44	0.56	-	-	-
	217	0.44	0.19	-	-	-
	219	-	-	-	-	0.07
	231	-	-	-	-	0.21
	null	-	-	-	-	0.36
BOT24	422	0.44	-	1.00	0.44	0.86
	423	-	-	-	0.50	-
	425	0.56	1.00	-	0.06	0.14
BOT35	221	-	-	-	-	0.50
	224	0.44	-	-	-	0.29
	227	-	-	-	-	0.14
	230	-	-	-	-	0.07
	237	-	0.25	1.00	0.06	-
	241	0.11	0.69	-	0.75	-
	245	-	-	-	0.13	-
	247	0.44	-	-	-	-
	258	-	0.06	-	-	-
	null	-	-	-	0.06	-
BOT37	305	0.11	-	-	-	-
	312	-	0.06	1.00	1.00	1.00
	320	0.67	-	-	-	-
	321	0.11	-	-	-	-
	323	-	0.13	-	-	-
	324	-	0.81	-	-	-
	null	0.11	-	-	-	-
No. of isolates		9	16	4	16	14
No. of alleles		20	17	7	14	20
No. of unique alleles		6	4	2	3	11

Table 2. *Botryosphaeria ribis* (R) and *B. parva* (P) genotypes as estimated from multilocus profiles generated from the 7 polymorphic SSR loci. Genotypes were distributed among populations collected Colombia (COL), Venezuela (VEN) and Hawaii (HAW).

Code	Genotype	RFLP profile	COL (P)	COL (R)	VEN (R)	HAW (P)	HAW (R)
COL1	ECCDDGF	P	3				
COL2	ECCDEEF	P	1				
COL3	ECCDEGF	P	5				
COL4	ECCDEOF	P	1				
COL5	ECNDFHF	P	1				
COL6	ECNDDGF	P	1				
COL7	FCCDDGF	P	2				
COL8	FCCDDHF	P	1				
COL9	FCCDEGF	P	1				
COL10	ECNDEIM	R		1			
COL11	FEAECBH	R		4			
COL12	GCACEIC	R		1			
COL13	GCADEIH	R		2			
COL14	HCADEGI	R		1			
VEN1	ECCEELF	R			1		
VEN2	GCADEGK	R			4		
VEN3	GCAEEFJ	R			1		
VEN4	GCAEEGK	R			5		
VEN5	GCAEEGJ	R			1		
VEN6	GCAFEEK	R			2		
VEN7	GCAFEGK	R			1		
VEN8	HCBEEEK	R			1		
HAW1	BCFIEBF	P				2	
HAW2	ECFGCAF	P				1	
HAW3	ECFKCCF	P				1	
HAW4	ECFACCF	P				1	
HAW5	ECFICDF	P				1	
HAW6	ECGKCAF	P				1	
HAW7	ECJKCAF	P				2	
HAW8	ECJDCAF	P				1	
HAW9	ECKDCAF	P				2	
HAW10	FCFKCBF	P				1	
HAW11	FCFACBF	P				1	
HAW12	KCDADEF	R					4
N			16	9	16	14	4
N(g)			9	5	8	11	1
G			5.83	3.52	5.12	8.99	1
G%			36	39	32	50	25

N = number of isolates
N(g) = number of genotypes
G = Genotypic diversity (Stoddart and Taylor, 1988)
$\hat{G}$ = G/N% = percent maximum diversity.

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Botryosphaeria ribis* from Venezuela (VEN) and Colombia (COL). For χ^2 values, stars indicate level of significance (*P<0.05), no stars indicate no significant differentiation between populations.

	Gene Diversity (H)			
Locus	VEN	COL	χ^2	df
BotF11	0.40	0.72	1.8	2
BotF15	0.00	0.31	1.7	1
BotF17	0.40	0.32	2.9	3
BotF21	0.53	0.56	6.3	3
BotF24	0.00	0.32	1.7	1
BotF35	0.59	0.56	9.4	4
BotF37	0.53	0.72	13.0*	6
N	8	5		
MEAN	0.35	0.50		

Table 4. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Botryosphaeria parva* from Hawaii (HAW) and Colombia (COL). For χ^2 values stars indicate level of significance (**P<0.01, ***P<0.001), no stars indicate no significant differentiation between populations.

	Gene Diversity (H)			
Locus	HAW	COL	χ^2	df
BotF11	0.43	0.44	1.3	2
BotF15	0.00	0.00	-	-
BotF17	0.54	0.35	20.0**	5
BotF21	0.76	0.00	13.4**	4
BotF24	0.16	0.59	16.8***	3
BotF35	0.68	0.61	20.0**	7
BotF37	0.00	0.00	-	-
N	11	5		
MEAN	0.37	0.50		

Fig. 1. Neighbour-joining tree for the distance DAD based on total nucleotide length at each of the 7 SSR-loci, showing the relationship between genotypes of *Botryosphaeria ribis* (R) and *B. parva* (P) from Venezuela (VEN), Colombia (COL) and Hawaii (HAW). Also included are two isolates of the ex-type of *B. ribis* from New York.

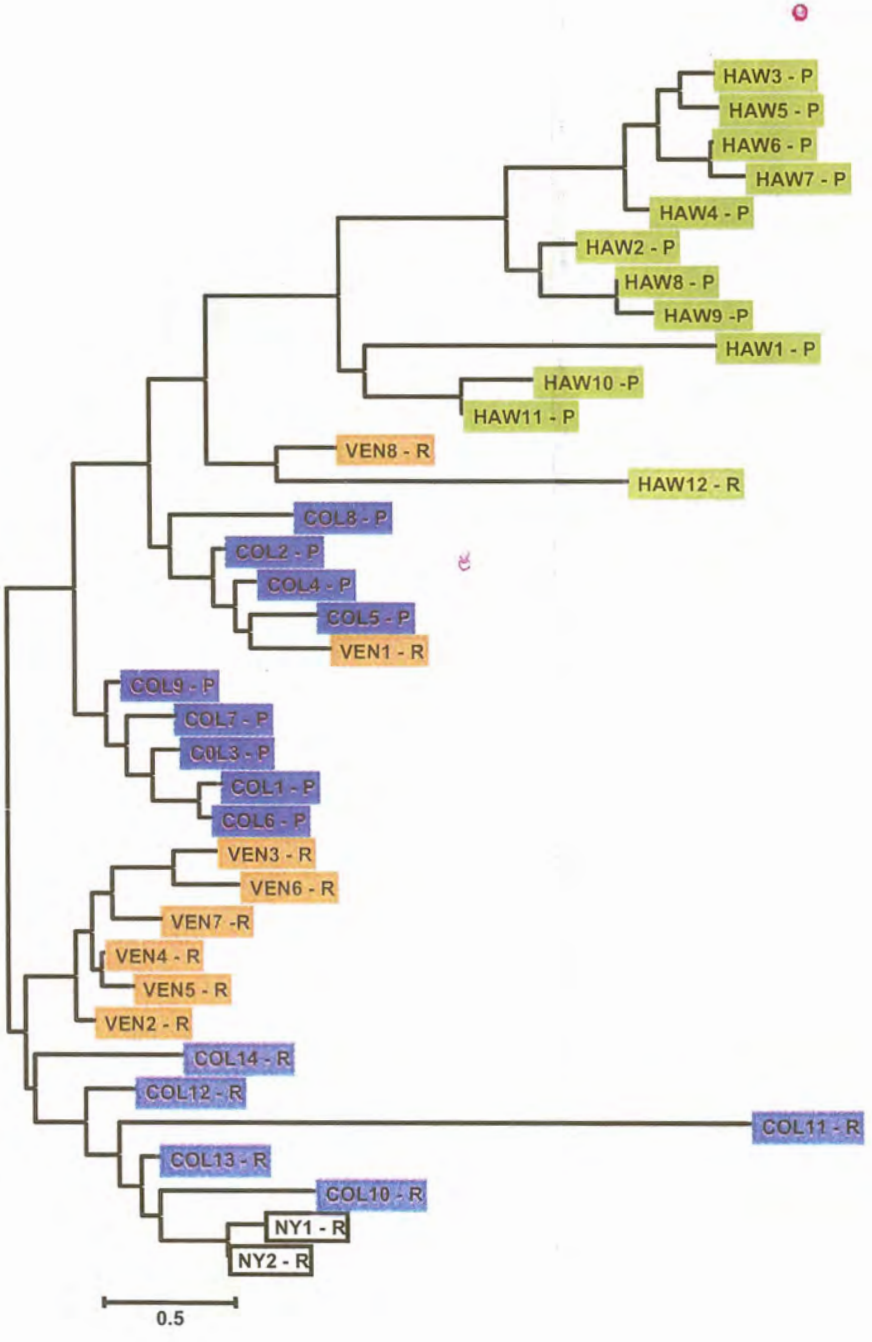


Fig. 2. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomized datasets of *Botryosphaeria parva*. (A) Colombia, (B) Hawaii Results were compared with the observed dataset (arrows).

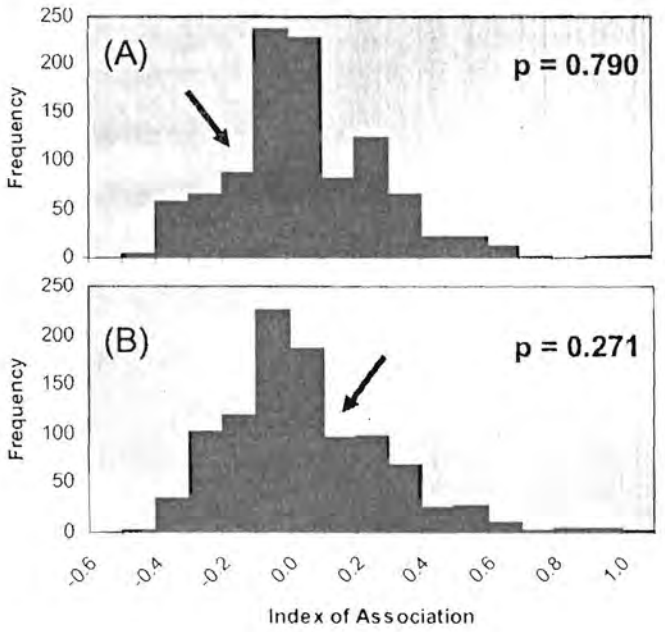
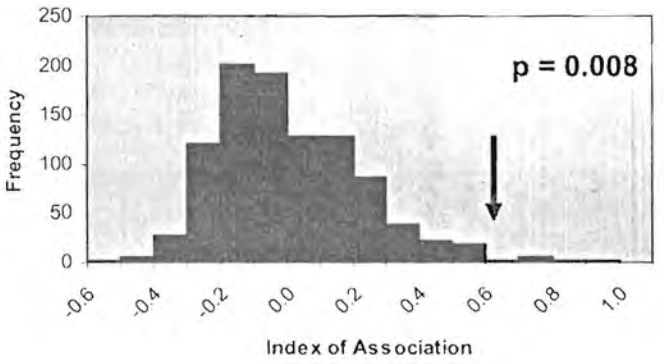
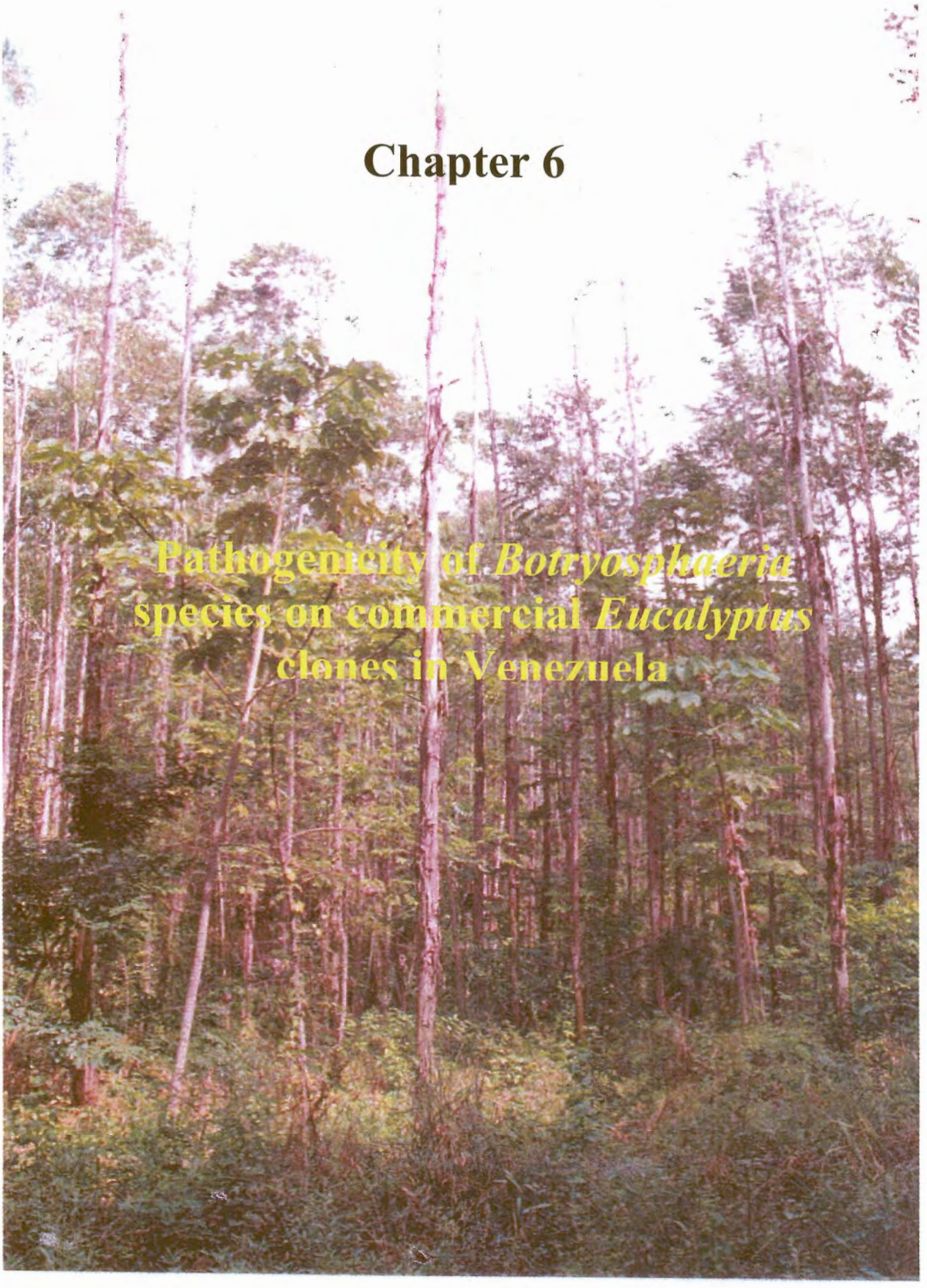


Fig. 3. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomised datasets of *Botryosphaeria ribis* (Venezuela and Colombia combined). Results were compared with the observed dataset (arrows).





Chapter 6

Pathogenicity of *Botryosphaeria* species on commercial *Eucalyptus* clones in Venezuela

Pathogenicity of *Botryosphaeria* species on commercial *Eucalyptus* clones in Venezuela

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Botryosphaeria spp. include a number of well-recognised *Eucalyptus* pathogens of which various species have recently been found on *Eucalyptus* spp. in Venezuela. An initial inoculation trial was conducted using seven *Botryosphaeria* species (*B. parva*, *B. ribis*, *B. mamane*, *B. rhodina*, *B. dothidea*, *Fusicoccum andinum* and *F. stromaticum*) on one commercial clone (256) of 2-year-old *Eucalyptus urophylla* x *E. grandis* hybrids in Venezuela. Stems of approximately between 25-35 cm in diam. and 7 m. in height were inoculated and lesion development recorded after seven weeks. Inoculations with *B. mamane*, *B. rhodina*, *B. dothidea*, *F. andinum* and *F. stromaticum* started to heal and produce callus around the wounds seven weeks after inoculation. *Botryosphaeria parva* and *B. ribis* caused bark swelling around the lesions and bleeding (black kino exudation) was observed when the outer bark was removed. A second inoculation trial was performed on four commercial clones to evaluate variation in their tolerance to infection, using *B. ribis* and *B. parva*, which were most pathogenic in the first trial. The clones differed in their tolerance to infection by *B. parva* and *B. ribis*. Clone 213 was the most tolerant, clones 113 and 276 were moderately tolerant, and clone 138 was least tolerant. *Botryosphaeria parva* was significantly more virulent than *B. ribis* in this clonal evaluation trial.

INTRODUCTION

The genus *Botryosphaeria* Ces. & De Not. represents a cosmopolitan group of fungi that have an exceptionally wide host range, particularly on woody plants (von Arx, 1987). These fungi are regarded as weak pathogens that attack stressed or wounded plants after drought, hail, wind, frost damage or insect infestation (Schoeneweiss, 1984; Old, 2000; Old & Davison, 2000). It has also been shown that some *Botryosphaeria* spp. live in asymptomatic tissue of hosts including *Eucalyptus* as latent pathogens, only expressing disease when the host defence mechanisms are inactivated due to stress (Fisher *et al.*, 1993; Smith *et al.*, 1994, 1996).

Infection by *Botryosphaeria* spp. has been reported to occur via wounds (von Arx & Müller, 1954; Ciesla *et al.*, 1996; Old, 2000; Old & Davison, 2000). On healthy plants, infection can also be directly through lenticels, stomata or other openings on healthy plants, without causing apparent damage or disease symptoms (Brown & Hendrix, 1981; Michailides, 1991; Smith *et al.*, 1996). After infection, these fungi also have the ability to invade the vascular system of woody hosts.

Eucalyptus species, which are native to Australia, are one of the most widely planted forest species worldwide (Poynton, 1979; Turnbull, 2000). *Botryosphaeria* spp. have been reported from both native and introduced *Eucalyptus* spp. and in many cases are amongst the most important pathogens of these trees (Davison & Tay, 1983; Shearer *et al.*, 1987; Old *et al.*, 1990; Smith *et al.*, 1994, 1996; Old, 2000; Old & Davison, 2000; Slippers *et al.*, 2004b). Common symptoms on *Eucalyptus* are dieback and cankers on stems and branches. The cankers are characterized by swelling of the stems, cracking of the bark around the lesions and copious exudation of red/black kino (Ciesla *et al.*, 1996; Smith *et al.*, 1994; Old, 2000; Old & Davison, 2000).

Various *Botryosphaeria* spp. have been reported from exotic *Eucalyptus* spp. (Sankaran *et al.*, 1995; Old, 2000; Old & Davison, 2000) where they are associated with a wide range of disease. Thus, *Botryosphaeria dothidea* has been isolated from basal cankers on *E. marginata* (Davison & Tay, 1983); seed capsule abortion and twig dieback on *E. camaldulensis* induced by *B. ribis* is known in Florida (Barnard *et al.*, 1987; Webb, 1983); stem canker on *E. tereticornis* caused by *Lasiodiplodia* (=*Botryodiplodia*) *theobromae* in Kerala, India (Sharma *et al.*, 1984); stick rot and canker caused by *B. ribis* is known on *E. grandis* and *E. citriodora* in Brazil (De Arruda Silveira, 2001); cankers on stems, branch and root disease on *Eucalyptus* sp. is caused by *B. ribis* in Argentina (Frezzi, 1952) and canker and die-back on *Eucalyptus* in South Africa, Congo and Uganda is caused by *Botryosphaeria dothidea, B. parva, B. eucalyptorum* and *Lasiodiplodia theobromae* (Smith *et al.*, 1994; Smith *et al.*, 2001; Roux, *et al.*, 2000, 2001, Slippers *et al.*, 2004b)

Recent studies have led to the identification of seven *Botryosphaeria* spp. from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela (Mohali *et al.*, 2005a, b). The aim of this study was to test the pathogenicity of the different species of *Botryosphaeria* in field inoculations on commercial *Eucalyptus* clones. A second aim was to determine whether a suite of commercially propagated clones are susceptible to infection by the most pathogenic of the *Botryosphaeria* spp. occurring on *Eucalyptus* in Venezuela.

MATERIALS AND METHODS

Fungal isolates

Isolates of the *Botryosphaeria* spp. used in this study (Table 1) emerged from a previous study (Mohali *et al.*, 2005a, b). These isolates originated from stems and branches of *Eucalyptus urophylla* S.T. Blake x *E. grandis* W. Hill ex Maiden hybrids and *Acacia mangium* Willd., growing in plantations in Acarigua, Portuguesa state, and from *Eucalyptus* sp. growing in the Cordillera Los Andes, Mérida state (Table 1). *Botryosphaeria* spp were collected from asymptomatic plant tissue, as well as from trees exhibiting blue stain or die-back, and from entirely dead trees. The isolates were grown on 2 % malt extract agar (MEA) (2% DIFCO Detroit, MI, USA) at 25 °C and stored on this medium at 4 °C. 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.

Inoculation tests

Inoculation experiments were conducted in January 2004. Inoculations were made on trees of approximately 7 m. in height, 25-35 cm in diam. and 2-years-old, in plantations of *E. urophylla* x *E. grandis* hybrids belonging to Smurfit Carton de Venezuela, Acarigua, Portuguesa state. For each tree, a piece of bark was removed with a cork borer (1.5 cm diameter) to expose the cambium. Bark discs were replaced by agar discs of the same size on which various test *Botryosphaeria* spp. grown. The wounds were covered with the original bark discs and sealed with masking tape to prevent desiccation. The lengths of the lesions that had formed in the cambium were measured seven weeks after the inoculations were made.

A total of seven *Botryosphaeria* spp. were used in the trial, of which six were isolated from the same area where this study was conducted. These included *B. ribis*, *B. parva*, *B. dothidea*, *B. mamane* Gardner, *B. rhodina*, *Fusicoccum stromaticum prov. nom.* and *F. andinum prov. nom.* from the mountain ranges in Mérida state (Table 1).

Two isolates of each of the seven *Botryosphaeria* spp. were used in inoculations (Table 1). A total of 280 trees (20 trees per isolate / 40 per taxon) of an *Eucalyptus urophylla* x *E. grandis* hybrid clone (256) were inoculated. Twenty trees of the same clone were inoculated with sterile MEA plugs to serve as controls. Prior to inoculations, the isolates were grown on 2 % MEA in Petri dishes at 25 °C for 14 days.

A second inoculation trial was made using only *B. ribis* and *B. parva*, which were the most pathogenic species in the trial used to assess pathogenicity of the different *Botryopshaeria* spp. This inoculation was made on four commercial clones (113, 138, 213, 276) representing *Eucalyptus grandis* x *urophylla* hybrids, and an equal number of trees (20 trees per isolate) were inoculated with sterile MEA plugs to serve as controls. Inoculations were made using the identical procedures to those used in the first inoculation trial. The lengths of the lesions were measured seven weeks after inoculation with the fungi.

Statistical analyses

Analyses of variance (ANOVA) were computed using the SPSS computer program (Nei *et al.*, 2005) for the lesion length in every treatment, and using Tukey's procedure for the comparison of means (P = 0.05).

RESULTS

There were significant differences ($P > 0.0001$, Table 2) in the lesion lengths for the different *Botryosphaeria* spp. inoculated onto clone 256. It was thus possible to separate the isolates into low, medium and high pathogenicity groups (Table 3, Fig. 1). The control inoculations with MEA plugs produced no lesions in the outer bark and had begun to heal and produce callus after seven weeks (Fig. 2).

Like the control, the isolates of *Botryosphaeria mamane* and *B. rhodina* did not produce lesions (Table 3, Fig. 1). *Botryosphaeria dothidea* produced very small lesions that did not differ significantly from the control and those of *B. mamane* and *B. rhodina* (Table 3, Fig. 1). Sites of inoculation with these three *Botryosphaeria* spp. had started to produce callus tissue around the wounds by the time the trial was terminated.

Fusicoccum andinum prov. nom. and *F. stromaticum prov. nom.* produced small lesions that were significantly larger than those for *Botryosphaeria mamane* and *B. rhodina* (Table 3, Fig.1). The sites of inoculation with *F. andinum* and *F. stromaticum* had started to heal and produce callus by the end of the trial.

Botryosphaeria ribis and *B. parva* produced significantly larger lesions than all the other *Botryosphaeria* spp. included in this study (Table 3, Fig. 1, 3). Both these species produced bark swelling around the lesions and bark cracks on some trees. Black kino exudation (Fig. 4, white arrow) was observed when the outer bark was removed.

In the second inoculation trial, on four commercial clones, only the isolates of *B. ribis* and *B. parva* were used. One isolate of *B. ribis* (CMW13409) and one of *B. parva* (CMW13355), was selected for this experiment. Bark swelling and occasional cracks and exudation of black kino were produced around the lesions on all of the clones

inoculated with these fungi. The control inoculations with sterile MEA plugs showed no lesion development.

Significant differences in lesion size were observed for lesions on the different clones ($P > 0.0001$, Table 4) after inoculations with *B. ribis* and *B. parva*. Clone 213 was the most tolerant to infection by either *B. ribis* or *B. parva* with an average lesion length of 30 mm. Clones 113 and 276 had average lesion length of 44 mm, and clone 138 was the least tolerant to infection with an average lesion length of 53 mm (Table 5, Fig. 5).

Significant differences in lesion size were also produced by *B. ribis* and *B. parva* on the different clones. *Botryosphaeria parva* was more virulent than *B. ribis* on the clones 113, 138 and 213, but on clone 276 *B. ribis* was more virulent (Fig. 6). On average across the clones, *B. parva* was more pathogenic (Fig. 6).

DISCUSSION

In this study stems of different *E. urophylla* x *E. grandis* hybrid clones were inoculated with seven different *Botryosphaeria* spp. recently identified in Venezuela (Mohali *et al.*, 2005a, b). Only some of the seven *Botryosphaeria* species were pathogenic on these trees and others failed to produce lesions. Clearly, those species that were pathogenic have the potential to damage trees sustaining an important forestry industry in Venezuela.

Botryosphaeria mamane did not produce lesions on stems of inoculated eucalypts. Prior to its recent discovery in Venezuela, this unusual species was known only from Hawaii. In that location, *B. mamane* has been associated with witches'-broom on the native leguminous forest tree, *Sophora chrysophylla* (Gardner, 1997), but these

symptoms have not been reported on *Eucalyptus*. In Venezuela, *B. mamane* was isolated as an endophyte from asymptomatic *Eucalyptus* tissue (Mohali *et al.*, 2005b). The origin of this fungus is unknown and it could be native to either Hawaii or Venezuela or it might have been introduced into either of these locations from elsewhere in the world.

Botryosphaeria rhodina did not produce lesions on *Eucalyptus* stems in this study. This result was somewhat unexpected as the fungus is associated with many diseases of trees. Some symptoms associated with this fungus include lesions in the inner wood, stem cankers, kino exudation and root disease on *E. tereticornis*, *E. grandis* and *Eucalyptus* spp. (Sharma *et al.*, 1984; Smith *et al.*, 1994; Roux *et al.*, 2000, 2001), as well as causing a reduction in the strength of tropical hardwoods of low density (Findlay, 1959). In Venezuela, *B. rhodina* has been associated with the death of *Pinus* and *Eucalyptus* trees, but only when plants are severely stressed by drought or other factors (Cedeño & Palacios-Pru, 1992; Mohali, 1993; Mohali *et al.*, 2002, 2005b). The isolates of *B. rhodina* used in this study were obtained from both asymptomatic and symptomatic tissue. These results suggest that *B. rhodina* is not a primary pathogen on healthy *Eucalyptus*, but based on its behaviour on other trees, it could be a serious opportunistic pathogen when trees are under environmental stress.

Botryosphaeria dothidea produced only small lesions on the inoculated *Eucalyptus* stems. This species has been associated with mortality of young transplants, dieback and cancer diseases on *Eucalyptus* in different parts of the world (Barnard *et al.*, 1987; Webb, 1983; Smith *et al.*, 1994). However, these studies were undertaken before recent studies characterizing *Botryopshaeria* spp. based on DNA sequence data, and the fungus might have represented one of a number of different species (Slippers *et al.*, 2004a). In this study, isolates were collected from asymptomatic plant tissue, as well as from trees exhibiting blue stain or dieback, and from entirely dead trees that were

stressed due to severe drought (Mohali, *et al.*, 2005b). As with *B. rhodina*, it appears that *B. dothidea* is not a primary pathogen on vigorously growing *Eucalyptus* in Venezuela, but the fungus has the potential to be a pathogen if trees are severely stressed.

Fusicoccum andinum prov. nom and *F. stromaticum prov nom.* produced only small lesions on inoculated *Eucalyptus* stems. These fungi represent newly described taxa that have only recently been discovered from Venezuela and we can not say, as yet, if they are native to the region or originate elsewhere (Mohali *et al.*, 2005a). *Fusicoccum andinum prov. nom.* was isolated from dried branches of old *Eucalyptus* trees without obvious diseases symptoms. *Fusicoccum stromaticum prov. nom.* originated from asymptomatic plant tissue, as well as from trees exhibiting blue stain or die-back, and from entirely dead trees (Mohali *et al.*, 2005a, 2005b). Currently there is no indication that these species are important pathogens and they rather appear to be weakly pathogenic endophytes.

Fusicoccum andinum was isolated from the mountain ranges (Mohali *et al.*, 2005a) where the temperature and humidity conditions are significantly different from those where this inoculation study was conducted. It is possible that the differences in conditions between the area of occurrence and the site of inoculation could have influenced the pathogenicity of this fungus in this study. In order to consider this question, pathogenicity trials would need to be conducted on eucalypts in the mountain ranges.

Botryosphaeria ribis and *B. parva* were the most pathogenic species in this study, where they produced the largest lesions as well as cracks in the bark and black kino exudation from the points of inoculation. *Botryosphaeria ribis* and *B. parva* are well-known pathogens of forest tree species, including *Eucalyptus* spp. (Frezzi, 1952;

Davison & Tay, 1983; Shearer *et al.*, 1987; Slippers *et al.*, 2004b; Ahumada 2003; Rodas, 2003). The pathogenicity of *B. ribis* and *B. parva* on inoculated *Eucalyptus* in Venezuela suggests that they have the capacity to cause diseases when the trees are under stress.

This study represents the first pathogenicity tests of different *Botryosphaeria* species of *Eucalyptus* in plantations in Venezuela. Future control efforts should clearly focus on the pathogenic species, *B. ribis* and *B. parva*. The tolerance of certain clones to inoculations with these fungi suggests that forestry enterprises will be able to capitalise on disease-tolerant *Eucalyptus* clones to avoid disease. This approach has been used in other countries such as South Africa (Wingfield *et al.*, 2001; Smith *et al.*, 2002). Based on the results of this study, it should be possible to initiate commercial disease screening trials to ensure that clones highly susceptible to infection by *Botryosphaeria* spp. are not planted on a wide scales.

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Table 1. Isolates of different *Botryosphaeria* species from Venezuela used in the inoculations trials. All isolates were collected by S. Mohali in 2003.

Isolates No	*Botryosphaeria* spp.	Host	Origin
CMW13355	*Botryosphaeria parva*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13419	*B. parva*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13362	*B. ribis*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13409	*B. ribis*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13373	*B. dothidea*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13381	*B. dothidea*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13370	*B. mamane*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13397	*B. mamane*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13487	*B. rhodina*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13488	*B. rhodina*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13366	*Fusicoccum stromaticum prov. nom*	*E. urophylla* x *E. grandis*	Acarigua, Portuguesa state
CMW13426	*F. stromaticum*	*Acacia mangium*	Acarigua, Portuguesa state
CMW13444	*F. andinum prov. nom*	*Eucalyptus* sp.	Mérida, Mérida state
CMW13455	*F. andinum*	*Eucalyptus* sp.	Mérida, Mérida state

Table 2. ANOVA for the lesion length associated with different *Botryosphaeria* spp. inoculated on *E. urophylla* x *E. grandis* hybrid (clone 256) in Venezuela.

Source	SS	df	MS	F	P
Species	382,830	7	54,690	62,483	0,0001
Isolates	4,705	1	4,705	5,375	0,0210
Species x Isolates	59,029	7	8,433	9,634	0,0001

Dependent Variable: Lesion length (mm)

Table 3. Mean lesion length (mm) of *Botryosphaeria* spp. inoculated on *E. urophylla* x *E. grandis* hybrid (clone 256) in Venezuela presented in groups representing three levels of pathogenicity (a, b, c).

Fungus	Low a	Medium b	High C
Control	0		
B. mamane	0		
B. rhodina	0		
B. dothidea	2		
F. andinum		6	
F. stromaticum		9	
B. ribis			27
B. parva			27

Tukey' multiple range test (P = 0.05)

Table 4. ANOVA for the lesion length associated with *Botryosphaeria parva* and *B. ribis* inoculated on different *Eucalyptus* commercial clones in Venezuela.

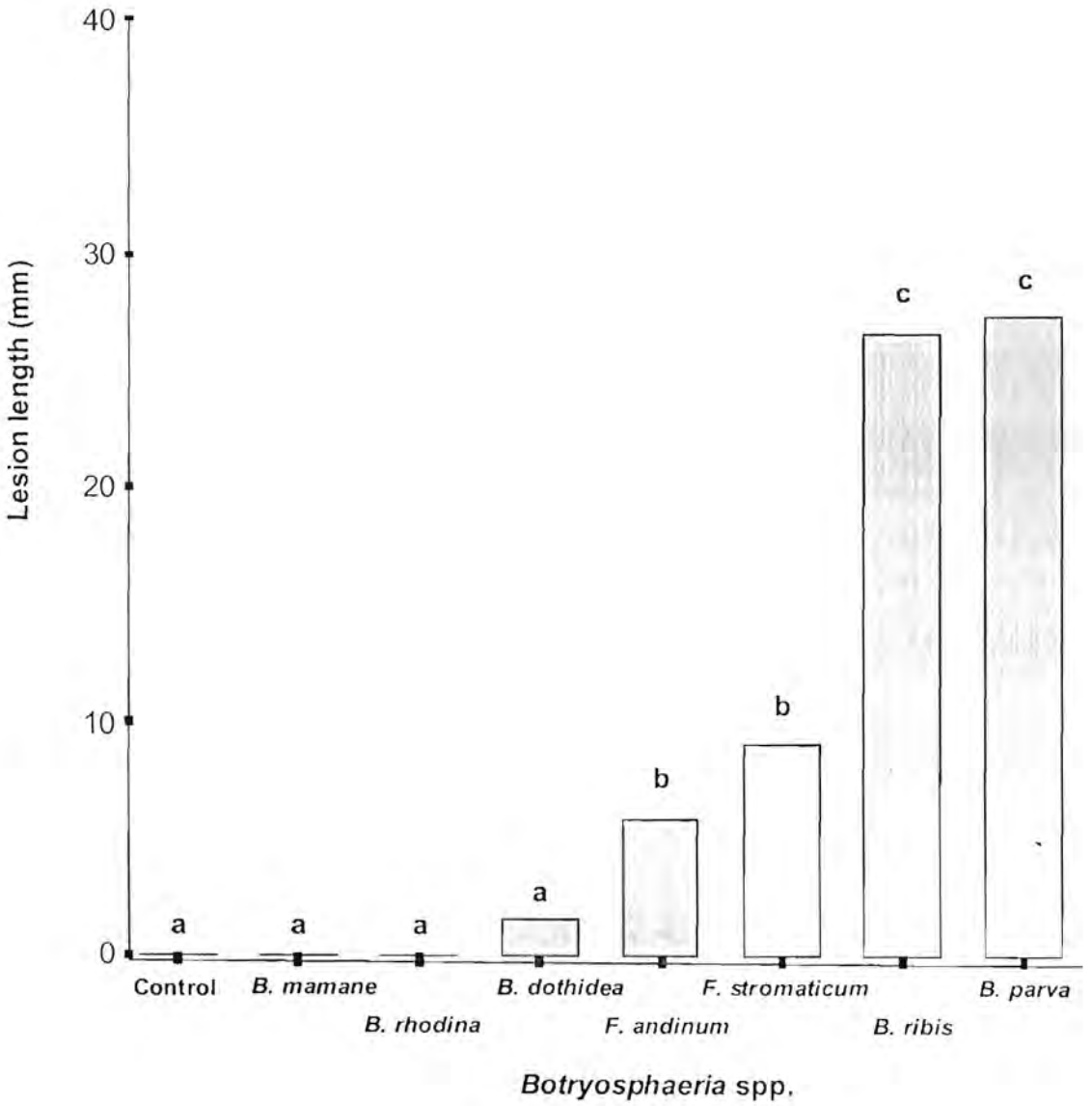
Source	SS	df	MS	F	P
Isolate	176,344	1	176,344	31,346	0,0001
Clones	145,732	4	36,433	6,476	0,0001
Isolate x Clone	179,784	4	44,946	7,989	0,0001

Table 5. Mean lesion length (mm) from commercial different clones inoculated with *Botryosphaeria parva* and *B. ribis* in Venezuela presented in groups according to level of pathogenicity (a, b, c).

Clones	Low a	Medium b	High c
Clone 213	30		
Clone 113		43	
Clone 276		45	
Clone 138			53

Tukey' multiple range test (P = 0.05)

Fig. 1 Mean lesion length values (mm) of different *Botryosphaeria* spp. inoculated on *E. urophylla* x *E. grandis* hybrid (clone 256) in Venezuela. Lesions were measured seven weeks after inoculation. The isolates of *Botryosphaeria* species differ significantly according to Tukey's multiple range test (P = 0.05).



Figs 2-4. Lesion development during inoculation trials. **Fig. 2.** Control inoculations on *Eucalyptus* hybrid stem with MEA plugs produced no lesions. **Fig. 3.** Lesion produced by *B. parva*. **Fig. 4.** Presence of black kino was observed when the outer bark was removed (white arrow) after inoculation with *B. parva*.

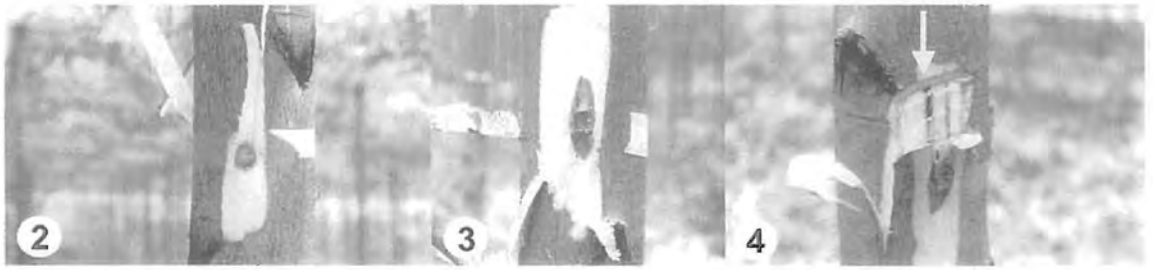


Fig. 5 Mean lesion length (mm) on stem of different clones of *Eucalyptus urophylla* x *E. grandis* hybrids inoculated to assess the tolerance of different commercial clones of this hybrid to *Botryospharia parva* and *B. ribis*.

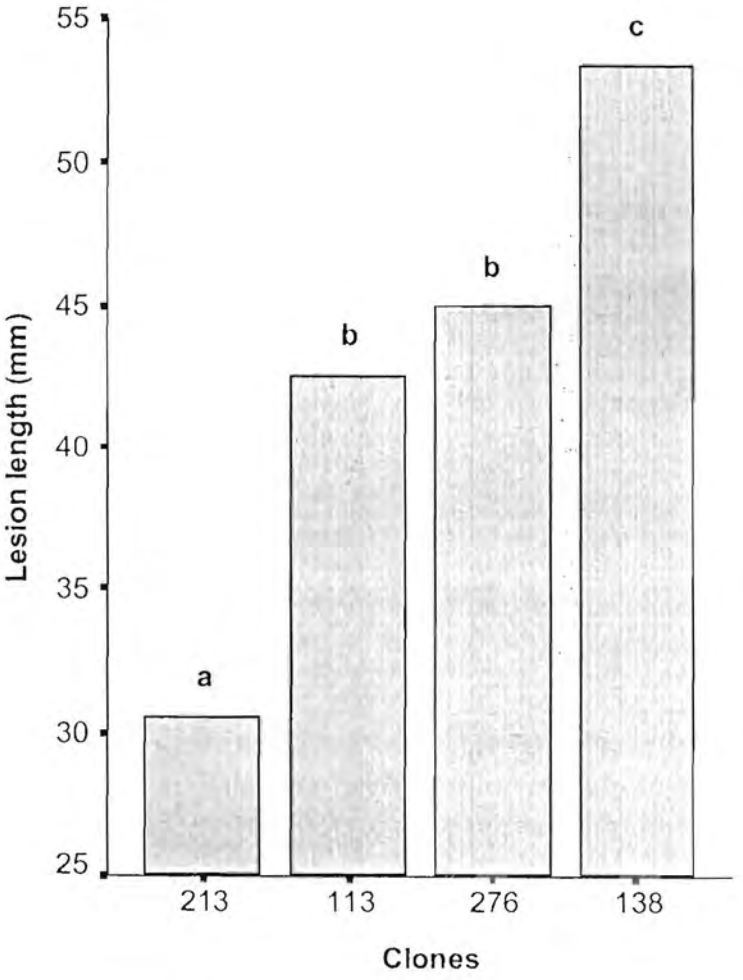


Fig. 6 Relative susceptibility (mean lesion length in mm) of 4 commercial clones of *Eucalyptus* to inoculation with *Botryosphaeria parva* and *B. ribis*. Values in the histogram differ significantly (Table 4, $P > 0.0001$). Relative virulence of *B. parva* and *B. ribis* on all commercial clones.