# The taxonomy, phylogeny and ecology of Botryosphaeriaceous fungi occurring on various woody hosts

This dissertation is presented in partial fulfillment of the requirements of the degree

***PHILOSOPHIAE DOCTOR***

in the Faculty of Natural and Agricultural Sciences, Department of Microbiology and
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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. This work has hitherto not been submitted for any degree at any other University.

Bernard Slippers

Bernard Slippers

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I dedicate this thesis to my wife, Jana.
You were with me through every bit of this experience, all the way to the end. You are truly my other half. I cannot wait for the rest of what lies ahead for us.

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# ACKNOWLEDGEMENTS

I am sometimes overwhelmed by the wonder of the biological world. This love for biology is deeply rooted and has been there since I was a child. I ended up studying fungi during the part of my life that this thesis represents, and have gained immense pleasure and satisfaction from it. Perhaps any part of biology would do, but fungi will no doubt always have a special place in my heart. I also truly believe the wonder of life was created, with a purpose, and I see myself as part of that creation. Therefore, looking back on this study, I am deeply grateful to the Creator for the purpose and love, the opportunity, the inspiration and guidance, and making me part of it all. I cannot wait to continue on this road and learn more.

The thesis was not produced on my effort alone, but was shaped by the social structure that I am part of. This includes family, friends, colleagues and other people that I have had contact with. Sometimes it is difficult to put boundaries around these categories of people – like some species studied during this project. Some friends are family, some colleagues are friends, etc. But no matter where you think you belong in the classification of relationships in my life, I need to and want to thank you.

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# PREFACE

Identification of *Botryosphaeria* spp. is difficult. The sexual state (teleomorph) is not commonly seen and there is considerable overlap in morphological characters across the species. Anamorphs are more common and often have more distinctive morphological features. However, closely related anamorph taxa also have overlapping morphological characters. Traditional identification using these data has, therefore, caused considerable confusion in the taxonomy of these fungi. In more recent years, comparisons of DNA sequence data have contributed significantly to *Botryosphaeria* taxonomy. Such studies, which are mostly based on single gene phylogenies or dominant molecular markers, have, however, not resolved some long-standing taxonomic controversies. It is evident from an overview of the current literature that this commonly encountered and economically important genus of fungi remains in taxonomic disarray. The main aims of this study were, therefore, to address questions relating to identification of species, as well as relationships between species and their geographic distribution.

The first question addressed in this thesis concerns the identity of the type species of the genus *Botryosphaeria*, namely *B. dothidea*. Since its description in 1863, this taxon has been applied to a wide diversity of species in the genus. The result is that there is considerable confusion over the use of this name, especially concerning its relationship to *B. ribis*. Many researchers use these taxa as referring to separate species, while others view them as conspecific. Further confusion arises from the morphological and ITS rDNA sequence similarity between isolates identified as *B. ribis* and *B. parva*. In the first chapter we consider the morphology of type specimens and original descriptions of these fungi. An attempt is also made to clarify confusion regarding these names by selecting neotypes, lectotypes and epitypes to represent these names. From ex-type and other morphologically characterized isolates, multiple gene sequence genealogies are produced in order to distinguish especially *B. ribis* and *B. parva*, which are difficult to distinguish using all other data.

*Botryosphaeria* species occur on a wide diversity of plants, world-wide. Some species are also important pathogens. Hosts include many economically important agricultural and forestry crops. A growing concern is that pathogenic *Botryosphaeria* spp. from introduced hosts can move onto native hosts, and *visa versa*. In Chapters 2 to 6 *Botryosphaeria* species and species complexes are identified from a number of

# SUMMARY

The identification of species is controversial in many groups of organisms. This is also true for species in the genus *Botryosphaeria*. One of the reasons is that phenotypic and ecological characters that have been traditionally used to identify species boundaries, tend to be interpreted subjectively. Modern techniques based on DNA sequence analyses can be used more objectively to make comparisons between species. The drawback of DNA based techniques is that the biology of an organism is not reflected by DNA, but in the expression of the information contained in the DNA and the eventual interaction with the physical and biological environment. Therefore, the combination of DNA and phenotypic data can be used very effectively to identify species boundaries and subsequently to characterize the populations that make up a species. This approach has thus been followed in developing chapters of this thesis focused on the taxonomy and identification of *Botryosphaeria* species.

Chapter 1. *Botryosphaeria dothidea* epitypification, *B. parva* and *B. ribis*

*Botryosphaeria dothidea* is the type and thus the anchor species for the genus. Controversy has persisted regarding the correct identity of this taxon, especially concerning closely related species such as *B. berengeriana, B. ribis* and *B. parva*. In order to clarify this situation, epitype material for *B. dothidea* was collected in 2001 from the same sites (Italy and Switzerland) and hosts as the original collections in 1863. Material for *B. ribis* was collected on our behalf in New York from where this species was first described in 1911. Material from the original collections was also obtained from herbaria from Rome, New York and New Zealand. Using these collections and combining morphology and multiple DNA genealogies, we were able to identify and describe each of the named species. *Botryosphaeria dothidea* and *B. berengeriana* were, however, synonymized. *Botryosphaeria ribis* and *B. parva* are shown to be distinct species, albeit very closely related and cryptic when studying individual gene phylogenies and morphology.

Chapter 2. *Botryosphaeria* on *Mangifera indica*

*Botryosphaeria* spp. cause die-back of mango trees, as well as severe pre- and post-harvest diseases of mango fruit. For a number of years a plethora of species in the anamorph genera *Dothiorella*, *Natrassia* and *Fusicoccum* have been applied to the

fungi isolated from this tree. Using DNA sequence data from two gene regions, we could identify four species involved in causing diseases of mango. These species are also easily distinguished using anamorph morphological characters. The four species group with other species of *Botryosphaeria*, but no specific teleomorph names could be linked to two of these taxa. They were identified as *B. parva*, *B. dothidea*, *F. mangiferum* (comb. nov.) and an unknown *Fusicoccum* sp.

### Chapter 3. *Botryosphaeria australis sp. nov.* and *B. lutea*

There are a number of *Acacia* spp. that are native to Australia. Some of these species are fast growing and are important commercial and informal forestry crops around the world. *Botryosphaeria* spp. are known to affect these trees where they have been introduced. Little is, however, known about potential pathogens of these trees in Australia. In this study, a group of isolates were identified that closely resembled *B. lutea* in morphology and ITS rDNA sequence. Concordance between phylogenies of the ITS rDNA and two other gene regions, however, confirmed the phylogenetic separation of *B. lutea* and the isolates from Australian *Acacia*. This previously unknown species is described here as *B. australis* sp. nov. (anamorph *F. australe*).

### Chapter 4. *Botryosphaeria* spp. infecting pome and stone fruit trees

A number of *Botryosphaeria* spp. affect pome and stone fruit trees world-wide. Confusion over names of these species complicates comparisons of results of different studies and the eventual control of the diseases. Sequence data and morphology were thus used in this study to compare South African collections with data from other parts of the world. *Botryosphaeria obtusa* was identified as the main species that affects these hosts in South Africa. The newly described *B. australis* is also identified from these hosts for the first time. Based on data from GenBank, *B. dothidea*, *B. stevensii* and fungi in the *B. ribis* – *B. parva* complex, were also identified from fruit trees in other parts of the world. A rapid PCR RFLP technique was developed to effectively identify all these *Botryosphaeria* species from fruit trees.

### Chapter 5. *Botryosphaeria* spp. from *Eucalyptus*

*Eucalyptus* trees are amongst the most important sources of fiber, especially for the production of paper, in South Africa and in other countries in the tropics and southern hemisphere. *Botryosphaeria* spp. are one of the most significant pathogens of

*Eucalyptus* trees in areas where they are planted as exotics. In this chapter, isolates from native *Eucalyptus* trees in Australia and exotic trees in South Africa, Hawaii and Uruguay were compared based on morphology and molecular data. A PCR RFLP technique was also developed to identify the fungi from this host. *Botryosphaeria dothidea*, which has commonly been reported from *Eucalyptus*, was shown to be rare on this host. This fungus and *B. ribis* have been mistaken for *B. parva*, which is commonly found on exotic *Eucalyptus*. A new and cryptic species closely related to *B. eucalyptorum* was also identified by studying concordance between genealogies of three gene regions. This new species was given the name *B. irregularis* sp. nov. (anamorph *F. irregulare*). This study also indicated that *B. eucalyptorum* and *B. irregulare* are most likely native to *Eucalyptus* in Australia, but have been introduced to new environments on this host.

### Chapter 6. *Botryosphaeria* spp. from southern hemisphere conifers

A number of rare coniferous tree species occur in the southern hemisphere, including *Wollemia nobilis* (Australia), *Widdringtonia* spp. (Africa) and some *Araucaria* spp. (widely distributed in the southern hemisphere). The small populations and limited genetic base of some of these species place them at high risk to diseases. Multiple gene genealogies and morphology were used to show that at least two Botryosphaeriaceous fungi infect these hosts. The first is most closely related to *B. ribis* and the second to *B. australis*. Both these fungi contain some unique DNA sequence polymorphism that necessitates further investigation.

### Chapter 7. Taxonomy of *B. quercuum, B. stevensii, B. obtusa* and their anamorphs

The taxonomy of *B. quercuum*, *B. obtusa* and *B. stevensii* was thoroughly treated in 1964. Despite this study confusion has persisted regarding the identities of these species. Recent studies using modern molecular tools to study phylogeny of these fungi have also been hampered due to this confusion. In this study, DNA sequence data and morphological characteristics of the *Diplodia* anamorphs in culture are combined to address the questions relating to this group of species. It was possible to show that conidial size is inordinately variable amongst these species, to be used as an isolated taxonomic character. When these data were combined with data relating to cell wall thickness, patterns of conidial septation and color, as well as host association, then *B. obtusa*, *B. stevensii* and *B. quercuum* could be more easily distinguished from each other.

### Chapter 8. Development of SSR and RFLP markers for *Botryosphaeria* spp.

Simple sequence repeat (SSR) or micro-satellite markers are some of the most powerful modern tools to study populations and species. A set of eight polymorphic, co-dominant micro-satellite markers was developed for *B. parva.* In addition loci with varying restriction sites were also identified as a source of polymorphic markers. The primers developed to amplify these regions were tested on other *Botryosphaeria* species and were shown to be useful for at least nine other species.

### Chapter 9. The *B. parva – B. ribis* complex

*Botryosphaeria parva* and *B. ribis* have been shown to be distinct species based on multiple gene genealogies, but their identification remains difficult. In this chapter, the genetic boundaries between these species were characterized by combining data from gene genealogies, SSR makers and PCR RFLP fingerprints. These data revealed variation within *B. parva* and *B. ribis* that are referred to as *sensu lato* and *sensu stricto* groups in these species. The RFLP profiles were particularly useful to provide a rapid and effective means to distinguish these species. Furthermore, it was shown that these fungi, especially *B. parva*, have been moved around the world on hosts such as *Eucalyptus.* Due to this movement, *B. parva* clearly represents a worldwide meta-population, where recombination is evident among the isolates from different hosts and continents. A number of clonal lines were, however, also identified that occurred across spatial and temporal boundaries. This indicates that asexual reproduction also plays an important role in structuring populations of this fungus.

### Chapter 10. Species identification in *Botryosphaeria*, 1863-2003

The data presented in this thesis and other recent publications, make it clear that *Botryosphaeria* species can no longer be identified solely on morphological characters. DNA sequence data are most useful to distinguish most *Botryosphaeria* species. Not all other DNA based tools are, however, phylogenetically informative, or they are difficult to interpret in isolation. This is especially true when attempting to identify cryptic species. In these cases SSR markers and multiple gene DNA sequence data are needed to distinguish the species. Techniques such as PCR RFLP's and species specific primers will facilitate more effective future identification of *Botryosphaeria* spp. These DNA data have also recently been successfully combined with phenotypic and ecological information to better understand and describe *Botryosphaeria* spp. I trust that the broader approach to the identification of *Botryosphaeria* spp. followed and advocated in this thesis will help to overcome problems relating to the systematics of this important group of fungi. I also hope that the information will promote accurate and reasonably rapid identification of species of *Botryosphaeria*.

# OPSOMMING

Spesie-identifikasie is in baie groepe organismes kontroversieel. Dit geld ook vir *Botryosphaeria*-spesies. Een van die redes is dat fenotipiese en ekologiese karakters, wat tradisioneel gebruik is om spesiegrense te identifiseer, subjektief geïnterpreteer kan word. Moderne tegnieke, gebaseer op DNS-volgordeanalises kan meer objektief gebruik word om spesies te vergelyk. Die tekortkoming van DNS-gebaseerde tegnieke is dat die biologie van 'n organisme nie hierin te vinde is nie, maar wel in die fenotipiese uitvloeisels van die inligting vervat in die DNS en die uiteindelike interaksie met die fisiese en biologiese omgewing. Daarom kan die kombinasie van DNS en fenotipiese data baie effektief gebruik word om spesiegrense te identifiseer en uiteindelik die populasies, waaruit die spesie bestaan, uit te beeld. Hierdie benadering is dus gevolg in die ontwikkeling van die proefskrif se hoofstukke wat fokus op die taksonomie en identifikasie van *Botryosphaeria*-spesies.

## Hoofstuk 1. *Botryosphaeria dothidea* epitipifisering, *B. parva* en *B. ribis*

*Botryosphaeria dothidea* is die tipe en dus anker-spesie van die genus. Daar is deurlopende kontroversie oor die identiteit van hierdie takson, veral met verwysing na nabyverwante spesies soos *B. berengeriana, B. ribis* en *B. parva.* Om hierdie situasie op te los is epitipe-materiaal vir *B. dothidea* in 2001 van dieselfde areas (Italië en Switzerland) en gashere as die oorspronklike versamelings in 1836 versamel. Materiaal van *B. ribis* is namens ons in New York versamel, waar hierdie spesie eerste in 1911 beskryf is. Materiaal van oorspronklike versamelings is ook vanaf herbaria in Rome, New York en Nieu-Seeland bekom. Deur die gebruik van hierdie versamelings en die kombinasie van morfologie en meervoudige DNS-geenfilogenie kon ons elk van die genoemde spesies identifiseer en beskryf. *Botryosphaeria dothidea* en *B. berengeriana* blyk sinoniem te wees. Daarenteen is *B. ribis* en *B. parva* afsonderlike spesies, alhoewel hulle baie naverwant is en kripties as individuele geenfilogene en morfologie bestudeer word.

## Hoofstuk 2. Botryosphaeria-*spesies* vanaf *Mangifera indica*

*Botryosphaeria*-spesies veroorsaak terugsterwing van mangobome en ook ernstige voor- en na-oessiektes van die mangovrugte. Vir 'n paar jaar reeds word verskeie spesiename in die anamorfgenera *Dothiorella, Natrassia* en *Fisicoccum* gebruik om

die swamme wat van hierdie bome geïsoleer word, te beskryf. Deur die gebruik van DNS-volgorde van twee geenareas kon ons vier spesies identifiseer wat siektes van mango veroorsaak. Hierdie spesies kan ook maklik op grond van hulle anamorf morfologie onderskei word. Al vier spesies word saam met die *Botryosphaeria*-spesie geklassifiseer, maar spesifieke teleomorfname kon slegs vir twee van hierdie spesies bepaal word. Hulle word dus as *B. parva, B. dothidea, F. mangiferun* (comb. nov.) en 'n onbekende *Fusicoccum*-spesie geïdentifiseer.

### Hoofstuk 3. *Botryosphaeria australis sp. nov.* en *B. lutea*

Australië het 'n hele aantal inheemse *Acacia*-spesies. Sommige van hierdie spesies groei vinnig en is wêreldwyd 'n belangrike kommersiële en informele bosbougewas. Dit is bekend dat *Botryosphaeria*-spesies hierdie bome, in areas waar hulle uitheems groei, besmet. Daar is egter min bekend oor die patogene van hierdie bome in Australië. In hierdie studie word 'n groep isolate geïdentifiseer wat baie na *B. litea* in morfologie en ITS rDNA-volgordes lyk. Ooreenstemming tussen die filogenie van die ITS rDNA en twee ander geenareas bevestig egter die filogenetiese skeiding van *B. litea* en die isolate van Australiese *Acacia*. Hierdie voorheen onbekende spesie word as *B. australis* sp. nov. (anamorf *F. australe*) beskryf.

### Hoofstuk 4. *Botryosphaeria*-spesies wat vrugtebome infekteer

'n Aantal *Botryosphaeria*-spesies veroorsaak wêreldwyd siektes van appel- en steenvrugbome. Die verwarring oor name van hierdie spesies bemoeilik die vergelyking van resultate tussen verskillende studies en uiteindelike beheer van die siektes. DNA-volgordedata en morfologie word in hierdie studie gebruik om Suid-Afrikaanse versamelings met data van ander wêrelddele te vergelyk. *Botryosphaeria obtusa* word as die vernaamste spesie wat hierdie gasheerplante in Suid-Afrika beïnvloed, geïdentifiseer. Die nuutbeskryfde *B. australis* word ook vir die eerste keer op vrugtebome geïdentifiseer. Gebaseer op data van GenBank word *B. dothidea*, *B. stevensii* en die *B. ribis* – *B. parva*-kompleks ook algemeen op vrugtebome van ander wêrelddele gevind. 'n Vinnige PKR RFLP-tegniek is ontwikkel om die *Botryosphaeria*-spesies effektief van mekaar te onderskei.

### Hoofstuk 5. *Botryosphaeria*-spesies vanaf *Eucalyptus*

*Eucalyptus*-bome is van die belangrikste veselbronne vir die vervaardiging van papier in Suid-Afrika en ander lande in die trope en suidelike halfrond. *Botryosphaeria*-spesies is van die belangrikste patogene van *Eucalyptus*-bome in die areas waar hulle uitheems geplant word. In hierdie hoofstuk word isolate van inheemse *Eucalyptus*-bome in Australië en uitheemse bome in Suid-Afrika, Hawaii en Uruguay op grond van morfologie en DNA-data met mekaar vergelyk. 'n PKR RFLP-tegniek is ontwikkel om die swamme van hierdie gasheer te identifiseer. Daar word aangetoon dat *B. dothidea* wat voorheen algemeen vanaf *Eucalyptus* beskryf is, eintlik raar op hierdie gasheer aangetref word. Hierdie swam en *B. ribis* is voorheen met *B. parva* verwar, wat algemeen op *Eucalyptus* voorkom. 'n Nuwe, kriptiese spesie wat naverwant aan *B. eucalyptorum* is, word geïdentisifeer deurdat ooreenstemming tussen drie geenareas gewys word. Hierdie nuwe spesie word as *B. irregularis* sp. nov. (anamorf *F. irregulare*) benoem. Die navorsing toon dat *B. eucalyptorum* en *B. irregularis* inheems op *Eucalyptus* in Australië is, maar dat hulle via hierdie gasheer na verskeie ander lande versprei het.

Hoofstuk 6. *Botryosphaeria*-spesies vanaf suidelike halfrond keëldraende bome

Daar is heelparty skaars, keëldraende bome in die suidelike halfrond, insluitende *Wollemia nobilis* (Australië), *Widdringtonia*-spesies (Afrika) en sommige *Araucaria*-spesies (wydversprei in die suidelike halfrond). Die klein populasies en beperkte genetiese basis van sommige van hierdie spesies veroorsaak dat daar 'n hoë risiko bestaan om deur siektes uitgewis te word. Meervoudige geenfilogenie en morfologie word gebruik om aan te toon dat daar ten minstens twee *Botryosphaeria*-agtige swamme is wat hierdie gasheer besmet. Hulle is onderskeidelik na aan *B. ribis en B. australis* verwant. Beide hierdie spesies bevat egter unieke DNS-volgorde polimorfismes wat verdere ondersoek vereis.

Hoofstuk 7. Taksonomie van *B. quercuum, B. stevensii, B. obtusa* en hul anamorwe

Die taksonomie van *B. quercuum, B. obtusa* en *B. stevensii* is deeglik in 1964 ondersoek. Ten spyte van hierdie studie het verwarring aangaande die identiteit van hierdie spesies voortgeduur. Onlangse studies wat moderne tegnieke gebruik om die filogenie van hierdie swamme te bestudeer, is deur hierdie verwarring gekortwiek. In hierdie studie word DNS-volgordedata en die morfologiese karaktereienskappe van *Diplodia*-anamorwe in kultuur gebruik om die vrae aangaande hierdie groep spesies te beantwoord. Daar word getoon dat die konidium grootte te veel varieer om in isoloasie as taksonomies onderskeidende karakter gebruik te word. As hierdie data egter gekombineer word met data van selwanddikte, die patroon van die konidium septering en -verkleuring en ook gasheerassosiasie kan *B. quercuum, B. obtusa* en *B. stevensii* meer akkuraat van mekaar onderskei word.

## Hoofstuk 8. Ontwikkeling van SSR en RFLP merkers vir *Botryosphaeria*-spesies

Mikrosatelietmerkers is van die mees kragtige, moderne molekulêre instrumente om spesies en populasies van organimes te bestudeer. 'n Stel van agt polimorfiese, ko-dominante mikrosatelietmerkers word vir *B. parva* ontwikkel. In ander loki word polimorfiese restruksiesnydingspunte, wat as bykomende merkers dien, geïdentifiseer. Dit word getoon dat die voorvoerders wat ontwikkel is om die verskillende loki te vermenigvuldig ook in nege ander *Botryosphaeria*-spesies benut kan word.

## Hoofstuk 9. Die *B. parva* - *B. ribis*-kompleks

Daar is vroeër aangetoon dat *B. parva* en *B. ribis* afsonderlike spesies is, maar hulle roetine-identifikasie bly problematies. In die hoofstuk word die genetiese grense tussen hierdie spesies deur die kombinasie van meervoudige geenfilogenie, mikrosatelietmerkers en PKR RFLP-patrone uitgebeeld. Hierdie data het variasie binne *B. parva* en *B. ribis* uitgewys wat as die *sensu lato* en *sensu stricto* groepe vir hierdie spesies beskryf word. Die RFLP-patrone was veral nuttig as vinnige en effektiewe manier om die spesies te onderskei. Verder word getoon dat hierdie swamme, veral *B. parva,* oor die wêreld op gashere, soos *Eucalyptus,* versprei is. As gevolg van hierdie verspreiding vorm *B. parva* 'n wêreldwye metapopulasie, waar genetiese herkombinasie tussen isolate van verskillende gashere en lande plaasvind. 'n Aantal klonale lyne word oor ruimtelike en tydsgrense geïdentifiseer. Hieruit kan afgelei word dat a-seksuele voortplanting 'n belangrike rol in die opbou van populasies van hierdie swam speel.

## Hoofstuk 10. Identifikasie van spesies in *Botryosphaeria*, 1863-2003

Die resultate van hierdie proefskrif en ander onlangse publikasies maak dit duidelik dat *Botryosphaeria*-spesies nie slegs op grond van morfologie geïdentifiseer kan word nie. DNS-volgordes is die mees bruikbare data om *Botryosphaeria*-spesies te onderskei. Nie alle ander DNS gebaseerde instrumente is egter filogeneties insiggewend nie, of hulle is moeilik om in isolasie te vertolk. Dít is veral die geval met die identifisering van kriptiese spesies. In hierdie gevalle word mikrosatelietmerkers en DNS-volgorde van meervoudige geenloki-data benodig om die spesies te onderskei. Tegnieke soos PKR RFLP en spesie spesifieke voorloperfragmente is ook nuttig as vinnige en effektiewe manier om spesies te identifiseer. Hierdie DNS-data is onlangs suksesvol met fenotipiese en ekologiese inligting gekombineer om *Botryosphaeria*-spesies te identifiseer en te omskryf. Ek vertrou dat dié breër benadering tot die identifisering van *Botryosphaeria*-spesies, wat in hierdie proefskrif gevolg en aanbeveel is, sal bydra om probleme in verband met die sistematiek van hierdie groep swamme te oorkom. Daarmee saam hoop ek dat die inligting met die akkurate en redelik vinnige identifisering van *Botryosphaeria*-spesies sal help.

# 1

# Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*

***Abstract:*** *Botryosphaeria dothidea* is one of the most commonly reported species in a genus of important pathogens of woody plants. This taxon is generally accepted to represent a species complex, and hence its identity remains unclear. Previous studies have either treated *B. dothidea* as the valid name for *B. ribis* and *B. berengeriana*, or argued for them to be separate entities. To add to the confusion, no ex-type cultures are available for either *B. dothidea* or *B. ribis*. The aim of the present study, therefore, was to recollect and characterize these fungi, and designate a set of reference cultures that can be used in future studies. To this end morphological, cultural, and multi-allelic DNA sequence data sets from the rDNA (ITS 1, 5.8S, and ITS 2), β-tubulin and EF1-α genes were used to fully characterize these species. *Botryosphaeria dothidea* was found to be distinct from *B. ribis*, while *B. berengeriana* was retained as synonym of the former name. Furthermore, *Fusicoccum aesculi* is accepted as anamorph of *B. dothidea*, while the anamorph of *B. ribis* is newly described as *F. ribis* sp. nov. *Botryosphaeria ribis* could be distinguished from *B. parva* based on β-tubulin and EF1-α sequence data. A combined phylogeny of the three gene regions used in this study also showed that the genus *Botryosphaeria* represents two distinct phylogenetic assemblages that correspond to species with *Diplodia* and *Fusicoccum* anamorphs.

## INTRODUCTION

*Botryosphaeria* Ces. & De Not. was described in 1863 (Cesati and De Notaris 1863). Cesati and De Notaris (1863) first included 12 species in the genus, but did not provide detailed morphological descriptions of the species. De Notaris (1863) added another 4 species, including *B. berengeriana* De Not., for which he provided detailed descriptions and sketches. Saccardo (1877) amended the initial generic descriptions of Cesati and De Notaris to exclude hypocreaceous species, which he transferred to two new genera, *Gibberella* and *Lisea*. Von Arx and Müller (1954, 1975), who did an extensive revision and key (respectively) of the genus, cite this amendment as part of the generic description.

Cesati and De Notaris (1863) did not select a type species for the genus. Barr (1972) rejected propositions that either *B. quercuum* (Schwein.) Sacc. or *B. berengeriana* be designated as the lectotype species of the genus because these species were not part of the original description of the genus. Barr (1972) therefore 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 species of the genus.

Despite obvious similarities between specimens, early researchers tended to describe new *Botryosphaeria* species, where these fungi occurred on different hosts (Cesati and De Notaris 1863, De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928). Von Arx and Müller (1954), however, synonymized many of these species under *B. quercuum* and *B. dothidea*, based on teleomorph herbarium material. Many researchers did not accept the extensive synonymies of von Arx and Müller (1954). For example, *B. dothidea* and *B. ribis* Grossenb. & Duggar have been viewed as distinct species by many due to differences in anamorph morphology (Punithalingam and Holliday 1973, Morgan-Jones and White 1987, Rayachhetry et al 1996, Smith and Stanosz 2001, Zhou and Stanosz 2001a, b), while others treated them as synonyms *sensu* von Arx and Müller (Witcher and Clayton 1963, Barr 1972, English et al 1975, Spiers 1977, Maas and Uecker 1984, Pennycook and Samuels 1985, Brown and Britton 1986, Smith et al 1994). A further basis for confusion is that von Arx and Müller (1975) considered *B. berengeriana*, which they had synonymized earlier with *B. dothidea* (von Arx and Müller 1954), as one of the most common species of the genus. According to von Arx (1987), the name *B. dothidea* should be restricted to isolates pathogenic to roses, while he considered *B. berengeriana* (including *B. ribis*) as polyphagous. Currently, the name *B. berengeriana* is not commonly used, except in Japan (Sassa et al 1998, Ogata et al 2000).

The *Botryosphaeria* teleomorph is seldom seen in culture, whereas the anamorphs are common. Species differences are manifested in the anamorph, while there is considerable overlapping in the continuous characters of the teleomorph, such as spore sizes. For these reasons, anamorph characters are often considered important to identify species in this genus (Shoemaker 1964, Pennycook and Samuels 1985). Denman et al (2000) recorded 18 anamorph genera that have been linked to *Botryosphaeria*, with *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Cda., *Lasiodiplodia* Ellis & Everh., and *Macrophoma* (Sacc.) Berl. & Voglino the most common. Of these, *Macrophoma* has been synonymized with *Sphaeropsis* Sacc. (Sutton 1980). Crous and Palm (1999) also showed that *Botryodiplodia* is a *nomen dubium* and that the type specimen of *Dothiorella* is best accommodated in *Diplodia*. The use of anamorph characters is also complicated by the overlapping characteristics between species and the effect of aging on conidium morphology (Pennycook and Samuels 1985, Jacobs and Rehner 1998, Smith and Stanosz 2001).

Phylogenetic studies, using both morphological and molecular data, have contributed significantly to *Botryosphaeria* taxonomy. Recent studies have used a combination of morphological and DNA sequence, RAPD or ISSR data to study relations among species and to define *Botryosphaeria* spp. (Jacobs and Rehner 1998, Denman et al 1999, Smith et al 2001, Smith and Stanosz 2001, Zhou et al 2001, Zhou and Stanosz 2001a). One of the main conclusions drawn from these studies is that *Botryosphaeria* spp. can be separated into two groups, namely those with dark-conidial diplodia-like anamorphs, and those with hyaline-conidial fusicoccum-like anamorphs. Denman et al (2000) revised the generic taxonomy of the anamorphs that have been linked to *Botryosphaeria* and concluded that those with hyaline conidia be included in *Fusicoccum* and those with conidia that are dark and opaque when mature be included in *Diplodia*. These findings were supported by Zhou and Stanosz (2001a), who referred the two anamorph genera to section *Hyala* and section *Brunnea*. Contrary to these studies, Zhou and Stanosz (2001b) found that these groups were not supported by partial mitochondrial (mt) SSU sequence data. These authors suggested that these contradictions might have been due to lack of resolution using this part the mitochondrial rDNA gene region. Alternatively, that it might have arisen through hybridization or horizontal gene transfer before the separation of the two groups mentioned above.

Despite their considerable contribution to *Botryosphaeria* taxonomy, single gene phylogenies and other molecular data have not resolved some long-standing taxonomic controversies. The morphological species *B. dothidea* is paraphyletic and divided into two clades based on molecular data (Jacobs and Rehner 1998, Denman et al 1999, Denman et al 2000, Smith et al 2001, Smith and Stanosz 2001, Zhou et al 2001, Zhou and Stanosz 2001a, b). These clades are regarded in some of the above studies as representing *B. dothidea* and *B. ribis*, raising a question regarding their synonymy. Furthermore, while rDNA sequence data and RAPD marker data could not distinguish *B. parva* Pennycook & Samuels and *B. ribis* (Smith and Stanosz 2001, Zhou and Stanosz 2001a), ISSR markers showed that they were distinct (Zhou et al 2001).

It is evident that the commonly encountered and economically important genus *Botryosphaeria* continues in taxonomic disarray. The objective of the present study is to test morphologically based hypotheses with data derived from multiple gene sequences. The need to use multiple gene phylogenies to distinguish closely related species has been emphasized before (O'Donnell and Cigelnik 1997, Taylor et al 2000). For this reason, rRNA [spanning the internal transcribed spacer region one (ITS 1), 5.8S gene and ITS 2 regions] sequence data were used in this study together with data from the partial β-tubulin and translation elongation factor 1-α (EF1-α) gene sequences to determine the phylogenetic relationships of *B. dothidea*, *B. ribis*, and *B. parva*.

## MATERIALS AND METHODS

*Isolates and type material*.--Thirty-one isolates representing nine *Botryosphaeria* spp. were used in this study (TABLE I). In an attempt to obtain representative specimens and isolates of *B. dothidea*, fresh material was collected from southern Switzerland and northern Italy in October 2000. This is the same time of year, area, and included the same hosts upon which Cesati and De Notaris based the original descriptions. Isolations were made from ascomata or pycnidia on dead or dying twigs of various hardwood species (TABLE I). Similarly, isolations were made from twigs of *Ribes* spp. showing symptoms of cane die-back from Ithaca, New York state. This is the host genus and area from which the original material for the description of *B. ribis* was collected by

Grossenbacher and Duggar. Ex-type isolates of *B. parva* Pennycook & Samuels were obtained from the International Collection of Microorganisms from Plants (ICMP), Landcare Research New Zealand Ltd., Auckland, New Zealand. Other isolates of representative *Botryosphaeria* spp. were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and the Culture Collection of the Tree Pathology Co-operative Programme (CMW), FABI, University of Pretoria, South Africa (TABLE I). *Botryosphaeria dothidea*, *B. ribis* and *B. parva* were compared based on morphological and molecular data. Other common *Botryosphaeria* spp. were used in molecular comparisons only.

Initial identification of the isolates was achieved based on conidial morphology. Isolates were grown on 2% water agar (WA; Biolab agar, Midrand, Johannesburg, S.A.) with sterilized pine needles, or halved twigs of *Malus* sp., *Eucalyptus* sp. or *Populus* sp. as substratum, at 25 C under near-UV light, to induce sporulation. Cultures were maintained on malt and yeast extract agar (MYA; 2% malt extract, 0.2% yeast extract and 1.5% agar; Biolab, Midrand, Johannesburg, S.A.) at 25 C and stored on this medium at 4 C. Colony morphology, color (Rayner 1970), and growth rates between 10 and 30 C, were determined on potato dextrose agar (PDA; 0.4% potato extract, 2% dextrose, 1.5% agar, Biolab, Midrand, Johannesburg, S.A.).

Type material or other representative specimens and cultures of *B. dothidea*, *B. ribis, B. parva* and *B. berengeriana* were obtained from various herbaria, including CUP, PDD, RO, S. Ascomata or pycnidia were mounted in lactophenol. Sections of herbarium, freshly collected and *in vitro* ascomata and pycnidia were cut with an American Optical Freezing Microtome or by hand. Morphological observations and measurements were made with a light microscope, an Axiocam digital camera and accompanying software (Carl Zeiss, Germany).

*DNA isolation and amplification.*--A modification of the phenol:chloroform DNA extraction method of Raeder and Broda (1985) was used to isolate DNA from the fungal isolates as described in Smith et al (2001). The primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al 1990) were used to amplify part of the nuclear rRNA operon in PCR reactions. The amplified region included the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene. A part of the β-tubulin gene region was amplified by use of the primers Bt2a (5' GGTAACCAAATCG GTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass and Donaldson 1995). Amplification of part of the EF1-α was done with the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTGAAGGA ACCCTTACC) (Carbone et al 1999). PCR reaction mixtures contained final concentrations of: 2.5 Units *Taq* DNA polymerase (Roche Molecular Biochemicals, Almeda, California, USA), 1X Buffer & $MgCl_2$ mixture (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP and 0.15 μM of each primer and made up to a final volume of 50 μL with water. During the PCR reaction, the DNA was first denatured at 94 C for 2 min, followed by 40 cycles of denaturation (94 C for 30 s), annealing (55 C for 45 s) and elongation (72 C for 1½ min) and ended with a final elongation step at 72 C for 5 min. Amplification of the EF1-α region was problematic for some species. In these cases amplifications were done by replacing the *Taq* polymerase with Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California, USA) with the same reaction concentrations as above, and PCR cycle conditions as indicated by the supplier. PCR amplicons were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Size estimates were made using 100bp or λ standard size markers.

*DNA sequencing and analysis.*--PCR products were cleaned using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, California, USA). Both strands of the amplicons were sequenced using the same primers that were used for the initial amplification. Reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied BioSystems, Foster City, California, USA) as indicated by the manufacturer and run on an ABI PRISM 377 autosequencer (Perkin-Elmer Applied BioSystems, Foster City, CA).

To compare the *B. dothidea*, *B. ribis, B. parva* and other *Botryosphaeria* isolates used in this study, with those from previous studies, 22 ITS rDNA sequences from GenBank were included in the analyses (TABLE I) (Jacobs and Rehner 1998, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001a). BLAST searches were used to find any other related sequences from GenBank, not referred to in these studies. Trees were rooted to sequence data of an isolate of *Guignardia philoprina* (Berk. &

M.A. Curtis) Aa, which was previously described in the genus *Botryosphaeria* before it was placed in the closely related genus, *Guignardia* Viala & Ravaz. Despite the close relationship between these last named genera, unambiguous alignment of outgroup sequence with that of the ingroup was not possible for all parts of intron and ITS regions.

ITS rDNA sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, California, USA) and manually aligned by inserting gaps. Phylogenetic analyses based on parsimony were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999). Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were found using heuristic searches, and including only informative characters in stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch supports, using 1000 bootstrap replicates (Felsenstein 1985), and estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were also determined in PAUP. Decay analyses of the branch nodes were determined using Autodecay (Eriksson 1998). Phylogenetic hypotheses were also tested using distance analyses with the Neighbor Joining algorithm and an uncorrected p-factor in PAUP.

Statistical congruence between the ITS rDNA, β-tubulin, and EF1-α sequence data sets was tested using partition homogeneity tests (Farris et al 1995, Huelsenbeck et al 1996) in PAUP. These tests revealed that the data were combinable. The data sets were subsequently analyzed together. Repetitive minisatellite regions in the intron of the EF1-α were coded to represent a single, rather than multiple evolutionary events.

## RESULTS

*Morphological characteristics and typification*.--The published description of *S. dothidea* (Fries 1823) refers to a fungus from fallen twigs of a *Fraxinus* sp. However, the herbarium specimen of *S. dothidea* in the Fries collection, collected by Mougeot, which has been cited as the type material (von Arx and Müller 1954), contains a sample with thorns that appears to be a *Rosa* sp. This sample can, thus, not be the holotype. No type specimen of *S. dothidea* on *Fraxinus* by Mougeot could be located in other herbaria that might have such a collection (BM, BR, K, LILLE, LIP, NCY, STR). Given that the holotype could not be located, the only remaining *S. dothidea* sample in the Fries herbarium is designated here as the neotype representing *B. dothidea.* This material is, however, not definitive of the species, because the specimens are immature and contain no spores.

In order to clarify taxonomic confusion surrounding *B. dothidea,* this taxon is epitypified here. An epitype is designated to complement the neotype and other authentic specimens, as well as their descriptions, which represent *B. dothidea.* The epitype also allowed isolation of cultures. To find an epitype, three samples were collected during the present study from a nearby locality (the border between Switzerland and Italy) to that of some of the collections of Cesati and De Notaris (TABLE II). The specimens were collected from *Fraxinus* sp., *Prunus* sp. and *Ostrya* sp. These samples contained ascomata that conformed to descriptions of *B. dothidea* by Fries (1823) and Cesati and De Notaris (1863). Thus, one of these samples (PREM57372, on *Prunus* sp.) is designated as the epitype specimen.

A taxonomic description of *B. dothidea* (FIGS. 1-7) based on the epitype material and cultures made from it, is given below. Cultures obtained from these samples produced an anamorph that matches descriptions of *F. aesculi* Corda by Pennycook and Samuels (1985) and Crous and Palm (1999).

A specimen labeled as *B. berengeriana* was obtained from the collection of De Notaris (RO). This specimen carries the signature of De Notaris and is from the same host (*Rhamnus frangula*) referred to in the original description (De Notaris 1863). It is likely that this is the material used by De Notaris for that description, or at least is similar to it. Ascomata on this material, as well as the original description of *B. berengeriana*, were not distinguishable from those of the lectotype or epitype specimens of *B. dothidea* (TABLE II).

Grossenbacher and Duggar (1911) described, but did not typify, *Botryosphaeria ribis*. Three collections used by Grossenbacher and Duggar for this description were located in CUP. We have designated one of these as lectotype for *B. ribis*. This material of *B. ribis* is mature and well preserved, and provides ample substance to characterize this species. This lectotype of *B. ribis* also contains a well-developed and preserved anamorph.

Specimens of *Ribes* sp. canes with die-back, that were collected on our behalf from the same geographical area as the original type material (New York state, USA), contained pycnidia of a *Fusicoccum* sp. and a *Diplodia* sp. The former species corresponds to the anamorph on the type material and to the original description of an anamorph associated with *B. ribis*. Isolates from this *Fusicoccum* sp. were used in cultural, morphological and molecular studies. In this paper, a description of *B. ribis* and its anamorph, based on this material and the lectotype specimens, is given to accompany the molecular characterization of this taxon (FIGS. 8-14).

Isolates residing in the clades that represent *B. dothidea* and *B. ribis* have similar ascomata, ascospores, conidial morphology, and cultural characteristics. However, these species can be distinguished from each other using average dimensions of these features, especially in culture (taxonomic description, key, FIGS. 1-14).

Both the type and corresponding ex-type cultures for *B. parva* are well preserved and representative of the description of this taxon. Using this material, *B. parva* and *B. dothidea*, as described here, were clearly distinguishable based on morphological features (key). There is no consistent morphological distinction between *B. ribis* and *B. parva*, other than some variation in septation in discharged and aged spores (key, FIGS. 8-15) (TABLE III).

*Phylogenetic sequence analyses*.--The ITS data set consisted of 563 characters after alignment, 418 uninformative characters were excluded and 145 parsimony-informative characters were used in the analyses. These data contained significant phylogenetic signal ($P < 0.01$; g1 = -0.644) (Hillis and Huelsenbeck 1992). After heuristic searches in PAUP, 71 most parsimonious trees of 325 steps were retained (CI = 0.757; RI = 0.933). (FIG. 16).

A partition homogeneity test of the full data set, combining ITS-rDNA, β-tubulin and EF1-α, indicated that the data sets could be combined (P value = 0.32). The combined data set consisted of 1344 characters after alignment (TreeBASE S861, M1396). A total of 968 characters were excluded, including 954 uninformative characters and 14 minisatellite characters from the EF1-α intron region that were coded to represent one evolutionary event. Using the 390 parsimony-informative characters (significant phylogenetic signal [<0.01; g1 = -0.851] [Hillis and Huelsenbeck 1992]), 10 most parsimonious trees of 858 steps were retained after heuristic searches in PAUP (CI = 0.795; RI = 0.91) (FIG. 17).

For both data sets, the same clades were identified by parsimony and distance analyses. The analyses of both datasets showed that the branch supports separating the main clades, which are identified as *B. lutea* A.J.L. Phillips, *B. eucalyptorum* Crous, H. Smith & M.J. Wingf., *B. dothidea*, *B. quercuum*, *B. stevensii* Shoemaker, *B. obtusa* (Schw.) Shoemaker and *B. rhodina* (Cooke) von Arx, were well supported (>d4/91% bootstrap) (FIGS. 16, 17). ITS rDNA sequences of *B. mamane* Gardner and *B. corticis* (Demaree & Wilcox) von Arx & Müller are significantly distinct, but were more closely related to each other than to any other species used in the analysis. These two species grouped in a sister clade to *B. dothidea*. *Botryosphaeria ribis* and *B. parva* could not be distinguished based on ITS rDNA data (FIG. 16), but were clearly separated in the combined datasets (FIG. 17).

*Botryosphaeria* spp. were divided into two main clades in the combined dataset. These correspond to the fusicoccum-like and diplodia-like anamorph types, respectively (FIG. 17). In the clade with *Fusicoccum* conidia, *B. dothidea* was clearly distinct, with the strongest support (d53/100% bootstrap in analyses of combined datasets) of all the clades. Similarly, among *Botryosphaeria* spp. with *Diplodia* anamorphs, the distinction of *B. rhodina* was strongly supported (d63/100% bootstrap). *Botryosphaeria rhodina* did not group with other isolates having *Diplodia* anamorphs in the rDNA dataset when using parsimony, but the branch separating these taxa was weakly supported (d2/60% bootstrap) (FIG. 16). Distance analysis of this rDNA dataset (tree not shown), however, also placed this species amongst other *Botryosphaeria* spp. with dark-spored (*Diplodia*) anamorphs, as we found in the analysis of the combined dataset.

## TAXONOMY

There are a number of published descriptions pertaining to the type material and other authentic specimens of *B. dothidea*, *B. ribis*, and their anamorphs (Fries 1823, Cesati and De Notaris 1863, De Notaris 1863, Winter 1886, Saccardo 1877, Grossenbacher and Duggar 1911, von Arx and Müller 1954, Punithalingam and Holliday 1973, Sutton 1980, Pennycook and Samuels 1985, Crous and Palm 1999). Due to the confusion regarding the use of these names in the descriptions, revised descriptions based on the type material and fresh collections made as part of this study are provided here. The morphological description of *B. parva*, which is also considered in this study, is not repeated here as this would be redundant and would not add substantially to the original description provided by Pennycook and Samuels (1985).

***Botryosphaeria dothidea*** (Moug.: Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital. 1:212. 1863. FIGS. 1-7

= *Sphaeria dothidea* Moug.: Fr. in Fries, Syst. Mycol. 2:423. 1823.

= *Botryosphaeria berengeriana* De Not., Sfer. Ital. 82. 1863 [1864].

*Anamorph*. ***Fusicoccum aesculi*** Corda in Sturm, Deutschl. Fl., Abth. 3, 2:111. 1829.

*Ascostroma* erumpent through the bark, 200--500 µm diam. *Ascomata* pseudothecial, forming a botryose aggregate of up to 100, sometimes solitary, globose with a central ostiole, ¼ to ½ emergent, rarely imbedded, papillate or not, brown to black; pseudothecial wall comprising 5--15 layers of *textura angularis*, outer region of dark brown or brown cells, inner region of 2--4 layers of hyaline cells lining the locule. *Asci* bitunicate, clavate, 63--125 x 16--20 µm, 8-spored, between numerous filiform, septate, rarely branched towards the tip, pseudoparaphyses, 2--4 µm wide. *Ascospores* fusoid to ovoid, sometimes with tapered ends giving a spindle shaped appearance, (17--) 19--24(--32) x (6--)7--8(--10) µm (average of 102 ascospores 22.7 x 7.8 µm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* pycnidial (anamorph structures were present only on the sample from *Ostrya* sp.), shape indistinguishable from the ascomata. *Conidia* narrowly fusiform or irregularly fusiform, base subtruncate to bluntly rounded, (17--)18--20(--22) x 4--5 µm (average of 35 conidia 19.6 x 4.8 µm, l/w 4.1), hyaline, unicellular, rarely forming a septum before germination, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 6--20 x 2--5 µm, proliferating percurrently with 1--2 proliferations and periclinical thickening. *Spermatia* unicellular, hyaline, allantoid to rod-shaped, 3--6 x 1.5--2 µm. *Spermatophores* hyaline, cylindrical to subcylindrical, 4--10 x 1--2 µm.

*Cultural characteristics*. Colonies olivaceous buff (21’’’d), becoming olivaceous grey (21’’’’i) to violaceous black (65’’’’k), with a sparse to moderately dense, appressed mycelial mat, occasional columns of aerial mycelium reaching the lid, margin smooth appearing crenulate as the colony darkens with age. Optimum temperature for growth 25(--30) C, colony reaching a 50 mm radius on PDA after 4 d at 25 C in the dark. *Pycnidia* (formed on WA on sterilized twigs of *Malus* sp., *Eucalyptus* sp., *Populus* sp., or needles of *Pinus* sp. within 7--14 d) superficial, globose, mostly solitary and covered by mycelium. *Conidia* produced in culture similar to those formed in nature, but regularly shaped, longer and appearing more narrowly fusiform, (20--)23--27(--30) x 4--5(--6) µm (average of 102 conidia 24.7 x 4.9 µm, l/w 5).

*Specimens examined.* FRANCE. *Rosa* sp., 1823, *Fries ex Mougeot* (NEOTYPE designated here, *Sphaeria dothidea*, herbarium S). SWITZERLAND. TICINO: Crocifisso, *Prunus* sp., October 2000, *B. Slippers* (EPITYPE designated here, PREM57372, culture CMW8000); *Ostrya* sp., October 2000, *B. Slippers* (PREM57373, culture CMW7999); Molinizza, *Fraxinus* sp., October 2000, *B. Slippers* (PREM57374, culture CMW7780). ITALY. Pusiano, *Populus* sp., 31 October 1846, *Cesati et De Notaris*; Pusiano, *Fraxinus* sp., 1846, *Cesati et De Notaris*; Locality unknown, *Rhamnus frangula*, 1863, *De Notaris*. GERMANY. Pr. Jever, *Fraxinus* sp., *Koch ex Cesati et De Notaris* (Rabenhorst. Herb. Mycol. 750, herbarium RO); Pr. Dreisen, *Robinia pseudoacacia*, *Lasch ex Cesati et De Notaris* (Rabenhorst. Herb. Mycol. 1330, RO).

***Botryosphaeria ribis*** Grossenb. & Duggar, Tech. Bull. N.Y. Agric. Exp. St. 18:128. 1911. FIGS. 8-14

*Anamorph.* ***Fusicoccum ribis*** Slippers, Crous, M.J. Wingf., sp. nov.

Ascomata aggregata 5--50-ni, pseudoperitheciales, botryosa, globosa ostiolo centrali, papillata vel non, brunnea vel nigra, 175--250 µm, pariete pseudothecii 5--15 stratis texturae angularis composita, stratis exterioribus atrobrunneis vel nigris, cum 2--4 stratis cellularum hyalinarum cavitatem saepientibus. Pycnidia in stromate eisdem quibus ascomatis, et illis simillimis, vel singularia, in surculis juvenibus hospitis inclusa. Conidia unicellularia, fusiformia, interdum irregulariter fusiformia, basin subtruncata vel obtuse rotundata, hyalina, granularia, superficiebus levibus, raro cum aetate septata, (16--)19--23(--24) x 5--6(--7) µm. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 6--22 x 2--5 µm, percurrenter cum 1--2 proliferationibus prolificentes. Pycnidia (in vitro in 'WA' in surculis sterilifactis specierum generum *Mali, Eucalypti, Populi* que, vel foliis *Pini* intra dies 7--14 facta) superficialia, globosa, plerumque solitaria vel bini ad quaterni aggregata mycelio tecta. Conidia a fructificationibus istis, illis in vivo factis similia sed breviora, late fusiformia vel ovoidea, forma regulariores, semel vel bis septata, aetate pallide brunnea, post emissionem (15--)16--20 x 5--6(--7) µm.

*Ascostroma* erumpent through the bark, pulvinate, 100--400 µm in diam. *Ascomata* pseudothecial, forming botryose aggregate of 5--50 structures, globose with central ostiole, papillate or not, brown to black, 175--250 µm, pseudothecial wall comprising 5--15 layers of *textura angularis*, outer region of dark brown or brown cells, inner region 2--4 layers of hyaline cells lining the locule. *Asci* bitunicate, clavate, 80--120 x 17--20 µm, 8-spored, between numerous filiform, septate, rarely branched towards the tip, pseudoparaphyses, 2--4 µm wide. *Ascospores* fusoid to ellipsoid, often round at the ends then broadly ellipsoidal, (14--)18--23(27) x 6--8(--10) µm (average of 80 ascospores 20.5 x 7.1 µm, l/w 2.9), hyaline, unicellular, smooth with granular contents, biseriate in the ascus. *Pycnidia* in same stromata as ascomata and morphologically indistinguishable from them, or solitary and imbedded in young host shoots. *Conidia* fusiform, sometimes irregularly fusiform, base subtruncate to blunt, (16--)19--23(--24) x 5--6(--7) µm (average of 90 conidia 20.8 x 5.5 µm, l/w 3.8), hyaline, unicellular, rarely septate with age, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 6--22 x 2--5 µm, proliferating percurrently with 1--2 proliferations with periclinical thickening. Spermatia not seen.

*Cultural characteristics.* Colonies white to olivaceous buff (21’’’d), becoming olivaceous grey (21’’’’’I) to violaceous black (65’’’’k), sectors often becoming rapidly darker and remaining darker than the rest of the culture, with very thick, felty mycelial mat from the surface to the lid, and smooth margin, but those of darker sections appearing lacinate. Optimum temperature for growth 25 C, colony reaching 65 mm radius on PDA after 4 d at 25 C in the dark. *Pycnidia* (formed on WA on sterilized twigs of apple (*Malus* sp.), *Eucalyptus* sp., *Populus* sp., or needles of *Pinus* sp. within 7--14 d) superficial, globose, mostly solitary or in aggregates of 2--4 and covered by mycelium. *Conidia* similar to those formed on the host, but shorter, broadly fusiform to ovoid and more regular in shape, occasionally 1--2 septate and light brown upon aging after discharge, (15--)16--20 x 5--6(--7) µm (average of 85 conidia 17.2 x 5.5 µm, l/w 3.1).

*Specimens examined.* USA. NEW YORK: Geneva, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [LECTOTYPE of teleomorph CUP-A-(F.Col. 3408)]; Milton, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [CUP-A-(F.Col. 3407)]; Milton, *Ribes vulgare*, 1911, *J.G. Grossenbacher & B.M. Duggar* [CUP-A-(F.Col. 3409)]; Ithaca, *Ribes* sp., 2000, *G. Hudler* (HOLOTYPE of anamorph, PREM57368 culture CMW7772); Ithaca, *Ribes* sp., 2000, *G. Hudler* (PREM57369, culture CMW7773).

## DISCUSSION

In this study, *B. dothidea*, *B. ribis* and *B. parva* are distinguished from each other and characterized based on morphological features. Epitype material is identified to complement lectotype and syntype material of *B. dothidea,* the type species of *Botryosphaeria*. Ex-type cultures from designated type specimens and other representative specimens has made it possible to confirm the identity of the groups of isolates that represent these taxa, through sequence data derived in this and other studies.

The type specimen of *B. dothidea* needed careful re-examination to clarify confusion regarding its name. In the original description by Mougeot (in Fries 1823, as *Sphaeria dothidea*), no specimen was designated as type, but reference was made to a collection from fallen branches of *Fraxinus* sp. This material appears to be lost, as the only material under this name from the Fries herbarium (which has been viewed as the type before; annotated by AJL Phillips and JA von Arx with the sample) contains only material from what appears to be a *Rosa* sp. The holotype material from *Fraxinus* could also not be located in other herbaria that house collections of Mougeot. Given that no type material exists, a neotype was designated here for the remaining *S. dothidea* sample from the Fries collection (Greuter et al 2000 ICBN articles 9.6 and 9.11). This material is, however, immature as noted by other researchers (von Arx and Müller 1954; note by AJL Phillips with the sample), and thus does not bear characteristics that would make it possible to clearly define the name.

As part of the description of *B. dothidea* by Cesati and De Notaris (1863), two additional specimens, one from *Fraxinus* sp. collected by Koch, and one from *Robinia pseudoacacia* collected by Lasch, were cited. The type sheet bearing *B. dothidea* samples collected by Koch and Lasch also contains samples from *Populus* sp. (the inscription indicating the host species is not clear) and *Fraxinus* sp. collected by them. All materials are, however, also immature, as is true for the collection from Fries' herbarium, or poor, and might even contain fruiting structures of more than one fungal species. This is not surprising, as spore morphology was not a critical characteristic used in descriptions of different species by either Fries (1823) or Cesati and De Notaris

(1863). Johnson (1992) also made reference to the immature and degraded state of the material, but reported seeing one ascospore.

The type and other early specimens of *B. dothidea* mentioned in this study are not sufficient to characterize this species. This is due to the poor state of development of structures on specimens, poor preservation of characters, and overlapping that exists in the morphological characteristics of *Botryosphaeria* spp. For these reasons, an epitype sample has been selected. Care has been taken with the selection of this epitype and the accompanying description to consider all aspects of the protologue, as well as to preserve the current usage of the name *B. dothidea* (Greuter et al 2000 ICBN article 9.7 and recommendation 9A). The epitype was selected from the same hosts and areas that the Cesati and De Notaris (1863) collections were made. Live cultures from this sample are deposited in culture collections (CMW, CBS).

We accept the synonymy of *B. berengeriana* and *B. dothidea* by von Arx and Müller (1954). In reviewing the original description, sketches and holotype material of *B. berengeriana*, and comparing these with the epitype of *B. dothidea*, no morphological or other reason could be found to resurrect *B. berengeriana*. The original description (De Notaris 1863) is very detailed and the ascomata on *Rhamnus frangula*, relatively well preserved. The original separation of this species from *B. dothidea* was most likely due to the variation in host and some variation in ascocarp morphology. Currently, however, we know that some *Botryosphaeria* spp. are not host specific. Furthermore, von Arx and Müller (1954) noted, and it is confirmed in this study, that variation can exist in the ascomatal and pycnidial morphology (e.g. size, aggregation and imbedding in tissue) of one *Botryosphaeria* sp. on different parts of one plant and between hosts.

*Fusicoccum aesculi* is generally accepted as the anamorph of *B. dothidea sensu* von Arx and Müller (1954). The separation of *B. dothidea* into at least two species, raises the question of the true identity of the anamorph of *B. dothidea*. The anamorph of the epitype of *B. dothidea* was thus studied *in vivo* and *in vitro*. The characteristics of the conidia and other morphological structures are in accordance with the amended description of *F. aesculi* (Crous and Palm 1999). The designation of *F. aesculi* as the anamorph of *B. dothidea*, as defined in this study, is thus accepted.

The type specimen of *B. ribis* is well preserved, providing ample material to define this taxon. There are, however, no cultures linked to this material. Freshly collected material from *Ribes* sp. in Ithaca, New York, the same host and area from which the lectotype of *B. ribis* was collected, contained only anamorph structures and conidia and could thus not be designated as epitype of the teleomorph (Greuter et al 2000 ICBN article 59.2). The structures on this material were, however, morphologically identical to anamorph structures and conidia on the lectotype specimen. The isolates from this freshly collected material are thus accepted as representing *B. ribis*.

Two cultural forms of *B. ribis* were described by Grossenbacher and Duggar (1911). The chromagena variant produced a reddish pigment when grown on starch media in diffuse daylight, while the achromagena variant did not. One isolate from *Ribes* sp. (CMW7054) was designated as a chromagena variant and produced a pigment similar to that described above, but not regularly, perhaps due to the age of the isolate as suggested by Witcher and Clayton (1963). No other *B. ribis* isolates used in this study produced such a pigment under artificial light or daylight and they would thus all be classified as achromagena variants. This characteristic was initially believed to relate to pathogenicity (Grossenbacher and Duggar 1911, Stevens and Jenkins 1924), but this notion was later rejected (Witcher and Clayton 1963). These designations are no longer used and are viewed as representative of intraspecific variation.

Grossenbacher and Duggar (1911) described a 'simple or *Macrophoma* stylosporic form' and a 'compound stylosporic or *Dothiorella* form' associated with *B. ribis*. These forms were separated based on whether the pycnidia were solitary, pycnidial and imbedded (on young succulent shoots) or botryose, stromatic and erumpent (on older more woody material). The former structures were also reported to have slightly smaller conidia. Re-examination of the material, however, revealed that the spores of these two morphological forms are of the same average dimension. *Botryosphaeria* spp. are known to display variation in the morphology of conidiomata on different parts or developmental stages of the same host (von Arx and Müller 1954, Phillips et al 2002). We, therefore, view these two forms described by Grossenbacher and Duggar (1911) as representing different characteristics of the same species.

The anamorph of *B. ribis* was not named in the description by Grossenbacher and Duggar (1911). These authors argued that the anamorph of *B. ribis* was not *Dothiorella ribis* (Fuckel) Sacc. or *D. ribicola* Ellis & Barthol., but did not provide a name for it. The morphological and molecular data provided in this and other studies (Morgan-Jones and White 1987, Rayachhetry et al 1996, Denman et al 2000), however, show that the anamorph of *B. ribis* is a species of *Fusicoccum*. We have chosen to provide the name

*F. ribis* for this element of the holomorph. Lectotype material of the teleomorph also contains anamorph structures. Freshly collected material is, however, designated here as holotype for the anamorph name, to also allow characterization of living isolates and so preserve all features of this taxon. Although it might be argued that an anamorph name is not strictly necessary, the fungus is most commonly seen as the anamorph in the laboratory. We believe that having a name for this state will be useful. This is especially true because many apparently new species of *Fusicoccum* are currently being discovered and only the anamorph is known for them.

In addition to *B. ribis*, we have obtained isolates of *B. obtusa* (*Diplodia* anamorph) from *Ribes* sp. in New York. Grossenbacher and Duggar (1911) encountered a dark-spored sphaeropsis-like fungus on *Ribes*, which they did not study. It is, however, possible that these researchers inadvertently isolated this fungus as an endophyte, because not all their cultures were from spores or conidia. *Botryosphaeria* spp. are known to occur commonly as endophytes in many woody plants (Fisher et al 1993, Smith et al 1996). It is thus possible that some of the variation in cultural morphology described in the experiments of Grossenbacher and Duggar (1911) could be due to the presence of a second species of *Botryosphaeria*, viz. *B. obtusa*.

Sequence data for the three gene regions used in this study show clearly that isolates of *B. ribis* and *B. parva*, respectively, reside in two clades. In contrast, data from the ITS, mt-SSU-rDNA gene sequence, and RAPD data (Smith and Stanosz 2001, Zhou and Stanosz 2001a), did not distinguish *B. parva* from *B. ribis*, and it was suggested that these species may be synonyms. Yet again, a study using Inter Simple Sequence Repeat (ISSR) markers separated these two species (Zhou et al 2001). Our data, based on a multiple gene genealogy, strongly support the view that *B. ribis* and *B. parva* are distinct and probably recently derived.

Some of the unique polymorphisms detected in the sequence data of this study, and that distinguish *B. ribis* from *B. parva*, are repetitive elements in the intron regions. Such elements can be highly polymorphic within species (Carbone et al 1999, Fisher et al 2000). Since the three isolates representing *B. ribis* were all collected from the same site and host (*Ribes* from New York), the variation that we observed at these sites might reflect the presence of a founder population. This is especially true given that preliminary data suggest that these fungi are non-outcrossing (H van Geuns, B Slippers, and S Denman unpubl.), and that *Ribes* spp. have been introduced into the New York area. Furthermore, there is also sequence variation among isolates in the *B. parva* clade.

These results call for a study of a wider collection of isolates, using co-dominant markers to determine possible gene flow and boundaries between groups of isolates that represent these species.

The morphological description of *B. parva* (Pennycook and Samuels 1985) is indistinguishable from that of *B. ribis* by Grossenbacher and Duggar (1911) and Punithalingam and Holliday (1973), except that the conidia and ascospores in the former species are reportedly wider and slightly shorter. These differences were not evident in the present study, and the characteristics overlapped between the species. Pennycook and Samuels (1985), however, did not consider the synonymy of these species, as they treated *B. ribis* as a synonym of *B. dothidea sensu* von Arx and Müller (1954). These authors and Punithalingam and Holliday (1973) refer to septation in older spores. Our observations show that the pattern of septation and discoloration in older, discharged conidia formed in culture is consistent with the separation of *B. ribis* and *B. parva* based on sequence data. Aging conidia of both species become one to two septate and light brown after being discharged from the pycnidium. Conidia of *B. ribis* are, however, commonly one-septate and dark walls are evenly spread. In contrast, conidia of *B. parva* are more regularly two septate, with conspicuously darker brown middle cells. However, care must be taken in making these observations because not all conidia darken and become septate, even after discharge and where cultures are left to age for two months.

Isolates residing in the *B. ribis*/*B. parva* clade reportedly form microconidia or spermatia (Pennycook and Samuels 1985, Rayachhetry et al 1996). These structures are not common and were not observed in the present study. Phillips et al (2002) also recently reported spermatia of similar dimensions to those described for *B. dothidea*, formed by some isolates of *B. lutea*. Spermatia have not been reported from *B. dothidea*. In the present study, microconidia were, however, formed in anamorph fruiting structures of *B. dothidea* from *Ostrya* sp. This characteristic appears to be insufficiently consistent to be useful in distinguishing between the *B. ribis, B. parva*, and *B. dothidea*.

The *Botryosphaeria* spp. considered in this study resided in two major and well resolved clades, based on the combined sequence datasets of the ITS rDNA, β-tubulin, and EF1-α. These clades correspond to the anamorph genera with hyaline conidia in *Fusicoccum* and those with dematiaceous conidia in *Diplodia sensu lato*. These two groups have also been identified in previous studies (Denman et al 2000, Zhou and

Stanosz 2001a). Despite a report to the contrary (Zhou and Stanosz 2001b), the fusicoccum- and diplodia-like anamorph conidium phenotype, therefore, seem to be consistent with major evolutionary events in *Botryosphaeria*.

Based on the combined sequence data sets used in this study, *B. rhodina* (anamorph = *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.) grouped with other species having *Diplodia* anamorphs. These combined and separately analyzed data sets also showed that this species groups separately within the larger *Diplodia* clade, in which *B. obtusa*, *B. stevensii*, and *B. quercuum* group closely. The conidial morphology of *B. rhodina* is similar to that of other *Diplodia* spp., but conidia are also unique in having conspicuous longitudinal striations. Our data thus suggest that conidial striations are definitive at the species level, and should not be used to distinguish genera within *Botryosphaeria* or its anamorphs.

*Botryosphaeria dothidea*, *B. ribis*, and *B. parva* can clearly be distinguished based on morphological and DNA sequence data. However, when considering morphology, care should be taken to examine a sufficiently large number of samples to compensate for the fact that some characteristics overlap significantly. For *in vitro* studies, sporulating cultures should be allowed to age for at least three weeks and preferably longer, because septation of discharged, aged spores is useful in separating some species. Recently collected isolates should preferably be used because cultures can lose their useful characteristics and ability to sporulate after repeated sub-culturing. We thus rely strongly on sequence data linked to morphologically defined groups to confirm their identity. For phylogenetic studies of closely related species such as *B. ribis* and *B. parva*, more than one gene region should be used. However, there is a preponderance of ITS rDNA sequence data for *Botryosphaeria* spp. in public databases and this appears to be sufficient to identify the major clades.

## KEY TO *B. DOTHIDEA, B. RIBIS, B. PARVA* AND *B. LUTEA*

*Botryosphaeria lutea* is included in the key, as it is closely related and commonly encountered in comparisons with the other species studied here (Jacobs and Rehner 1998, Zhou and Stanosz 2001a, Smith and Stanosz 2001, Phillips et al 2002). Data for *B. lutea* in this key are derived from Pennycook and Samuels (1985) and Phillips et al (2002) and were confirmed in this study.

1. Conidia in culture averaging <18 µm long, l/w ±3, colony on MEA or PDA thick felt of grey aerial mycelium ..........................................................................2

1. Conidia in culture averaging ≥20 µm long, l/w >3, colony on MEA or PDA appressed with occasional tufts of grey to buff aerial mycelium.....................................3

 2. Conidia 15--20 x 5--7 µm, becoming light brown and septate after discharge..................................................................................***B. ribis***

 2. Conidia 12--23 x 4--6 µm, frequently becoming light brown and one to two septate with a darker brown middle cell after discharge.................................***B. parva***

3. Conidia fusiform to irregularly rod-shaped, 15--30 x 5--8 µm (average 22 x 6 µm), l/w 3--4, colony on MEA or PDA producing distinct yellow pigment after three days, becoming dull brown to buff with age..........................................................***B. lutea***

3. Conidia narrowly fusiform, 19--30 x 4--6 µm (average 25 x 5 µm), l/w3.5--6, colonies on MEA or PDA not producing yellow pigment and becoming grey to black with age..............................................................................***B. dothidea***

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TABLE I. Isolates of *Botryosphaeria* and *Guignardia* species considered in the phylogenetic study.

| **Culture no.**$^{1,2}$ | **Other no.**$^{1}$ | **Identity**$^{3}$ | **Host** | **Location** | **Collector** | **GenBank**$^{4}$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | **ITS** | **$\beta$ tubulin** | **EF-1α** |
| **CMW7772** | | *Botryosphaeria ribis* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236935 | AY236906 | AY236877 |
| **CMW7773** | | *B. ribis* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236936 | AY236907 | AY236878 |
| **CMW7054** | CBS121 | *B. ribis* | *R. rubrum* | New York, USA | N.E. Stevens | *AF241177* | AY236908 | AY236879 |
| **CMW994** | ATCC58189 | *B. parva* | *Malus sylvestris* | New Zealand | G.J. Samuels | *AF243395* | AY236912 | AY236883 |
| **CMW9077** | ICMP7924 | *B. parva* | *Actinidia deliciosa* | New Zealand | S.R. Pennycook | AY236939 | AY236913 | AY236884 |
| **CMW9078** | ICMP7925 | *B. parva* | *A. deliciosa* | New Zealand | S.R. Pennycook | AY236940 | AY236914 | AY236885 |
| **CMW9079** | ICMP7933 | *B. parva* | *A. deliciosa* | New Zealand | S.R. Pennycook | AY236941 | AY236915 | AY236886 |
| **CMW9080** | ICMP8002 | *B. parva* | *Populus nigra* | New Zealand | G.J. Samuels | AY236942 | AY236916 | AY236887 |
| **CMW9081** | ICMP8003 | *B. parva* | *P. nigra* | New Zealand | G.J. Samuels | AY236943 | AY236917 | AY236888 |
| CMW10122 | BOT21 | *B. parva* | *Eucalyptus grandis* | Mpumalanga, S. Africa | H. Smith | *AF283681* | AY236911 | AY236882 |
| CMW1130 | | *B. parva* | *Sequoia gigantea* | Hogsback, S. Africa | W. Swart | AY236945 | AY236919 | AY236890 |
| CMW10123 | BOT19 | *B. parva* | *E. smithii* | Mpumalanga, S. Africa | H. Smith | *AF283683* | AY236910 | AY236881 |
| CMW10124 | BOT681 | *B. parva* | *Heteropyxis natalensis* | Kwazulu-Natal, S. Africa | H. Smith | *AF283676* | | |
| CMW4049 | | *B. parva* | *E. grandis* | Sumatra, Indonesia | M.J. Wingfield | AY236937 | | |
| CMW9071 | | *B. parva* | *Ribes* sp. | Australia | M.J. Wingfield | AY236938 | AY236909 | AY236880 |
| CMW7885 | | *B. parva* | *Eucalyptus* sp. | Hawaii | M.J. Wingfield | AY236944 | AY236918 | AY236889 |
| | KJ94.09 | *B. ribis / B. parva* | *Melaleuca quinquenervia* | Florida, USA | M.B. Rayachhetry | *AF027743* | | |
| | KJ93.03 | *B. ribis / B. parva* | *Cercis canadensis* | District of Columbia, USA | K.A. Jacobs | *AF027742* | | |
| CMW10125 | BOT24 | *B. eucalyptorum* | *E. grandis* | Mpumalanga, S. Africa | H. Smith | *AF283686* | AY236920 | AY236891 |
| CMW10126 | BOT16 | *B. eucalyptorum* | *E. grandis* | Mpumalanga, S. Africa | H. Smith | *AF283687* | AY236921 | AY236892 |
| | KJ93.12 | *B. dothidea* | *Prunus* sp. | District of Columbia, USA | K.A. Jacobs | *AF027746* | | |
| | KJ94.26 | *B. dothidea* | *P. persica* | Japan | P.L. Pusey | *AF027749* | | |
| | KJ93.23 | *B. dothidea* | *Syringa vulgaris* | Maryland, USA | K.A. Jacobs | *AF027751* | | |
| CMW991 | ATCC58188 | *B. dothidea* | *P. nigra* | New Zealand | G.J. Samuels | *AF241175* | AY236924 | AY236895 |

TABLE I. Continued.

| Culture no.[1,2] | Other no.[1] | Identity[3] | Host | Location | Collector | GenBank[4] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ITS | *β* tubulin | EF-1α |
| CMW9075 | ICMP8019 | *B. dothidea* | *P. nigra* | New Zealand | G.J. Samuels | AY236950 | AY236928 | AY236899 |
| **CMW7780** | | *B. dothidea* | *Fraxinus excelsior* | Molinizza, Switzerland | B. Slippers | AY236947 | AY236925 | AY236896 |
| **CMW7999** | | *B. dothidea* | *Ostrya* sp. | Crocifisso, Switzerland | B. Slippers | AY236948 | AY236926 | AY236897 |
| **CMW8000** | | *B. dothidea* | *Prunus* sp. | Crocifisso, Switzerland | B. Slippers | AY236949 | AY236927 | AY236898 |
| | ATCC58194 | *B. lutea* | *Malus x domestica* | New Zealand | G.J. Samuels | *AF243396* | | |
| CMW992/3 | KJ93.52 | *B. lutea* | *A. deliciosa* | New Zealand | G.J. Samuels | *AF027745* | AY236923 | AY236894 |
| CMW9076 | ICMP7818 | *B. lutea* | *Malus x domestica* | New Zealand | S.R. Pennycook | AY236946 | AY236922 | AY236893 |
| | ZS97-59 | *B. mamane* | *Sophora chrysophylla* | Hawaii | D. Gardner | *AF246930* | | |
| | ATCC22929 | *B. corticis* | *Vaccinium* sp. | North Carolina, USA | R.D. Milholland | *AF243397* | | |
| | KJ93.29 | *B. quercuum* | *Quercus* sp. | California, USA | E. Hecht-Poinar | *AF027753* | | |
| CMW7062 | CBS177.89 | *B. quercuum* | *Q. cerris* | Italy | A. Vannini | *AF243399* | | |
| CMW7060 | CBS 431 | *B. stevensii* | *F. excelsior* | Netherlands | H.A. van der Aa | AY236955 | AY236933 | AY236904 |
| | ATCC60259 | *B. stevensii* | *M. pumila* | Unknown | H.J. Boesewinkel | *AF243406* | | |
| CMW7774 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236953 | AY236931 | AY236902 |
| CMW7775 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236954 | AY236932 | AY236903 |
| | KJ93.56 | *B. obtusa* | Hardwood shrub | New York, USA | G.J. Samuels | *AF027759* | | |
| | KJ93.41 | *B. rhodina* | *Pistacia* sp. | California, USA | T.J. Michailides | *AF027762* | | |
| CMW10130 | BOT977 | *B. rhodina* | *Vitex donniana* | Uganda | J. Roux | AY236951 | AY236929 | AY236900 |
| CMW9074 | | *B. rhodina* | *Pinus* sp. | Mexico | T. Burgess | AY236952 | AY236930 | AY236901 |
| CMW7063 | CBS447 | *Guignardia philoprina* | *Taxus baccata* | Netherlands | H.A. van der Aa | AY236956 | AY236934 | AY236905 |

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[1] Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection, Fairfax, VA, USA; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; ZS = Zhou and Stanosz (2001a).

[2] Isolates in bold are ex-type (CMW7772; CMW8000; CMW9081) or from samples that have been linked morphologically to type material of the species.

[3] Identities as determined in this study.

[4] ITS sequences represented by the 22 numbers in italics were obtained from GenBank. The remaining 75 sequences were determined in the present study.

TABLE II. A comparison of the epitype specimens with holotype material and descriptions of *Sphaeria dothidea*, *Botryosphaeria dothidea* and *B. berengeriana*.

| | Fries 1823[1] | Cesati and De Notaris 1863[2] | De Notaris 1863 | Pennycook and Samuels 1985[3] | Epitype |
|---|---|---|---|---|---|
| *Ascostromata* | | | | | |
| Position in Substratum | Erumpent | Erumpent | Erumpent | Erumpent | Erumpent |
| Shape or Appearance | Oblong | Cushion- or disk like or elongate | Disk-like | Caespitose clusters (2--5 mm) | Botryose clusters or cushion-like when young |
| Color | 'Bleek' dark-brown | Lavender blue | Dark | Black | Black |
| *Perithecia* | | | | | |
| Position | Initially imbedded becoming sub-emerged | Top always free | Erumpent | ¼ emergent | ¼ to ½ emerged, but sometimes imbedded with only ostiole visible |
| Number | N/a | N/a | N/a | 5--50(--100) per cluster | Clusters up to 100 or solitary (less common) |
| Colour | White contents | N/a | Black with lighter base and white contents | Black with white contents | Black with white contents |
| Shape | Round when immature becoming globose | N/a | Ovate-sphaerical | Globose, smooth, non-collapsing when dry | Globose, rarely irregular, non-collapsing, except in old material |
| Opening | N/a | Small open ostiole | Papillate with small open ostiole | Non-papillate or short conical papilla | Small open ostiole or short conical papilla |
| Size | N/a | | | 150--250 µm | 100--250 µm |

TABLE II. Continued.

| | Fries 1823[1] | Cesati and De Notaris 1863[2] | De Notaris 1863 | Pennycook and Samuels 1985[3] | Epitype |
|---|---|---|---|---|---|
| *Asci* | | | | | |
| Description | N/a | 8-spored | 8-spored | Bitunicate, 8-spored | Bitunicate, 8-spored |
| Shape | N/a | Slender, clavate | Clavate, obtuse | Clavate | Clavate |
| Size | N/a | N/a | N/a | (65--)75--112(--140) µm | 63--125 µm |
| Paraphyses | N/a | N/a | N/a | N/a | Interspersed between asci |
| *Ascospores* | | | | | |
| Description | N/a | Unicellular, 4 locules | Granular nuclues | Unicellular, smooth | Unicellular, smooth, granular contents |
| Colour | N/a | Hyaline | Pale | Hyaline | Hyaline |
| Shape | N/a | Ovoid to oblong | Subclavate | Ellipsoid to fusoid | Fusoid (rarely ovoid) to spindle shaped |
| Size | N/a | 25.5 x 7 µm | <30 µm in length | (13--)19--27(--35) x (6--)8--11(--14) µm | (17.2--)19--24(--26.4) x (6.2--)7--8(--9.4) µm [21.9 x 7.8 µm; l/w 2.8] |
| *Host* | Fallen branches of *Fraxinus* | *Fraxinus* sp., *Rosa* sp. and *Robinia* sp. | *Rhamnus frangula* | *Populus nigra, Actinidia deliciosa* | *Fraxinus* sp., *Ostrya* sp., *Prunus* sp. |
| *Area* | Unknown | Northern Italy | Italy | New Zealand | Italy, Switzerland |

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[1] The type material in the Fries herbarium is from *Rosa* sp., while that from the description is from fallen branches of *Fraxinus*. This information is based on the description of *S. dothidea* (Fries 1823).

[2] Ascospore size from one spore found by Johnson (1992) on the samples from Cesati and De Notaris.

[3] The description of *B. dothidea* given by Pennycook and Samuels (1985) is not based on type specimens, but this description is included here as isolates from this study are used in phylogenetic analyses in this study.

TABLE III. A comparison of the holotype material and descriptions of *Botryosphaeria ribis* (Grossenbacher and Duggar 1911) and *B. parva* (Pennycook and Samuels 1985)[1].

| | *B. ribis* | *B. parva* |
|---|---|---|
| *Ascostromata* | | |
| Position in substrate | Erumpent | Erumpent |
| Shape or appearance | Botryose clusters (1--4 mm) | Caespitose clusters (2--5 mm) |
| *Ascomata* | | |
| Position | 1/3 emergent, but sometimes submerged or wholly emerged | ¼ emergent |
| Number | N/a | 5--50(--100) per cluster |
| Colour | Black with white contents | Black with white contents |
| Shape | Round when immature, becoming globose | Globose, smooth, non-collapsing when dry |
| Opening | Papillate ostiole | Non-papillate or short conical papilla |
| Size | 175--250 µm | 150--250 µm |
| *Asci* | | |
| Description | N/a | Bitunicate, 8-spored |
| Shape | Clavate | Clavate |
| Size | 80--120 x 17--20 µm | 75--143(--210) µm |
| Paraphyses | Filiform, interspersed between asci | |
| *Ascospores* | | |
| Description | Unicellular | Unicellular, smooth |
| Color | Hyaline | Hyaline |

TABLE III. Continued.

| | *B. ribis* | *B. parva* |
|---|---|---|
| Shape | Fusoid | Broadly ellipsoid to fusoid |
| Size | (14--)18--22(--27) x 6--8(--10) µm [20.5 x 7.1 µm, l/w 2.9] | (14--)18--23(--26) x (7--)8--10(--11) µm [20.8 x 9.2 µm, l/w 2.2] |
| *Anamorph* | | |
| General | Same stroma as teleomorph | Same stroma as teleomorph |
| Pycnidia (on material) | Same as ascomata or depressed globular and imbedded | Same as given for ascomata |
| Pycnidia (in culture) | N/a | Globose, non-papillate, single or aggregate (up to 0.5 mm diam) |
| Conidia | Fusoid to ellipsoid, obtuse apex and flat base, unicellular, hyaline, rarely becoming light brown with 1--2 septa | Ellipsoid with obtuse apex and flat base, unicellular, hyaline, becoming light brown with 1--2 septa with age, middle section often darker brown |
| Conidial size (*in vivo*) | (16--)19--22(--24) x 5--6(--7) µm [20.8 x 5.5 µm, l/w 3.8] | N/a |
| Conidial size (*in vitro*) | (15--)16--19(--20) x 5--6(--7) µm [17.2 x 5.5 µm, l/w 3.1] | (12--)15--19(--24) x 4--6 µm [16.9 x 5.4 µm, l/w 3.1] |
| *Host* | *Ribes* spp. | *Populus nigra*, *Malus x domestica*, *Actinidia deliciosa*, *Sequoia* sp., *Eucalyptus* spp., *Ribes* sp. |
| *Geographic region* | New York, USA | Australia, New Zealand, Hawaii, South Africa, Sumatra |

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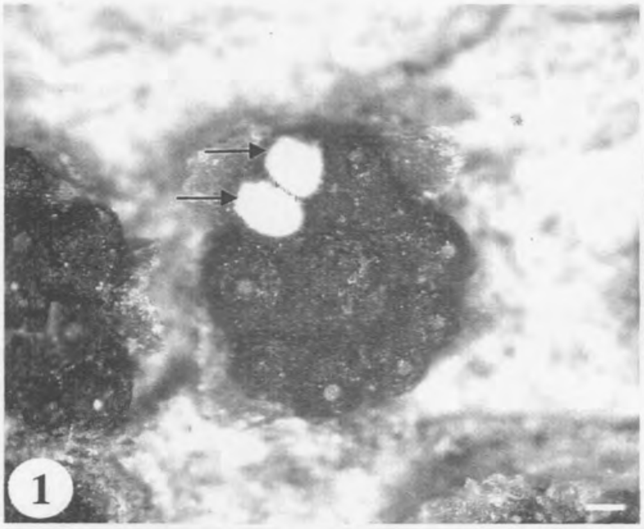
[1] All information is as given in the original description and was confirmed during the current study and left unchanged, except ascospore and conidial measurements, host and area, which are given as determined in this study.

FIGS. 1-7. *Botryosphaeria dothidea*, dissecting microscope and DIC compound-microscope micrographs. 1. Botryose ascomata, from which the tops of two have been removed to show the typical white centrum contents (arrows). 2. Median, longitudinal section through a mature ascoma. Bars = 100 µm. 3. Asci and ascospores. 4. Conidiogenous cells (arrows). 5. Conidia from nature. 6. Conidia produced in culture on WA and pine needles. 7. Spermatiophores (arrows) and spermatia. Bars = 10 µm.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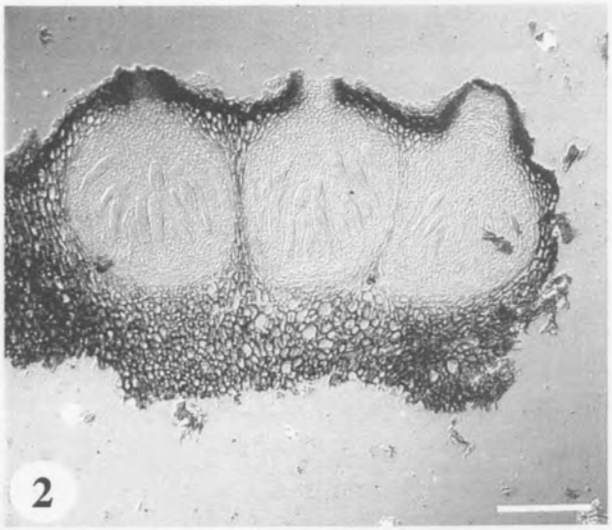
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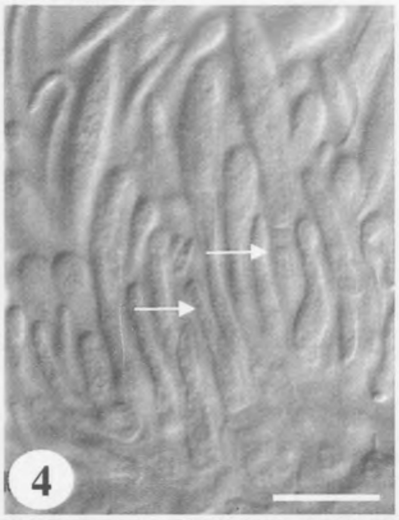
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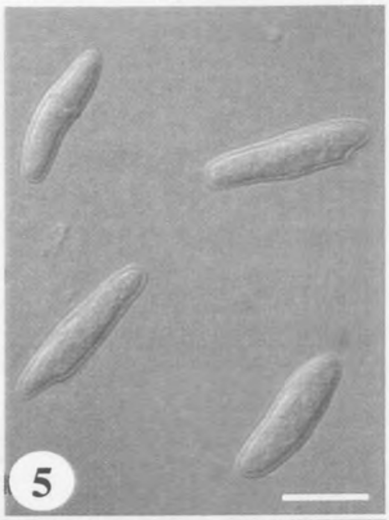
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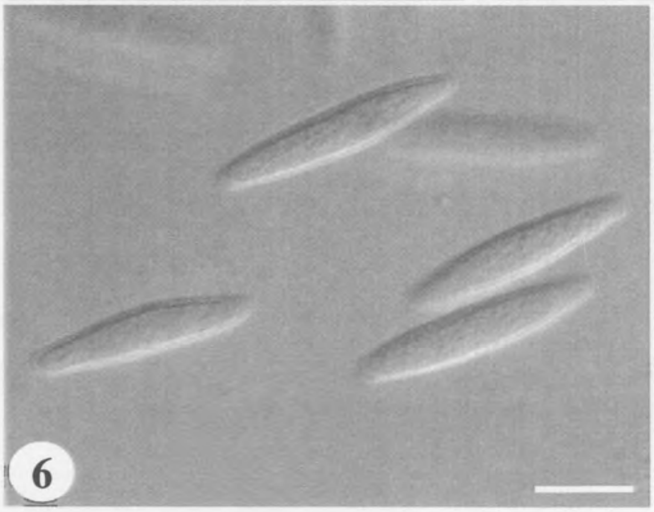
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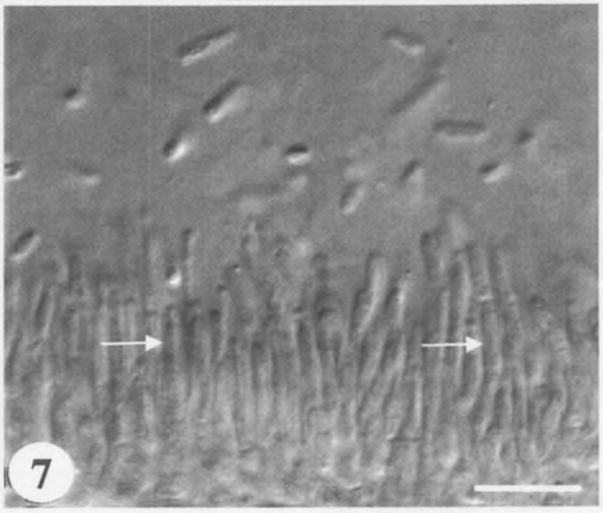
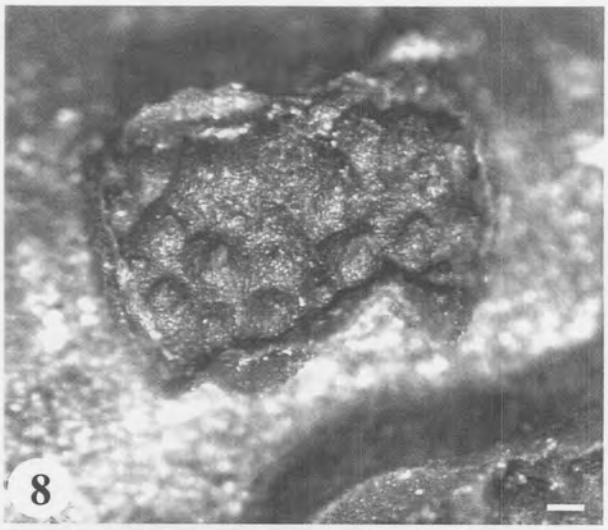

7

FIGS. 8-14. *Botryosphaeria ribis*, dissecting microscope and DIC compound-microscope micrographs. 8, 9. Ascomata. Bars = 100 µm. 10. Asci and ascospores. 11. Conidiogenous cells (arrows). 12. Conidia from nature. 13, 14. Conidia produced in culture on WA and pine needles; older conidia septate (arrows). Bars = 10 µm.

FIG. 15. *B. parva*, DIC compound-microscope micrographs. Conidia produced in culture on WA and pine needles; older conidia septate (arrows). Bar = 10 µm.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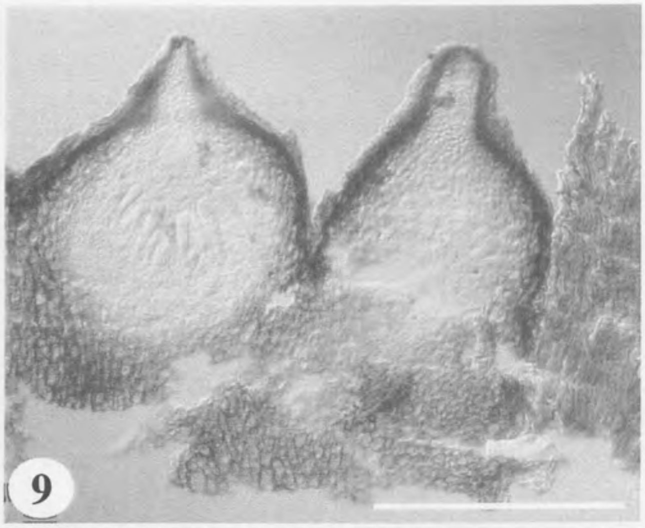
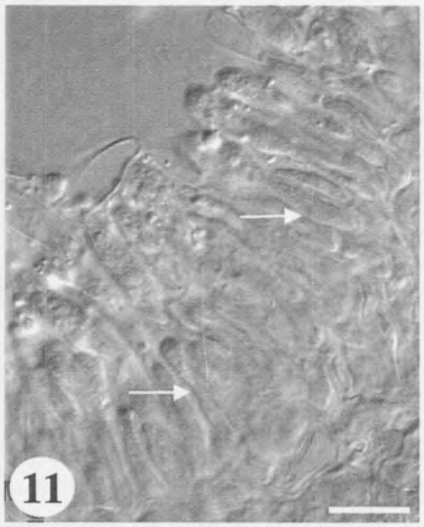
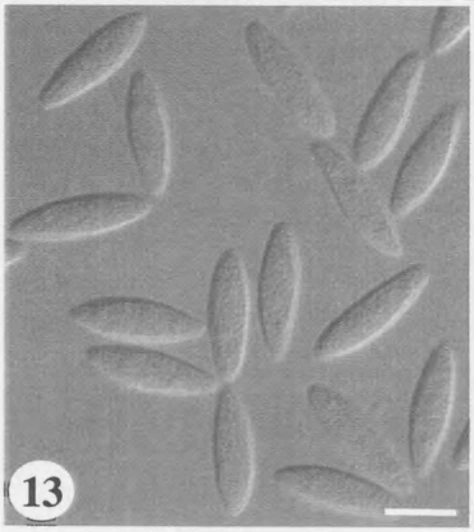

9


10


11


12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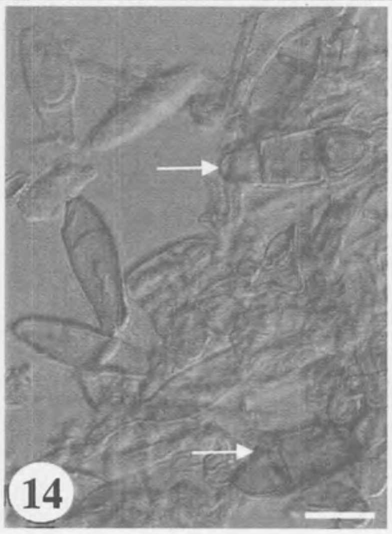
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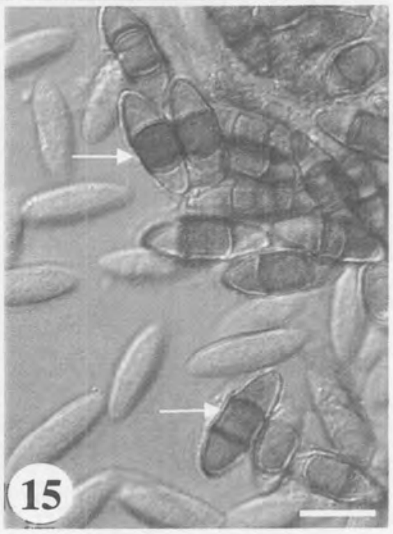
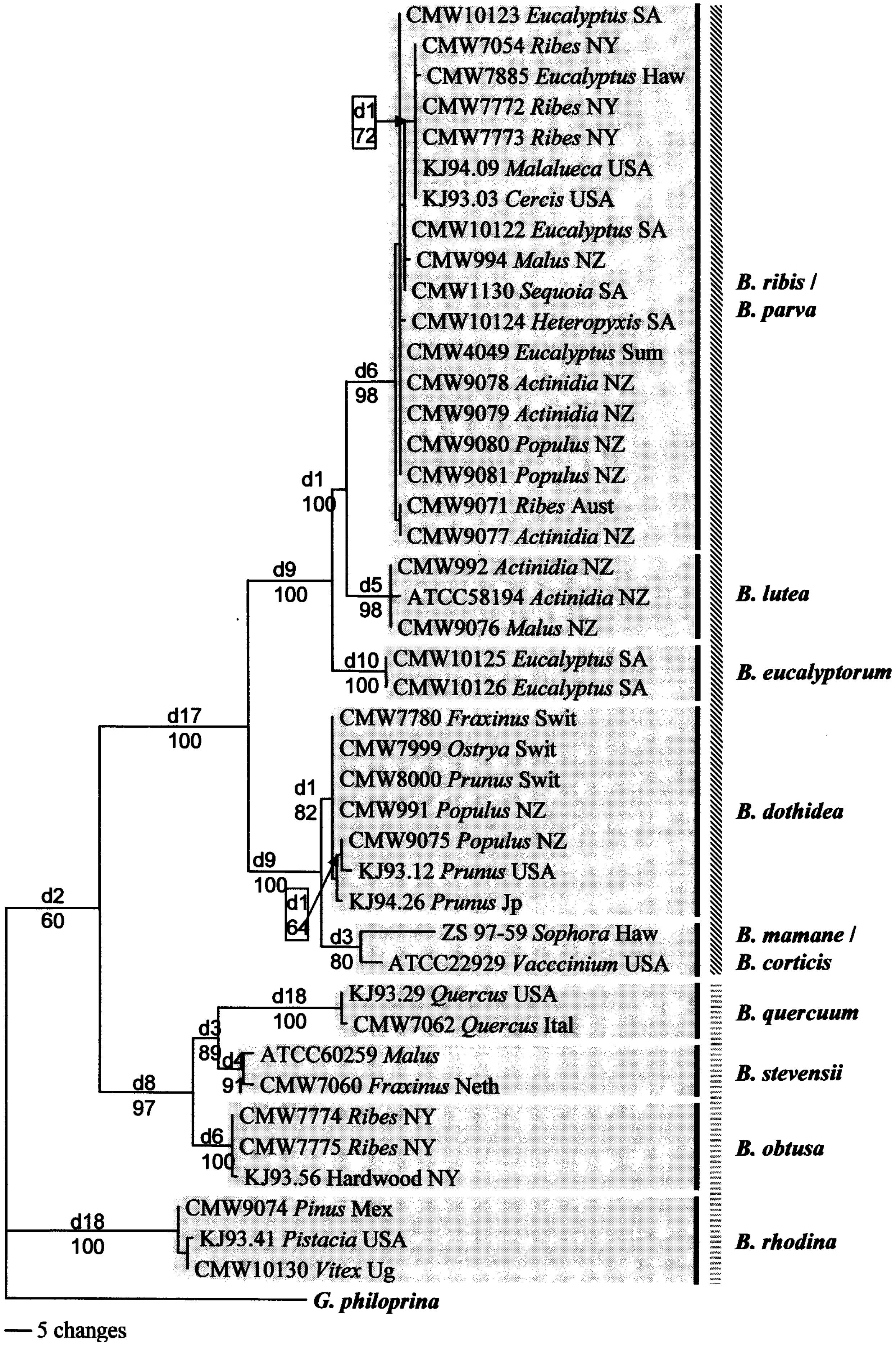

15

FIG. 16. Most parsimonious tree of 325 steps obtained from ITS1, 5.8S and ITS2 rDNA sequence data. Branch supports are indicated by decay indices above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to the outgroup, *Guignardia philoprina*. Clades are shaded individually and their identities are as used in this study. Host and origin (Aust = Australia, Haw = Hawaii, Ital = Italy, Jp = Japan, Neth = Netherlands, NY = New York, NZ = New Zealand, Mex = Mexico, SA = South Africa, Sum = Sumatra, Swit = Switzerland, Ug = Uganda), of each isolate are also indicated. ▨ = *Botryosphaeria* spp. with *Fusicoccum* anamorphs and ▤ = *Botryosphaeria* spp. with *Diplodia* anamorphs.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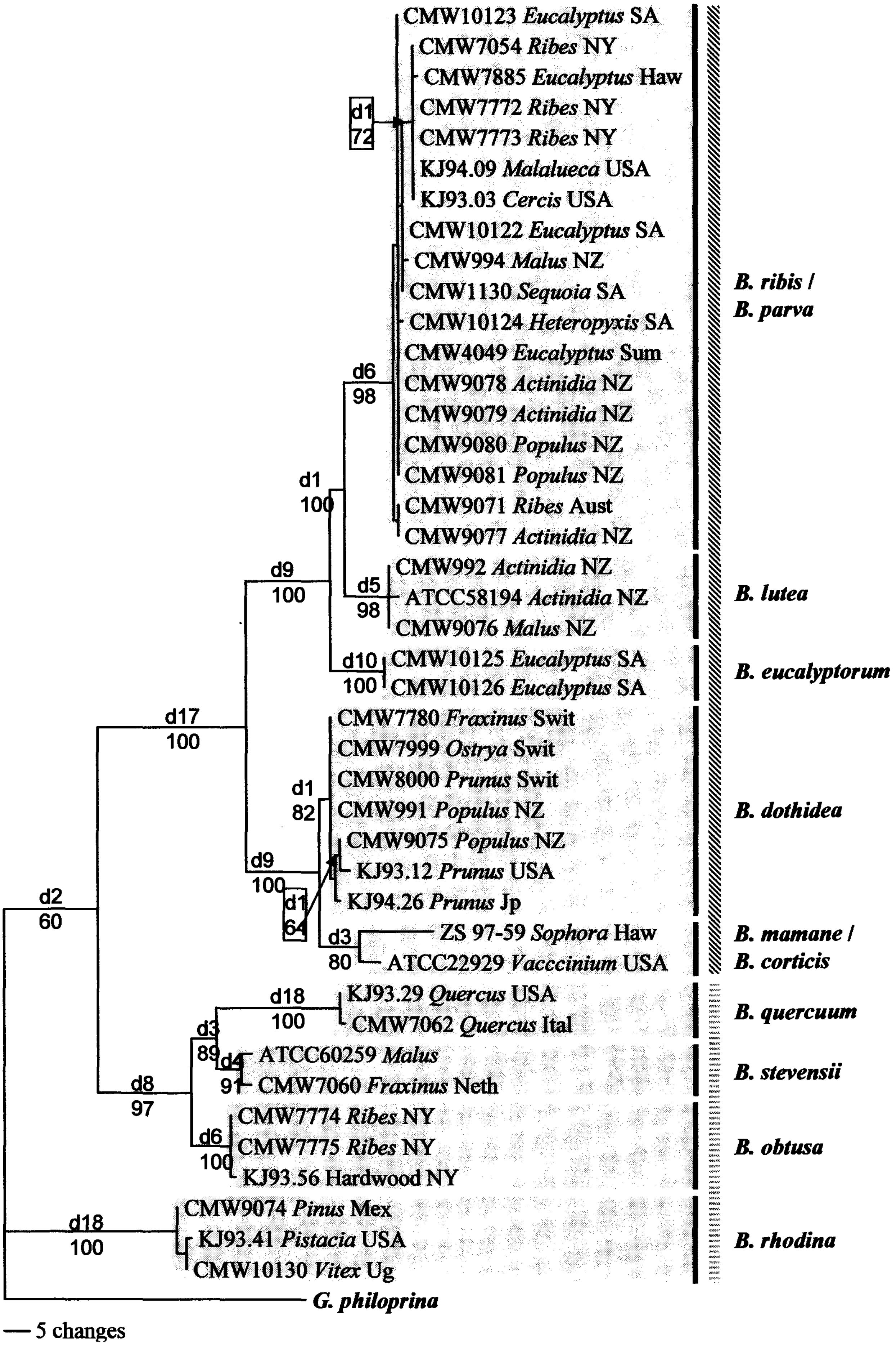
CMW7054 *Ribes* NY
CMW7885 *Eucalyptus* Haw
CMW7772 *Ribes* NY
CMW7773 *Ribes* NY
KJ94.09 *Malalueca* USA
KJ93.03 *Cercis* USA
CMW10122 *Eucalyptus* SA
CMW994 *Malus* NZ
CMW1130 *Sequoia* SA
CMW10124 *Heteropyxis* SA
CMW4049 *Eucalyptus* Sum
CMW9078 *Actinidia* NZ
CMW9079 *Actinidia* NZ
CMW9080 *Populus* NZ
CMW9081 *Populus* NZ
CMW9071 *Ribes* Aust
CMW9077 *Actinidia* NZ
CMW992 *Actinidia* NZ
ATCC58194 *Actinidia* NZ
CMW9076 *Malus* NZ
CMW10125 *Eucalyptus* SA
CMW10126 *Eucalyptus* SA
CMW7780 *Fraxinus* Swit
CMW7999 *Ostrya* Swit
CMW8000 *Prunus* Swit
CMW991 *Populus* NZ
CMW9075 *Populus* NZ
KJ93.12 *Prunus* USA
KJ94.26 *Prunus* Jp
ZS 97-59 *Sophora* Haw
ATCC22929 *Vacccinium* USA
KJ93.29 *Quercus* USA
CMW7062 *Quercus* Ital
ATCC60259 *Malus*
CMW7060 *Fraxinus* Neth
CMW7774 *Ribes* NY
CMW7775 *Ribes* NY
KJ93.56 Hardwood NY
CMW9074 *Pinus* Mex
KJ93.41 *Pistacia* USA
CMW10130 *Vitex* Ug
*G. philoprina*
d1 72
d6 98
d1 100
d5 98
d9 100
d10 100
d17 100
d1 82
d9 100
d1 64
d3 80
d18 100
d3 89
d4 91
d8 97
d6 100
d2 60
d18 100
**B. ribis / B. parva**
**B. lutea**
**B. eucalyptorum**
**B. dothidea**
**B. mamane / B. corticis**
**B. quercuum**
**B. stevensii**
**B. obtusa**
**B. rhodina**
5 changes

FIG. 17. Most parsimonious tree of 858 steps obtained from 5.8S and ITS2 rDNA and partial β-tubulin and EF1-α gene sequence data. Support for the branching points are given in decay values above the nodes and bootstrap values (1000 replicates) below the nodes. The tree is rooted to the outgroup *Guignardia philoprina*. Clades are shaded individually and their identities are as used in this study. Isolates' identities are given in the order, number, host and origin (Aust = Australia, Haw = Hawaii, Neth = Netherlands, NY = New York, USA, NZ = New Zealand, Mex = Mexico, SA = South Africa, Swit = Switzerland, Ug = Uganda). ▨ = *Botryosphaeria* spp. with *Fusicoccum* anamorphs and ▤ = *Botryosphaeria* spp. with *Diplodia* anamorphs.


CMW10123 Eucalyptus SA
CMW7054 Ribes NY
CMW7885 Eucalyptus Haw
CMW7772 Ribes NY
CMW7773 Ribes NY
KJ94.09 Malalueca USA
KJ93.03 Cercis USA
CMW10122 Eucalyptus SA
CMW994 Malus NZ
CMW1130 Sequoia SA
CMW10124 Heteropyxis SA
CMW4049 Eucalyptus Sum
CMW9078 Actinidia NZ
CMW9079 Actinidia NZ
CMW9080 Populus NZ
CMW9081 Populus NZ
CMW9071 Ribes Aust
CMW9077 Actinidia NZ
CMW992 Actinidia NZ
ATCC58194 Actinidia NZ
CMW9076 Malus NZ
CMW10125 Eucalyptus SA
CMW10126 Eucalyptus SA
CMW7780 Fraxinus Swit
CMW7999 Ostrya Swit
CMW8000 Prunus Swit
CMW991 Populus NZ
CMW9075 Populus NZ
KJ93.12 Prunus USA
KJ94.26 Prunus Jp
ZS 97-59 Sophora Haw
ATCC22929 Vaccinium USA
KJ93.29 Quercus USA
CMW7062 Quercus Ital
ATCC60259 Malus
CMW7060 Fraxinus Neth
CMW7774 Ribes NY
CMW7775 Ribes NY
KJ93.56 Hardwood NY
CMW9074 Pinus Mex
KJ93.41 Pistacia USA
CMW10130 Vitex Ug
G. philoprina
d1 72
d6 98
d1 100
d5 98
d9 100
d10 100
d17 100
d1 82
d9 100
d1 64
d3 80
d2 60
d18 100
d3 89
d4 91
d8 97
d6 100
d18 100
B. ribis / B. parva
B. lutea
B. eucalyptorum
B. dothidea
B. mamane / B. corticis
B. quercuum
B. stevensii
B. obtusa
B. rhodina
5 changes

# 2

# Phylogenetic and morphological re-evaluation of the *Botryosphaeria* anamorphs causing diseases of *Mangifera indica* in Australia

***Abstract:*** Species of *Botryosphaeria* are among the most serious pathogens that affect mango trees and fruit. Several species occur on mangoes and these are identified mainly based on the morphology of the anamorphs. Common taxa include *Dothiorella dominicana*, *D. mangiferae* (= *Natrassia mangiferae*), *D. aromatica* and an unidentified species, *Dothiorella* 'long'. The genus name *Dothiorella* is, however, acknowledged as a synonym of *Diplodia*. The aim of this study was to characterise and name the *Botryosphaeria* spp. associated with disease symptoms on mangoes. To achieve this, isolates representing all four *Dothiorella* spp. mentioned above were compared with the anamorphs of known *Botryosphaeria* spp., based on conidial morphology and DNA sequence data. Two genomic regions were sequenced and analyzed, namely the ITS rDNA and β-tubulin regions. The morphological and molecular results confirmed that the fungi previously identified from mango as species of *Dothiorella*, all belong to *Fusicoccum*. *Dothiorella dominicana* isolates were identical to isolates of *F. parvum* (teleomorph = *B. parva*). A new epithet, namely *F. mangiferum*, is proposed for isolates previously treated as *D. mangiferae* or *N. mangiferae*. Isolates of *D. aromatica* were identified as *F. aesculi* (teleomorph = *B. dothidea*). A fourth *Fusicoccum* sp. was also identified as those isolates previously known as *Dothiorella* 'long'. A key is provided to distinguish these species based on anamorph morphology in culture. This study provides a basis for the identification of *Botryosphaeria* species from mango, which is important for disease control and to uphold quarantine regulations.

## INTRODUCTION

Stem end rot of mango (*Mangifera indica* L.) fruit is one of the most serious diseases affecting this industry worldwide (Prakash and Srivastava 1987, Cappellini et al 1988, Prusky 1991, Mitra and Baldwin 1997). This disease is caused by a complex of fungal pathogens, of which various *Botryosphaeria* spp. are among the most dominant (Darvas 1991, Johnson et al 1991a, b, 1992, Sangchote 1991). Apart from fruit diseases, *Botryosphaeria* spp. also cause tip- and branch die-back and cankers on mango trees (Stevens 1926, Ramos et al 1991). These fungi live endophytically in healthy tissue, and mostly cause disease after stress to the trees or fruit after harvest (Johnson et al 1991a, 1992, Sangchote 1991).

Botryosphaeriaceous fungi considered as pathogens of mango trees and fruit are best known by their anamorph states. These include species of *Natrassia* B. Sutton & Dyko, *Dothiorella* Sacc., *Fusicoccum* Corda, *Diplodia* Fr. and *Lasiodiplodia* Ellis & Everh. (Johnson 1992). Some of the most commonly used names, and those considered in this study are *Dothiorella dominicana* Petr. & Cif., *D. mangiferae* Syd. & P. Syd., *D. aromatica* (Sacc.) Petr. & Syd. and an unnamed species, *Dothiorella* 'long' (Johnson 1992). These names are, however, in need of revision. *Dothiorella mangiferae* has been reduced to synonymy under *Nattrassia mangiferae* (Syd. & P. Syd.) Sutton & Dyko (Sutton and Dyko 1989). This synonymy has been recognised by some researchers (Londsdale 1992, Roux 1993), but disputed by others (Johnson 1991a, b, 1992). In addition the type species of *Dothiorella* was recently synonymised under *Diplodia*, raising questions about the correct generic affinities of all species presently placed in *Dothiorella* (Crous and Palm 1999).

Not all the *Dothiorella* spp. are of equal importance as pathogens of mango. *Dothiorella dominicana* is the most common pathogen and causes significant losses annually (Darvas 1991, Johnson et al 1991a). *Dothiorella mangiferae* is another common *Botryosphaeria* anamorph associated with mango fruit and trees world-wide, especially in Australia and Thailand (Sydow et al 1916, Johnson et al 1991a, 1992, Mitra and Baldwin 1997). *Dothiorella aromatica* (Sacc.) Petr. & Syd., and an unnamed species, *Dothiorella* 'long', have occasionally been recorded from mango in Thailand and Australia, but are of less importance (Johnson et al 1991a, Johnson 1992). *Dothiorella aromatica* has been reported from mango, but is better known as a pathogen of avocado (Johnson 1992, Johnson et al 1992, Hartill 1991).

It has been suggested that the *Dothiorella* spp. occurring on mango should all be accommodated in the genus *Fusicoccum* (Johnson 1992). In that study it is suggested that *D. dominicana* is a synonym of *F. aesculi* Corda (*B. dothidea* (Fr.: Moug.) Ces. & De Not.), and that *D. aromatica* should be renamed as *F.* "*aromaticum*", having *F. luteum* Pennycook & Samuels as synonym. He also suggested that *D. mangiferae* should be renamed as *F. 'mangiferam'*, which he considered to be the anamorph of *B. parva* Pennycook & Samuels, and that *Dothiorella* 'long' is a synonym of *F. cajani* (Syd., P. Syd. & E.J. Butler) Samuels & Singh (teleomorph *B. xanthocephala* (Syd., P. Syd. & E.J. Butler) Theissen). These synomymies were, however, never formally proposed.

Other researchers have reported a species of *Fusicoccum* from mango and avocado. Hartill (1991) examined Botryosphaeriaceous fungi from avocado in New Zealand, which have previously been described as *Dothiorella* species. He concluded that these fungi should reside in the genus *Fusicoccum* and identified *F. aesculi*, *F. parvum* Pennycook & Samuels and *F. luteum* from New Zealand collections. In California, Ramos et al (1991) reported on the presence of *Fusicoccum* anamorph of *B. ribis* Grossenb. & Duggar from mango plants.

Anamorph morphology is commonly used to identify species of *Botryosphaeria* (Shoemaker 1964, Pennycook and Samuels 1985, Jacobs and Rehner 1998, Slippers et al 2003). The morphological distinctions between some of the closely related anamorph species are, however, not always clear. Recent studies, using DNA sequence data have highlighted taxonomic groups and relationships in *Botryosphaeria* (Jacobs and Rehner 1998, Denman et al 2000, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001, Slippers et al 2003). These data combined with morphological characteristics could clarify the current taxonomic confusion. There is a clear need to use the same approach to clarify the relationships and identities of the stem end rot pathogens of mango.

The aim of this study was to re-evaluate the status of the anamorph names of *Botryosphaeria* species from mango in Australia, and determine their relatedness to other *Botryosphaeria* spp. DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and the β-tubulin gene were used, in combination with morphological characteristics to characterise and name the different '*Dothiorella*' spp. The taxonomy of *B. rhodina* (Berk. & Curt.)

Arx (anamorph = *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.), another *Botryosphaeria* sp. that commonly occurs on mango in Australia, is not considered in this study.

## MATERIALS AND METHODS

*Isolates and morphological characterization.*--A total of 14 single spore isolates from stem-end rot lesions on mango fruit or from necrotic twigs, were used in this study (TABLE 1). These isolates had previously been characterized based on morphology by Johnson (1992). In the current study, the isolates were induced to sporulate on water agar amended with pine needles as substrate, and exposed to near UV light for a 12 h cycle at 20--25 C for up to one month. Fruiting structures and spores were mounted in lactophenol. Observations and measurements of conidial characteristics were made with a light microscope and an Axiocam digital camera (Carl Zeiss, Germany). At least 50 conidia were measured for each isolate.

*Molecular characterization.*--A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA, as described in Raeder and Broda (1985) and Smith et al (2001). Partial sequences from two housekeeping gene regions were used for phylogenetic comparisons between isolates. Firstly, the region spanning the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene, was amplified using the primers ITS1 (5' TCCGTAGGTGAAC CTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al 1990). Secondly, a part of the β-tubulin gene was amplified using the primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGA CCCTTGGC 3') (Glass and Donaldson 1995). PCR reaction mixtures, PCR conditions and visualization of amplicons were as described in Slippers et al (2003). ITS and β-tubulin PCR amplicons were purified and sequenced as described in Slippers et al (2003).

To compare the sequence data determined in this study with those of known taxa, 15 ITS rDNA sequences and 15 β-tubulin sequences obtained from GenBank were included in the analyses (TABLE 1). These sequence data included those of *B.*

*dothidea*, *B. ribis* and *B. parva* from a study of type material and ex-type cultures (Slippers et al 2003), as well as other sequence data of related *Botryosphaeria* spp. (Jacobs and Rehner 1998, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001). BLAST searches were done to identify any other related sequence data to the fungi studied here. *Guignardia philoprina* (Berk. & M.A. Curtis) Aa, which is closely related to *Botryosphaeria*, was included as an outgroup taxon in the analyses. Despite the close relationship between the outgroup and the ingroup taxa, unambiguous alignment of intron regions of the outgroup sequence with the ingroup was not always possible, due to the high degree of sequence variation within these regions. Analysis with and without these regions did not affect the relationships of isolates of the ingroup taxa and were thus left as is.

Sequence data determined in this study were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA). These data were manually aligned with each other and with the data obtained from GenBank, by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Partition homogeneity tests (Farris et al 1995, Huelsenbeck et al 1996) were run in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), to determine whether the ITS rDNA and β-tubulin sequence data sets were congruent and, therefore, combinable. These data were then analyzed together to determine possible phylogenetic relationships between the taxa using parsimony in PAUP. To construct maximum parsimonious trees from the data, heuristic searches were done using informative characters and stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were determined. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985) and decay analysis of the branch nodes using Autodecay (Eriksson 1998).

# RESULTS

*Isolates and morphological characterization.*--The isolates used in this study had been maintained in culture for an extended period of time and sub-cultured extensively. Many of the cultures grew poorly and isolates did not sporulate as readily on pine needles as observed previously with freshly isolated strains (Slippers et al 2003). Nevertheless, all species sporulated on the needles after one to four weeks. Pycnidia were spherical (150--400 µm), with an apical pore, with or without a conical neck (50--200 µm), semi-immersed to superficial on the needle surfaces and mostly occurred singly (FIG. 1). The apical pore was often inconspicuous due to dense growth of grey mycelium covering the pycnidia.

The four taxa represented by the isolates, were distinguishable based on conidial size and shape (TABLE II) (KEY) (FIGS. 2-7). Isolates previously identified as *D. dominicana* had fusiform to ellipsoid, hyaline conidia (average of 56 conidia = 19 x 5.2 µm). These conidia were infrequently observed to become 1--2 eu-septate, often with a darker brown middle cell. Such septate, versicolored spores were usually observed after discharge from the pycnidia or on material that had been left to dry. These conidia are similar to those reported to be *F. parvum* (TABLE II). Conidia of isolates previously identified as *D. mangiferae*, were similar in shape, septation and color to those of *D. dominicana*, but were smaller (average of 54 conidia = 13.6 x 5.4 µm). Dark brown mycelial, toruloid cells were infrequently observed on pine needles or in culture. Isolates identified as *D. aromatica* produced long, fusiform conidia (average of 59 conidia = 23 x 5.1 µm). These isolates were similar to those reported as *F. aesculi*, the anamorph of *B. dothidea* (TABLE II). Isolates previously identified as *D.* 'long' also produced long conidia, but differed from the last named taxon by their broader, rod-shaped conidia (average of 59 conidia = 26.6 x 6 µm).

*Molecular characterization.*--Amplicons of about 550 bp were obtained using the primers ITS1 and ITS4, and approximately 450 bp using the primers Bt2a and Bt2b. Approximately 25 bp of the terminal end sequence data were excluded in each case in the final alignments. The total aligned sequence data set had 1016 characters. Only the 260 parsimony informative characters were included in the analysis.

A partition homogeneity test showed that the ITS rDNA and β-tubulin datasets were congruent (P value = 0.2). Evaluation of random trees showed that the combined datasets contained significant phylogenetic signal ($P < 0.01$; $g1 = -0.72$) (Hillis and Huelsenbeck 1992). Heuristic searches found two equal, most parsimonious trees (Tree length = 544 steps; CI = 0.746; RI = 0.885) (FIG. 8). The nine clades in these trees were identified as follows: clade I = *B. ribis*, clade II = *B. parva*, clade III = *F. mangiferum*, clade IV = *B. eucalyptorum* Crous, H. Smith & M.J. Wingf., clade V = *B. lutea* A.J.L. Phillips, clade VI = *Fusicoccum* sp., clade VII = *B. dothidea*, clade VIII = *B. obtusa* (Schwein.) Shoemaker and *B. stevensii* Shoemaker, and clade IX = *B. rhodina*. All these clades were supported by high bootstrap values (>99%).

Clades I-VII all represent *Botryosphaeria* spp. with *Fusicoccum* anamorphs, and formed a monophyletic group supported by a 100% bootstrap value. Within this group, clades I-II (*B. ribis*, *B. parva*) grouped together (100% bootstrap), and these two clades, were most closely related to clades III-V (*F. mangiferum*, *B. eucalyptorum* and *B. lutea*) with 85 % bootstrap support. Clade VI (undescribed *Fusicoccum* sp.) and clade VII (*B. dothidea*) grouped apart from the other groupings. There was sequence variation among isolates within each of clades VI and VII. The variation in clade VI is in the β-tubulin region in only one isolate (CMW7023). The variation within clade VII is in two bases located in a repetitive G (nine repeats) and C (10 repeats) rich area in the ITS1 region. Clade VIII and IX represent *Botryosphaeria* spp. with *Diplodia*-like conidia.

## TAXONOMY

Four botryosphaeriaceous fungi were identified in this study from mango. Two species have known teleomorphs, *B. parva* and *B. dothidea*. Johnson (1992) provisionally suggested a new combination in the genus *Fusicoccum* for the fungus reported as *D. mangiferae* or *N. mangiferae* from mango and other hosts. That proposal is supported by molecular and morphological data obtained in this study and a new combination is formally proposed here. The fourth distinct species is only identified as a species of *Fusicoccum*.

***Botryosphaeria parva*** Pennycook and Samuels, Mycotaxon 24:455. 1985.

*Anamorph.* ***Fusicoccum parvum*** Pennycook and Samuels, Mycotaxon 24:455. 1985. FIG. 2

*Notes.* The anamorph state of *B. parva* has commonly been identified from mango as *D. dominicana*. The conidia from putative *D. dominicana* isolates collected in Australia (Johnson 1992) are exactly the same as those reported from the type of *F. parvum* (Pennycook and Samuels 1985, Slippers et al 2003). These data, and that emerging from this study (TABLE II), show that this taxon can be distinguished from other Botryosphaeriaceous fungi on mango, based on conidial characteristics. The most recognizable characteristics of these conidia are that they are aseptate, hyaline, granular, broadly ellipsoid to fusoid, on ave. 17--19 x 5--6 µm (see KEY). Older, discharged conidia sometimes become 1--2 septate and light brown with darker middle cells. Septate conidia with distinctly darker middle cells in this fungus have been confused with *N. mangiferae* and *D. mangiferae* (Sutton and Dyko 1989, Roux 1993).

Although the '*D. dominicana'* isolates considered here are conspecific with *B. parva*, the true identity of the type of *D. dominicana* remains unclear. The dimensions reported in the original description of *D. dominicana* from mango leaves by Petrak and Ciferri (1930) fall within the range of *F. parvum*. Johnson (1992) re-examined and described the type material of *D. dominicana*, which he considered to be synonymous with *B. dothidea.* The conidia reported by Johnson (1992) from the *D. dominicana* type material are, however, smaller than the anamorphs of either *B. dothidea* or *B. parva*. Despite this uncertainty, it is clear that the name *D. dominicana* is not appropriate for isolates associated with stem end rot and other diseases of mango in Australia.

***Fusicoccum mangiferum*** (Syd. & P. Syd.) Johnson, Slippers & M.J. Wingf. comb. nov. FIG. 3-5

*Basionym. Dothiorella mangiferae* Syd. & P. Syd., Ann. Mycol. 14:192. 1916.

*Synonyms. Natrassia mangiferae* (Syd. & P. Syd.) B. Sutton & Dyko, Mycol. Res. 93:484. 1989.

*Hendersonula toruloidea* Natrass, Trans. Br. Mycol. Soc. 18:197. 1933.

*Hendersonula cypria* Natrass, Cypress fungi, Nicosia: 43. 1937.

*Teleomorph*. ***Botryosphaeria*** sp.

*Notes*. Various morphological descriptions have been given for this taxon. The holotype was first described by Sydow et al (1916) from *M. indica* in India. Sutton and Dyko (1989) reviewed all previous type material connected with this taxon, including the holotype, and provided a very thorough description. Other clear descriptions are found in Natrass (1933), Punithalingam and Waterston (1970) and Johnson (1992).

Duplication of previous descriptions is avoided here, but the most distinctive features are highlighted. The conidia are distinct from other *Fusicoccum* spp. by their shorter average length (ave. ~13--14 μm) and smaller length/width ratio (2--2.5) (see KEY). The conidia often become one to two septate, light brown with distinctly darker middle cells. This feature is also shared with *F. parvum*. *Fusicoccum mangiferum* produces vegetative, toruloid cells in culture and in nature. This species also produces fluffy, evenly grey-coloured aerial mycelium, lacking the white tufts found in other similar species such as *F. parvum*.

Sydow et al (1916) described *D. mangiferae* from mango, but noted only the aseptate conidia. Re-examination of the type material, however, confirmed the presence of 1--2 septate, pigmented conidia (Sutton and Dyko 1989). It is possible that the spores on the type material aged and became septate after the description by Sydow. Natrass (1933) studied the taxon from pome and stone fruit trees and first noticed the pigmented conidia, which led him to describe it as *Hendersonula toruloidea* Natrass. He also studied the fungus in culture and noted the characteristic brown one or two celled, toruloid, vegetative cells. Sutton and Dyko (1989) revised the genus *Hendersonula* and synonymized both *D. mangiferae* and *H. toruloidea*, amongst other genera and species, with the newly described *N. mangiferae*.

Natrass (1933) and Sutton and Dyko (1989) reported fragmented mycelial cells or toruloid cells in culture and in nature. In the last named study, this form was described as the synanamorph *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko. Johnson (1992) reported no toruloid state, but referred only to these cells as fragmented mycelia, and he did not use the last named epithet. Such cells as described in Natrass (1933) and Sutton and Dyko (1989) were rarely observed in this study and when seen, resembled fragmented, thick-walled hyphae.

Sutton and Dyko (1989) reduced *Fusicoccum eucalypti* Sousa da Câmara and *H. agathi* to synonymy with *F. mangiferum* (as *N. mangiferae*). These synonymies are not accepted here, as the taxa both have conidia that differ from those of *F. mangiferum* in length and in length/width ratio (Young 1948, Sutton and Davison 1983, Sutton and Dyko 1989). The conidial sizes reported in the last named studies for *F. eucalypti* and *H. agathi* were more similar to those of *F. parvum*.

The teleomorph of *F. mangiferum* is a *Botryosphaeria* sp. The DNA sequence data presented here group this species with the type species, *B. dothidea*, and other *Botryosphaeria* sp. Johnson (1992) also reported *Botryosphaeria* ascomata and ascospores forming in cultures of *F. mangiferum* (Johnson 1992). Sufficient material was, however, not available to formally describe a specific name for the teleomorph.

***Botryosphaeria dothidea*** (Moug.: Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital. 1:212. 1863.

*Anamorph*. ***Fusicoccum aesculi*** Corda in Sturm, Deutschl. Fl., Abth. 3, 2:111. 1829. FIG. 6

*Notes*. Previous reports of this fungus from mango and avocado listed it as *D. aromatica*. Conidial morphology of this species is similar to that described for the anamorph of *B. dothidea* (Pennycook and Samuels 1985, Slippers et al 2003). The most distinctive feature of this taxon is its conidia, which are aseptate, hyaline, fusiform to narrowly fusiform and on average 23-25 x 4-5 µm (see KEY).

The type specimen of *D. aromatica* has not been compared with that of *F. aesculi* in this or any other study. Johnson (1992) reports that this material is not available and we have been unable to locate it. The synonymy of *D. aromatica* and *F. aesculi* is, therefore, not considered. The fungus occurring as a pathogen of mango and avocado and generally identified as *D. aromatica* is, however, *F. aesculi.*

***Fusicoccum* sp.** FIG. 7

*Notes*. Johnson et al (1991a) and Johnson (1992) identified an unknown *Dothiorella* or *Fusicoccum* sp. from mango in Australia and Thailand, which was referred to only as *Dothiorella* 'long'. Our DNA sequence and morphological data confirm that this is a *Fusicoccum* sp., and that it probably represents an undescribed species.

Johnson (1992) considered this *Fusicoccum* sp. as possibly synonymous with *F. cajani* (teleomorph = *B. xanthocephala*). Samuels and Singh (1986) described *F. cajani* from *Cajanus* spp. (pigeon pea) from India, Fiji and the USA. Conidial measurements of *F. cajani* [(17-) 21.6--27.8 (-32) x (5-) 6.5--8 (-9) µm] and the *Fusicoccum* sp. [average = 26.6 x 6 µm, see TABLE II] considered here, overlap. This alone is, however, not sufficient evidence for synonymy. For example, the measurements and the shape of the conidia of *F. cajani* overlap with the anamorphs of a number of other *Botryosphaeria* spp., such as *B. lutea* (Pennycook and Samuels 1985, Phillips et al 2002), *B. eucalyptorum* (Smith et al 2001), *B. protearum* Denman and Crous (Denman et al 2003), and others. Furthermore, the host and geographical differences make this view impossible to confirm without further evidence. No isolates of *B. xanthocephala* could be located to further test this hypothesis, using molecular or cultural characters.

## KEY TO *BOTRYOSPHAERIA* SPP. AND THEIR ANAMORPHS FROM MANGO IN AUSTRALIA

Conidial characters are used to separate the Botryosphaeriaceous fungi treated here. The anamorph state is most frequently encountered in nature and is also readily induced *in vitro* on nutrient poor medium (e.g. water agar) supplemented with sterilized pine needles. Differences among the species are more pronounced in anamorph than teleomorph features. Teleomorphs have not been described or even observed for all the species treated here, but these names are used preferentially where they are known. The unnamed species of *Fusicoccum* refers to the fungus previously known as *Dothiorella* 'long'.

1. Conidia in culture on average <18 µm in length, l/w 2--3.5, unicellular, but occasionally becoming light brown and 1--2 septate with a darker brown middle cell after discharge, colony on MEA or PDA thick felt of grey aerial mycelium............2

1. Conidia in culture on average >18 µm in length, l/w >4, unicellular, colony on MEA or PDA appressed with only occasional tufts of grey to buff aerial mycelium...3

2. No toruloid cells, conidia 12--23 x 4--6 µm (average 19 x 5.2 µm), l/w 3--3.5 ........................................................................................***B. parva***

2. Toruloid cells, conidia 12--14 x 4--6 µm (average 13.6 x 5.4 µm), l/w 2--3...... ........................................................................................***F. mangiferum***

3. Conidia hyaline, unicellular, rod-shaped, 20--32 x 5--7 µm (average 26.6 x 6 µm), l/w 3.5--4.5....................................................................***Fusicoccum* sp.**

3. Conidia hyaline, unicellular, narrowly fusiform, 19--30 x 4--6 µm (average 23 x 5.1 µm), l/w 4--5..........................................................................***B. dothidea***

## DISCUSSION

Four *Fusicoccum* spp. were identified as endophytes and pathogens of Australian mango fruit and trees in the present study. Identification of these species is based on a combination of morphological and molecular phylogenetic analyses. These species are *F. parvum*, *F. mangiferum*, *F. aesculi*, and an undescribed *Fusicoccum* sp. They were all previously known as species of *Dothiorella* or *Natrassia*. The data emerging from this study show that all these taxa should be seen as species of *Fusicoccum*, and that their teleomorphs, although some are presently still unknown, should all reside in *Botryosphaeria*.

The description of *Dothiorella* spp. from mango as *Fusicoccum* spp. is in accordance with recent proposals for the correct use of these two generic names (Crous and Palm 1999, Denman et al 2000). *Fusicoccum* and *Dothiorella* have often been confused, because both have commonly been used to describe anamorphs of *Botryosphaeria* (Saccardo 1882, Petrak 1922, Von Arx and Müller 1954). The common use of the name *Dothiorella* for *Botryosphaeria* anamorphs from mango, follows the preference of this name for botryosphaeriaceous fungi from this host by Sydow et al (1916) and Petrak (1922). Recently, *D. pyrenophora* Sacc., the type species of *Dothiorella*, was re-described as *Diplodia pyrenophora* (Sacc.) Crous & M.E. Palm (Crous and Palm 1999). These authors suggested that all *Botryosphaeria* anamorphs that are placed in *Dothiorella* should be re-examined. Denman et al (2000) argued that all hyaline, thin walled fusiform conidial *Botryosphaeria* anamorphs should reside in *Fusicoccum*.

Results of this study and those of Johnson (1992) show clearly that *B. parva* (reported as *D. dominicana*) is one of the most common pathogens that cause stem end rot, die-back and blossom blight on mango. The species was first described by Pennycook and Samuels (1985) from *Populus*, *Malus* and *Actinidia* species in New Zealand. Subsequently, it was shown that this species occurs world-wide on a number of hardwood species, including native Australian flora, such as *Eucalyptus* spp. (Slippers et al 2003). *Botryosphaeria parva* has often been misidentified as *B. ribis* and *B. dothidea*, due to overlapping host ranges, morphological similarities and taxonomic confusion over the use of the names (Slippers et al 2003). It is thus also likely that the fungus described as *B. ribis* from mango in Florida (Ramos et al 1991) is *B. parva*. These identifications from Florida were done based on conidial dimensions, which overlap between *B. ribis* and *B. parva* (Slippers et al 2003).

The name *F. mangiferum* has been proposed in this study for the mango pathogen that was previously identified as *D. mangiferae* and *N. mangiferae*. Johnson (1992) first suggested that *D. mangiferae* and *N. mangiferae* should be described in the genus *Fusicoccum*. This proposal is supported in the present study by the phylogenetic monophyly of this taxon with the type species, *F. aesculi*, and other *Fusicoccum* spp. Isolates used for sequence analyses were not ex-type cultures. The conidia of these isolates were, however, similar to those from the type specimens of *D. mangiferae* and *N. mangiferae*, which are unique in morphology (Sutton and Dyko 1989, Sydow et al 1916).

There are obvious similarities in the septation and pigmentation of conidia of *F. mangiferum* and *F. parvum*. This has led to confusion between these taxa in the past. These species can, however, be separated based on conidial size, as the conidia of the former species are smaller in average length and width. Moreover, in culture *F. parvum* has more fluffy aerial mycelium than the appressed grey aerial mycelium of *F. mangiferum*.

*Botryosphaeria dothidea* is of little importance as a pathogen of mango in Australia or other parts of the world. It is less common on mango than *B. parva* and *F. mangiferum* and is often omitted from lists of important pathogens of this host (Johnson et al 1991a, b, 1992, Johnson 1992). This name is, however, one of the most commonly used for *Botryosphaeria* pathogens on a wide variety of other hosts (McGlohon 1982, Pennycook and Samuels 1985, Brown and Britton 1986, Hartill 1991, Jacobs and Rehner 1998, Smith et al 2001). Some of these identifications, however, need to be viewed with care, as many species have incorrectly been relegated to the name *B. dothidea*. This followed the extensive synonymy of many species with *B. dothidea* by Von Arx and Müller (1954). Due to this synonymy, *B. ribis* was treated as a synonym of *B. dothidea*. *Botryosphaeria parva* was often not distinguished from *B. ribis*, and was consequently also treated under *B. dothidea* (Slippers et al 2003).

Reports of *B. dothidea* from Australasia and other southern hemisphere countries are from exotic hosts (Pennycook and Samuels 1985, Hartill 1991, Slippers et al 2003). Studies of pathogens of native hosts in Australasia have not reported this pathogen (Denman et al 2003). This species is, however, common on both cultivated and indigenous hosts in the northern hemisphere (Zhou and Stanosz 2001, Slippers et al 2003). This suggests a northern hemisphere origin for this fungus and implies that it was introduced into the southern hemisphere with planting material of agricultural and ornamental crops.

The taxon previously known from mango as *Dothiorella* 'long' is identified in this study as an undescribed species of *Fusicoccum*. This fungus was identified from mango from Australia and Thailand by Johnson et al (1991a) and Johnson (1992). This species was rarely found in extensive surveys during these studies and is not considered important in causing pre- or post-harvest diseases of mango. This species of *Fusicoccum* is also not known from any other hosts. The suggested synonymy of this taxon with *F. cajani* (Johnson 1992) is rejected based on the distinctive hosts of each species and the lack of any further evidence to their conspecificity.

Johnson (1992) both suggested that *F. luteum* (teleomorph *B. lutea*) occurs on mango in Australasia. Based on DNA sequence data produced in this study, none of the *Fusicoccum* spp. from mango in Australia group with this taxon. This finding is surprising as *F. luteum* seems to be common in the Australasian region. *Fusicoccum luteum* was initially described from *Actinidia*, *Malus* and *Pyrus* in New Zealand (Pennycook and Samuels 1985) and subsequently also from avocado (Hartill 1991).

Sequence variation was observed among isolates of clade VI (*Fusicoccum* sp.) and clade VII (*B. dothidea*) that was not phylogenetically informative. Among the three isolates identified in clade VI one isolate had sequence variation only in the β-tubulin region. In clade VII the three isolates from mango grouped together based on two variable bases in the ITS1 region in a highly repetitive region. In both cases these variable characters were thus found only in one of the two sequenced regions.

Additional data and a larger number of isolates are required to determine the extent of variation and its phylogenetic relevance to populations of the above clades.

The many misidentifications of Botryosphaeriaceous fungi from mango in the past illustrate aptly how confusing morphological characterisation of these fungi has been. This problem results from the fact that sizes of structures for these species overlap. There is also some variation between morphological features in nature and in culture, which has added to the confusion (Slippers et al 2003, Johnson 1992). Furthermore, conidial septation and colour, which has been used to characterize species, is not always consistent. Spores tend to age only after discharge from the pycnidia and their color and septation changes with age.

This study provides a basis on which future identifications of *Botryosphaeria* and its anamorphs from mango can be made. The combination of molecular data and average conidial size and shape, as well as cultural characteristics, has successfully been used here to identify these fungi from mango. Correct identifications of these pathogens are becoming more crucial due to increased quarantine requirements. These data will also facilitate studies to better understand the epidemiology of the different fungal species.

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TABLE I. Isolates from mango fruit and trees considered in the phylogenetic study.

| **Culture no.**[1] | **Other no.**[1] | **Identity**[2] | **Host** | **Location** | **Collector** | **GenBank**[3] | |
|---|---|---|---|---|---|---|---|
| | | | | | | **ITS** | **β-tubulin** |
| CMW7772 | | *Botryosphaeria ribis* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236935 | AY236906 |
| CMW7054 | CBS121 | *B. ribis* | *Ribes* sp. | New York, USA | N.E. Stevens | AF241177 | AY236908 |
| CMW9078 | ICMP7925 | *B. parva* | *Actinidia deliciosa* | New Zealand | S.R. Pennycook | AY236940 | AY236914 |
| CMW9081 | ICMP8003 | *B. parva* | *Populus nigra* | New Zealand | G.J. Samuels | AY236943 | AY236917 |
| CMW7798 | BRIP23348 | *B. parva* | *Mangifera indica* | Australia | G.I. Johnson | | |
| CMW7796 | BRIP23349 | *B. parva* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7799 | BRIP23300 | *B. parva* | *Persea americana* | Australia | G.I .Johnson | | |
| CMW7026 | BRIP19684 | *B. parva* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7025 | BRIP24083 | *B. parva* | *M. indica* | Australia | G.I. Johnson | | |
| CMW78701 | BRIP23396 | *Fusicoccum mangiferum* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7024 | BRIP24101 | *F. mangiferum* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7797 | BRIP23350 | *F. mangiferum* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7802 | BRIP23491 | *Fusicoccum* sp. | *M. indica* | Australia | G.I. Johnson | | |
| CMW7023 | BRIP19560 | *Fusicoccum* sp. | *M. indica* | Australia | G.I. Johnson | | |
| CMW7022 | BRIP19782 | *Fusicoccum* sp. | *M. indica* | Australia | G.I. Johnson | | |
| CMW7803 | BRIP23750 | *B. dothidea* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7020 | BRIP24286 | *B. dothidea* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7027 | BRIP24172 | *B. dothidea* | *M. indica* | Australia | G.I. Johnson | | |
| CMW7780 | | *B. dothidea* | *Fraxinus excelsior* | Switzerland | B. Slippers | AY236947 | AY236925 |
| CMW8000 | | *B. dothidea* | *Prunus* sp. | Switzerland | B. Slippers | AY236949 | AY236927 |
| CMW9076 | ICMP7818 | *B. lutea* | *Malus* x *domestica* | New Zealand | S.R. Pennycook | AY236946 | AY236922 |
| CMW992 | KJ93.52 | *B. lutea* | *A. deliciosa* | New Zealand | G.J. Samuels | AF027745 | AY236923 |
| CMW10125 | BOT24 | *B. eucalyptorum* | *Eucaluptus grandis* | South Africa | H. Smith | AF283686 | AY236920 |
| CMW10126 | BOT16 | *B. eucalyptorum* | *E. grandis* | South Africa | H. Smith | AF283687 | AY236921 |
| CMW7774 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | AY236953 | AY236931 |
| CMW7060 | CBS431 | *B. stevensii* | *F. excelsior* | Netherlands | H.A. van der Aa | AY236955 | AY236933 |
| CMW10130 | BOT977 | *B. rhodina* | *Vitex donniana* | Uganda | J. Roux | AY236951 | AY236929 |
| CMW9074 | | *B. rhodina* | *Pinus* sp. | Mexico | T. Burgess | AY236952 | AY236930 |
| CMW7063 | | *Guignardia philoprina* | *Taxus baccata* | Netherlands | H.A. van der Aa | AY236956 | AY236934 |

[1] Abbreviations for culture collections and isolates: BRIP = Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; BOT = *Botryosphaeria* sub-collection of CMW; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998).

[2] Identities are given as determined in this study. Isolates from *M. indica* were previously known by the anamorph names *D. dominicana* (*B. parva*), *D. mangiferae* (*F. mangiferum*), *D.* 'long' (*Fusicoccum* sp.) and *D. aromatica* (*B. dothidea*).

[3] Sequences obtained from GenBank are from Jacobs and Rehner (1998), Smith et al (2001) and Slippers et al (2003).

TABLE II. Conidial measurements for *Botryosphaeria* spp. and their *Fusicoccum* anamorphs associated with mango.

| **Identity**[1] | **Previously used name**[2] | **Conidial size *in vitro* (µm)** | **L/W** | **Source of data**[3] |
|---|---|---|---|---|
| *B. parva* / *F. parvum* | *D. dominicana* | (12-)15-19(-24) x 4-6 [Ave. 16.9 x 5.4] | 3.1 | Pennycook & Samuels 1985 |
| | | 14.5-18.7(-17.1) x 4.6-7 [Ave. 17.1 x 4.7] | 3.6 | Johnson 1992 |
| | | (14.7-)17-21(-25.5) x 4.5-6(-7) [Ave. 19 x 5.2] | 3.7 | This study |
| *B. dothidea* / *F. aesculi* | *D. aromatica* | (20-)23-27(-30) x 4-5(-6) [Ave. 24.7 x 4.9] | 5 | Slippers et al 2003 |
| | | 19.9-26.2 x 3.9-5.5 [Ave. 22.8 x 4.6] | 4.9 | Johnson 1992 |
| | | (18.8-)21-24(-30.4) x 4.5-6(-7) [Ave. 23 x 5.1] | 4.5 | This study |
| *F. mangiferum* | *D. mangiferum* or *N. mangiferum* | 9-13 x 3.5-5 | | Sydow et al 1916 |
| | | 10-16 x 3.5-6.5 | N/a | Sutton & Dyko 1989 |
| | | 11-14 x 4.5-5.5 [Ave. 12.8 x 5] | 2.6 | Johnson 1992 |
| | | (11-)12-15(-17.3) x 5-6.6 [Ave. 13.6 x 5.4] | 2.5 | This study |
| *Fusicoccum* sp. | *D.* 'long' | 18.6-37.2 x 4.6-7 [Ave. 22.4 x 5] | 4.5 | Johnson 1992 |
| | | (20.2-)23-30(-35.5) x (4.7-)5.5-7 [Ave. 26.6 x 6] | 4.4 | This study |

[1] Teleomorph (where known) and anamorph names as used in this study.

[2] Common anamorph name used for this taxon from mango in earlier literature.

[3] The isolates studied in this study and by Johnson (1992) are the same, but measurements from both studies are given because of the difference in the technique used to induce sporulation.

FIGS. 1-7. Anamorphs of various *Botryosphaeria* spp. formed in culture. 1. Pycnidium forming on a pine needle. Bar = 100 µm. 2. *Fusicoccum parvum* conidia. 3, 4. Young, aseptate conidia of *F. mangiferum*, which become septate and discolored after discharge. 5. Toruloid cells of *F. mangiferum*. 6. Fusiform conidia of *F. aesculi*. 7. Rod-shaped conidia of a *Fusicoccum* sp. Bars = 10 µm.


①


2


3

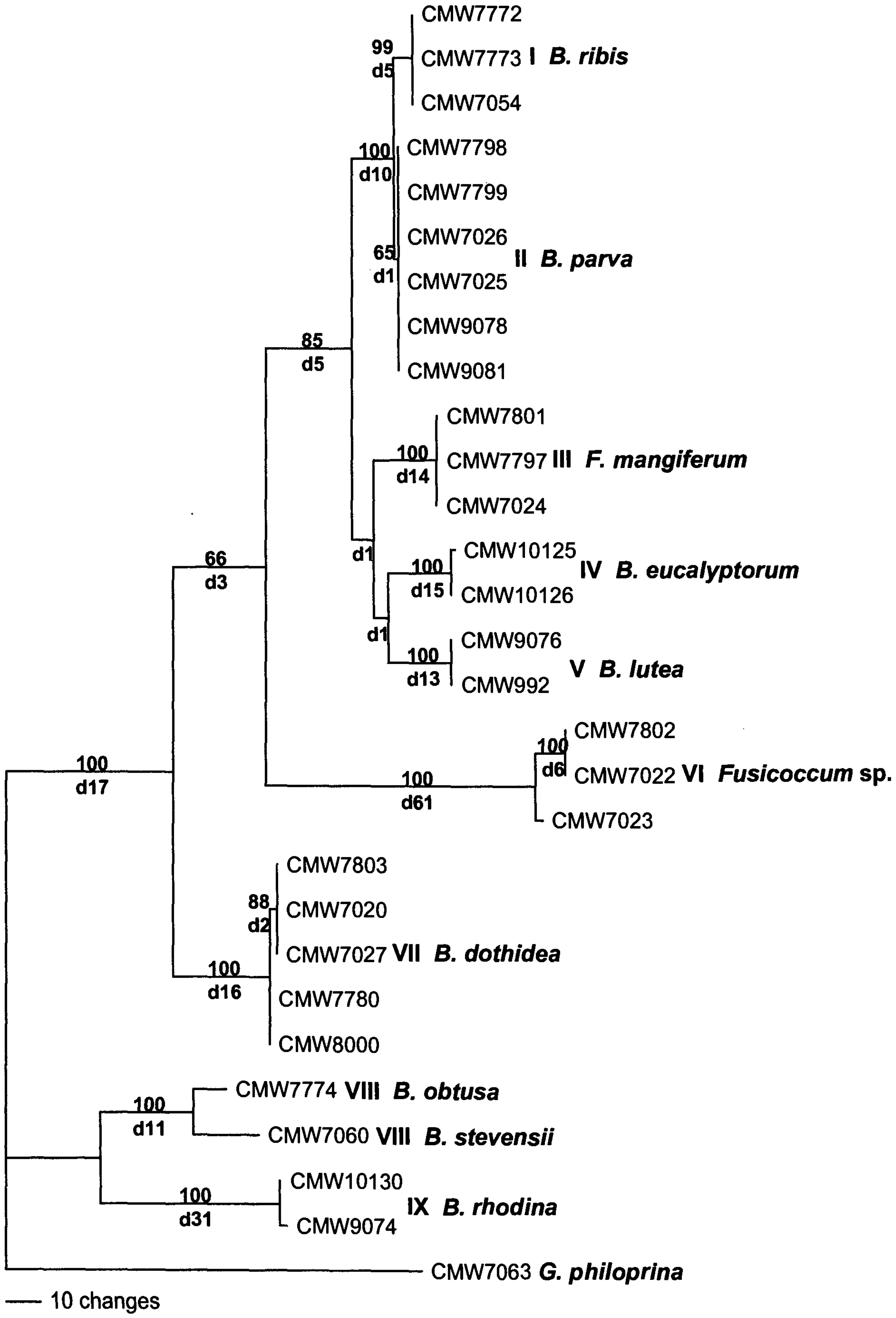

4


5


6


7

FIG. 8. One of the two equal, most parsimonious trees obtained by heuristic searches of the ITS rDNA and β-tubulin sequence datasets in PAUP. Branch supports are indicated by decay indices below and bootstrap values above the branches. Nine clades or taxa are identified. Clade VI represents an unknown *Fusicoccum* sp. (previously identified as *Dothiorella* 'long').


CMW7772
CMW7773 I B. ribis
CMW7054
99
d5
100
d10
CMW7798
CMW7799
CMW7026
65
d1
CMW7025
II B. parva
CMW9078
CMW9081
85
d5
CMW7801
CMW7797 III F. mangiferum
CMW7024
100
d14
d1
CMW10125
100
d15
CMW10126
IV B. eucalyptorum
d1
CMW9076
100
d13
CMW992
V B. lutea
66
d3
CMW7802
100
d6
CMW7022 VI Fusicoccum sp.
CMW7023
100
d61
100
d17
CMW7803
88
d2
CMW7020
CMW7027 VII B. dothidea
100
d16
CMW7780
CMW8000
CMW7774 VIII B. obtusa
100
d11
CMW7060 VIII B. stevensii
CMW10130
100
d31
CMW9074
IX B. rhodina
CMW7063 G. philoprina
10 changes

# 3

## Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. as a sister species to *B. lutea*

***Abstract:*** *Botryosphaeria lutea* (anamorph *Fusicoccum luteum*) is most easily distinguished from other *Botryosphaeria* spp. by a yellow pigment that is formed in young cultures. This fungus has been reported from a number of cultivated hosts in New Zealand and Portugal. During a survey of *Botryosphaeria* fungi that occur on native *Acacia* species in Australia, a yellow pigment was observed in some cultures. These isolates were morphologically similar to *B. lutea*, but the pigment differed slightly compared to authentic *B. lutea* isolates. Preliminary data also revealed small differences in ITS rDNA sequence data. The aim of this study was to determine whether these small differences were indicative of separate species, or merely variations within *B. lutea.* Anamorph, teleomorph and culture morphology were compared between *B. lutea* and authentic isolates from Australia. Sequence data of two other genome regions, namely the β-tubulin and EF1-α gene and intron regions, were combined with ITS rDNA sequence data to determine the phylogenetic relationship between these isolates. Isolates of *B. lutea* and those from Australian *Acacia* species were not significantly different in spore morphology. The yellow pigment was, however, much more distinct in cultures of *B. lutea* than in cultures from *Acacia*. There were only a few base pair variations in each of the analyzed gene regions, but these variations were fixed in the two groups in all regions. By combining these data it was clear that *B. lutea* and the isolates from *Acacia* were distinct species, albeit very closely related. We, therefore, propose the new epithet, *B. australis,* for the fungus from Australia. *Botryosphaeria australis* was also isolated in this study from exotic *Sequoiadendron* trees in Australia. Re-analyses of GenBank data in this study showed that *B. australis* also occurs on other native Australian hosts, namely a *Banksia* sp. and a *Eucalyptus* sp., as well as a native *Protea* sp. in South Africa and on *Pistachio* in Italy. These records from GenBank have previously been identified as *B. lutea*. The common occurrence of *B. australis* on a variety of native hosts across Australia suggests that this fungus is native to this area.

## INTRODUCTION

Various native Australian woody plants, such as *Acacia* spp., *Eucalyptus* spp., species of Proteaceae and others, make up commercial plantations world-wide (Evans 1984, Wingfield et al 2001a, b, Denman et al 2003). In order to establish and maintain these plantations, germplasm must be introduced into different countries, and this can result in the accidental introduction of exotic pathogens to new environments (Palm 1999, Wingfield et al 2001a). In this regard, a group of pathogens that are easily overlooked are *Botryosphaeria* spp., which live as endophytes in healthy plants and seeds for part of their life cycle (Smith et al 1996, Burgess and Wingfield 2002).

Once introduced into a new environment, *Botryosphaeria* spp. can threaten both native and exotic hosts (Burgess and Wingfield 2002). This is because many of these fungi have a wide host range (Wingfield et al 2001a). In order to reduce this threat, it is necessary to obtain a clear knowledge of the taxonomy and ecology of *Botryosphaeria* spp., both in their areas of natural occurrence, and in countries where these trees are commercially planted (Palm 1999, Wingfield et al 2001b).

Members from the genus *Botryosphaeria* are commonly accepted to be problematic to identify to species level. For a number of years after the circumscription of the genus, ascomatal morphology and host range were considered characteristic for different species (Cesati and De Notaris 1863, Saccardo 1877, Trotter 1928). This resulted in considerable difficulty, because the teleomorph is not frequently found in nature, and is only rarely produced in culture. It is also currently understood that some *Botryosphaeria* spp. can infect a wide variety of hosts (Stevens and Jenkins 1924, Punithalingham and Holliday 1973, Punithalingham and Waller 1973). Furthermore, teleomorph characters vary on different hosts, and are often not distinctive at species level (von Arx and Müller 1954, Slippers et al 2003).

Conidial and cultural morphology are often used to distinguish different *Botryosphaeria* spp. (Shoemaker 1964, Pennycook and Samuels 1985, Denman et al 2000, Slippers et al 2003). The anamorphs of these fungi are commonly encountered, both in nature and in culture. Distinctive features of conidia are the shape, size, length/width ratio, septation, content, color, wall thickness and ornamentation. The general growth pattern, speed and color of a colony on agar are also sometimes informative for species identification (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003).

In recent years, various DNA based techniques have been used to distinguish between *Botryosphaeria* spp. These techniques include dominant and co-dominant molecular markers such as RAPD's, ISSR's and microsatellites (Burgess et al 2001, Smith and Stanosz 2001, Zhou et al 2001), and sequence data for a number of DNA regions (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001a, b, Phillips et al 2002, Slippers et al 2003). These data have, however, not always been sufficient to distinguish boundaries between closely related or cryptic species. In such cases, multiple gene genealogies have been used (De Wet et al 2003, Slippers et al 2003). These molecular data, combined with morphological and ecological data, allow for robust identification of *Botryosphaeria* spp.

Pennycook and Samuels (1985) described an anamorph of a *Botryosphaeria* species, *Fusicoccum luteum* Pennycook & Samuels, from New Zealand. This species could most easily be distinguished from other Botryosphaeriaceous fungi from *Malus* sp., *Populus* sp. and *Actinidia deliciosa* by a yellow pigment produced in young cultures. The teleomorph was unknown. A few years later, Phillips et al (2002) also noticed a yellow pigment in cultures derived from *Botryosphaeria* ascomata on *Vitis vinifera* in Portugal. Using ribosomal DNA (rDNA) sequence and SSCP, RAPD and morphological data, the Portugese fungus was shown to be similar to *F. luteum*, for which the teleomorph, *B. lutea* A.J.L. Phillips, was described. *Botryosphaeria lutea*, thus, seems to be a more important pathogen of fruit and forestry crops than was previously recognized. This pathogen has evidently also been mistaken for *B. dothidea* (Fr.: Moug.) Ces. & De Not. in the past (Phillips et al 2002).

*Botryosphaeria rhodina* (Berk. & Curt.) Arx and *B. dothidea* have both been reported from Australian native *Acacia* spp., where these trees are planted as exotics (Roux 1998, Roux et al 2001). However, during a recent survey of *Botryosphaeria* spp. on native Australian flora, some cultures from an *Acacia* sp. produced a yellow pigment in culture, similar to that described for *B. lutea*. Initial ITS rDNA sequence data confirmed a close relationship with *B. lutea*, but some sequence divergence was also obvious. The aim of this study was, therefore, to determine the relationship between these isolates from *Acacia* in Australia and *B. lutea* isolates from New Zealand and Portugal. In order to evaluate the phylogenetic significance of the sequence variation seen in the ITS region, sequence data from three gene regions (ITS rDNA, β-tubulin and Elongation factor 1α (EF-1α)) were compared. Teleomorph, anamorph and cultural characters were also considered.

## MATERIALS AND METHODS

*Isolates*.--Thirteen isolates of a *Botryosphaeria* species resembling *B. lutea* and its anamorph *F. luteum*, were collected from diseased or dying stems of *Acacia* spp. in Australia. Collections were made by J Roux from *A. mearnsii* during 1999 and by the senior author during 2001 (TABLE I). Four similar isolates were also obtained from samples of diseased *Sequoiadendron gigantea* growing as an exotic in Canberra (TABLE I). Two isolates of *B. lutea* (one ex-type) were provided by AJL Phillips (CAP002, CAP037) (TABLE I). Two isolates originating from the original description of *F. luteum* (one ex-type) by Pennycook and Samuels (1985) were also included (TABLE I). Isolates were maintained on malt yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, S.A.) at 25°C in the dark or under near UV light. Isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

*Molecular phylogenetic characterization*.--A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA as described in Raeder and Broda (1985), and Smith et al (2001). Four isolates from *Acacia* and *Sequoiadendron* from Australia were used in phylogenetic comparisons with two isolates from each of the studies of Pennycook and Samuels (1985) and Phillips et al (2002), including the ex-type cultures of *B. lutea* and *F. luteum* from these studies (TABLE I). DNA sequences of other *Botryosphaeria* spp. that were included in the analysis for comparative purposes were from Slippers et al (2003) (TABLE I). Furthermore, Smith and Stanosz (2001) and Denman et al (2003) used ITS rDNA sequence comparisons to identify *B. lutea* or *F. luteum* isolates from native *Banksia* spp., *Eucalyptus marginata* and a *Protea* sp. in Australia and South Africa. ITS sequences from these studies were obtained from GenBank and compared with the isolates examined in the present study (TABLE I).

Three housekeeping gene sequences were used for phylogenetic comparisons between isolates. The region spanning the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene, was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTA

TTGATATGC) (White et al 1990). A region of the β-tubulin gene was amplified using the primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5' ACCCTCA GTGTAGTGACCCTTGGC) (Glass and Donaldson 1995). In addition, a part of the elongation factor 1-α was amplified using the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTGAAGGAACCCT ACC) (Carbone et al 1999). PCR reaction mixtures, PCR conditions and visualization of amplicons are as described by Slippers et al (2003). The amplicons of all three DNA regions were also cleaned and sequenced as described by Slippers et al (2003), using the same primers used to generate the amplicons.

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. A partition homogeneity test was done to determine whether the datasets could be combined (Farris et al 1995, Huelsenbeck et al 1996). Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were determined. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), with heuristic searches of only informative characters and tree bisection and reconstruction (TBR) as branch swapping algorithm (random stepwise addition). Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985) and decay analysis of the branch nodes using Autodecay (Eriksson 1998). Phylogenetic species hypotheses were also tested using distance analyses with the Neighbor Joining algorithm, using both an uncorrected p-factor and HKY85 parameters alternatively in PAUP.

To test the consistency of branches in the combined dataset, the three partial gene sequence datasets were also analyzed separately, but in the same way as described above. The ITS rDNA dataset was also expanded and analyzed separately to include isolates for which only ITS rDNA data were available from GenBank.

*Morphological characterization*.--All isolates were grown on sterilized pine needles that were placed on water agar (WA) (2% agar; Biolab Midrand, Johannesburg, S.A.) at 25°C under near UV light, to promote sporulation. Fruiting structures from *in vivo* and *in vitro* collections were sectioned with an American Optical Freezing Microtome or by hand, and mounted in clear lactophenol. Morphological observations and photographs were done on an Axiocam digital camera (Carl Zeiss, Germany). The morphology of these samples was compared with material from of *B. lutea* [LISE94070 (holotype) and LISE94073] and *F. luteum* (PDD45400).

Growth rate, colony morphology and color (Rayner 1970) of isolates obtained during this study, as well as of ex-type isolates of *B. lutea* and *F. luteum*, were determined. Two isolates of each species were incubated on potato dextrose agar (PDA) (0.4% potato extract, 2% dextrose, 1.5% agar; Biolab, Midrand, Johannesburg, S.A.) at 5 C intervals ranging from 10 to 35 C in the dark. Growth rate was measured at the leading edge in 24 hr intervals. The experiment was repeated for the new species described below.

## RESULTS

*Molecular phylogenetic characterization.*--PCR products of approximately 580 bp (ITS rDNA), 450 bp (β-tubulin) and 300 bp (EF1-α) were amplified for all the isolates. Sequence data at the 5' and 3' ends were deleted from the dataset if they were doubtful. The partition homogeneity test of the ITS-rDNA, β-tubulin and EF1-α data sets, indicated that they could be combined (P value = 0.53). The total data set consisted of 1324 characters after alignment. A seven base pair region in the EF1-α region was repeated twice in most species. Isolates of *B. ribis* contained two extra of these repeats, which were coded to represent only two evolutionary events and not fourteen as was the case before the coding. Of the remaining characters, 322 were parsimony-informative and used in the analyses. This combined data set contained significant phylogenetic signal compared to random trees ($P < 0.01$; g1 = -0.97) (Hillis and Huelsenbeck 1992). After heuristic searches in PAUP, 3 most parsimonious trees of 540 steps were retained (CI = 0.843; RI = 0.909) (FIG. 1). Trees obtained using distance methods were the same as those obtained using parsimony.

Based on the combined analysis of the sequence data from the three gene regions, *B. lutea* and the *Botryosphaeria* sp. from *Acacia* and *Sequoiadendron* in Australia grouped into two distinct clades (FIG. 1). Although the branches separating these two clades were short, compared to the branches separating other well-defined species in the analysis, they were strongly supported (d5/96% and d5/100% decay values and bootstrap support). The separation was also supported in the individual analyses of the sequence datasets of the three gene regions (analyses done using the same parameters as for the combined dataset) (FIG. 2A-C). Analysis of the sequences between these two groups showed that at the alleles were fixed in the two groups at 14 of the 15 polymorphic loci (TABLE II). These data also showed a significant bias towards transitions (only one transversion recorded) in these polymorphic sites. This factor is considered in the distance analysis (HKY85 parameters) and does not change the interpretation of the results. Among the isolates studied here, these two clades are considered phylogenetically separate and the fungus from Australia is described as new.

GenBank sequences of the ITS rDNA region from isolates that were previously identified as *B. lutea* or *F. luteum* (Smith and Stanosz 2001, Denman et al 2003), separated into both the *B. lutea* and *B. australis* clades, although the bootstrap values for both groups were low (63% and 73% respectively) (FIG. 3). Sequences from *Actinidia* (New Zealand) and *Buckinghamia* (Australia) grouped with *B. lutea*. Isolates from Australian native hosts including *Eucalyptus* and *Banksia*, and from a South African native *Protea,* grouped with the newly identified *Botryosphaeria* sp. Three isolates from *Banksia* in Australia (Denman et al 2003) resided in a sister group to the *B. lutea* and *B. australis* clades.

Isolates residing in the clades representing *B. lutea* and the new species described in this study were more closely related to each other (d14/100% bootstrap) than to any other taxon included in the analysis. These two species were also more closely related to *B. ribis*, *B. parva* and *B. eucalyptorum* (d28/100% bootstrap) than to *B. dothidea.* All these species have *Fusicoccum* anamorphs and group together (100% bootstrap), as opposed to isolates of *B. obtusa*, *B. stevensii* and *B. rhodina*, which all have *Diplodia* or *Lasiodiplodia* anamorphs. DNA sequence of the latter species were used as outgroup taxa in the analyses.

*Morphological characterization.*--The isolates from *Acacia* spp. and *Sequoiadendron giganteum* produced anamorph structures on pine needles on WA within two to three weeks. Teleomorph and anamorph structures of this fungus from field samples and from conidia formed in culture were very similar in morphology to those of *B. lutea* and *F. luteum* (FIGS. 4-12). This new species also produced a yellowish pigment in young cultures, as was true for *B. lutea*. The fungus from *Acacia* and *Sequoiadendron* could, however, be distinguished from *B. lutea* and *F. luteum* by its longer conidia with a higher l/w ratio (TABLE III). Isolates of *B. lutea* also produce a much brighter yellow pigment in culture than the Australian isolates, especially at 25 C and higher. Unlike *B. lutea*, no yellow pigment was produced between 25--30 C by the Australian isolates.

## TAXONOMY

The fungus collected and isolated from *Acacia* spp. and *S. giganteum* in Australia is phylogenetically and morphologically distinct from *B. lutea*. The currently known host ranges of these taxa also do not overlap. The fungus is thus described as new as follows:

***Botryosphaeria australis*** Slippers, Crous & M.J. Wingf., sp. nov. FIGS. 4-14

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

*Etymology*. Referring to the origin in the southern hemisphere.

Ascostromata per corticem erumpentia, 1.2 mm diametro. Ascomata pseudothecialia, 2--10 botyroide aggregata, interdum solitaria, globosa ostiolo centrale, papillata vel glabra, inclusa cum solum papillis emergentibus usque ad 2/3 emergentia, nigra, 100--300 µm; paries pseudothecii e 5--8 stratis texturae angularis, extus e cellulis atrobrunneis vel brunneis composita, intus e cellulis hyalinis revestimentum loculi facientes. Asci bitunicati, clavati, 60--125 x 16--25 µm, inter pseudpoaraphyses multas, filiformes, septatas, raro apicem versus ramosas, 3--4 µm latas. Ascosporae fusoideae vel ovoideae, 20--23(--25) x 7--8(--9) µm, unicellulares, hyalinae, laeves, contentis granularibus, in asco biseriatae. Conidiomata (in "WA" in acis pinorum sterilifactis in 7--21 diebus formata) pycnidialia, superficialia, globosa, plerumque solitaria mycelio tectaque. Conidia fusiformia, basibus subtruncatis vel obtuse rotundatis, (18--)23--26(--30) x 5--6(--7.5) µm, hyalina, unicellularia raro septum ante germinationem facientia, laevia contentis granularibus. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 10--14 x 2--3 µm, percurrenter proliferantes cum 1--2 proliferationibus, inspissatione periclinali. Spermatia non visa.

*Ascostromata* erumpent through the bark, 1.2 mm diam. *Ascomata* pseudothecial, forming botryose aggregates of 2--10, sometimes solitary; globose with a central ostiole, papillate or not, imbedded with only papilla emerging up to 2/3 emergent, black, 100--300 µm; pseudothecial wall comprising 5--8 layers of *textura angularis*, outer region of dark brown or brown cells, inner region 3--6 layers of hyaline cells lining the locules. *Asci* bitunicate, clavate, 60--125 x 16--25 µm, 8-spored, between numerous filiform, septate pseudoparaphyses, rarely branched towards the tips, 3--4 μm wide. *Ascospores* fusoid to ovoid, 20--23(--25) x 7--8(--9) μm (average of 50 ascospores 21.9 x 7.6 μm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* (formed on WA on sterilized twigs within 7--21 d) pycnidial, superficial, globose, mostly solitary and covered by mycelium. *Conidia* fusiform, base subtruncate to bluntly rounded, (18--)23--26(--30) x 5--6(--7.5) μm (average of 240 conidia 24.7 x 5.1 μm, l/w 4.8), hyaline, unicellular, rarely forming a septum before germination, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 10--14 x 2--3 μm, phialidic with periclinal thickening, or proliferating percurrently with 1--4 proliferations. *Spermatia* not observed. *Cultures* having buff (19''f) to light primrose (23''b) colonies, light yellowish pigment most noticeable between 15--20 C in the dark, becoming olivaceous buff (21'''d) to olivaceous gray (21''''i) after 5--6 days, with a sparse to moderately dense, appressed mycelial mat in center with sparse tufts of aerial mycelium around edges, margin smooth. Optimum temperature for growth 25 C, colony reaching 48 mm in diam on PDA after 4 days at 25 C in the dark.

*Specimens examined.* AUSTRALIA. VICTORIA: Batemans Bay, *Acacia* sp., *M.J. Wingfield* (HOLOTYPE PREM57589) (culture CMW6838); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57590); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57592); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57593); ACT: Canberra, *Sequoiadendron* sp., *M.J. Wingfield* (PREM57594); Canberra, *Sequoiadendron* sp., *M.J. Wingfield* (PREM57595)

## DISCUSSION

In this study *Botryosphaeria australis* and its anamorph, *F. australis*, are newly described. This fungus is phylogenetically closely related to *B. lutea.* The branches of the clades representing these two species are short subdivisions of a deeply branched and well-defined clade. This indicates a relatively recent speciation event.

The genetic and subsequent taxonomic separation of the closely related species, *B. lutea* and *B. australis*, was confirmed using the phylogenetic species concept. Sequence variation within the ITS, β-tubulin and EF1-α regions were small, and were considered insignificant if compared to the divergence between other well defined *Botryosphaeria* spp. The alleles were, however, distributed in the same manner, or fixed for the two groups, in each of the three gene regions. These fixed alleles over multiple gene regions indicate a barrier to genetic exchange and are considered indicative of phylogenetic species (Taylor et al 2000). This phylogenetic separation confirmed the taxonomic value of small but distinct phenotypic variation that would otherwise have been overlooked. The combination of these DNA based and phenotypic data are considered sufficiently robust evidence to treat these fungi as separate taxa.

The distinction between *B. lutea* and *B. australis* was not recognized in previous studies based only on ITS rDNA sequence data (Smith and Stanosz 2001, Zhou and Stanosz 2001a, Denman et al 2003). This was due to the small ITS sequence divergence between them. The fact that we had access to a much larger collection of isolates also facilitated the discovery of the new taxon. ITS data alone can thus obscure the true diversity in *Botryosphaeria*. This is similar to *B. ribis* and *B. parva* (Slippers et al 2003), or *Diplodia pinea* (Desm.) Kickx. (= *S. sapinea*) and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (De Wet et al 2003), that were considered to represent single species based on their ITS data alone. Multiple gene genealogies, however, showed that these species are phylogenetically distinct (De Wet et al 2003, Slippers et al 2003). These case studies provide good evidence to show that single gene genealogies are insufficient to distinguish cryptic *Botryosphaeria* spp.

Internal transcribed spacer sequences provided by Smith and Stanosz (2001) and Zhou and Stanosz (2001a) for one isolate from a *Eucalyptus* sp. and one from a *Banksia* sp. were included in this study. Comparisons show that these isolates, previously treated as *B. lutea,* actually represent *B. australis*. In the studies of Smith and Stanosz (2001) and Zhou and Stanosz (2001a), *B. australis* isolates could, however, not be separated from ex-type isolates of *F. luteum* based on RAPD data. These authors were also not able to separate two other cryptic *Botryosphaeria* sp., namely *B. ribis* and *B. parva* using RAPD data. Another similar example where RAPD data have been insufficient to define species, has been with the three recognized "morphotypes" of *D. pinea*. These three groups are distinguished using RAPD data, but correspond to only two phylogenetic species (De Wet et al 2000, 2003).

*Botryosphaeria lutea* and *B. australis* cannot be distinguished from each other based on teleomorph fruiting structures or ascospores. The conidial dimensions are equally misleading, because they are similar in form and general appearance, and their length and width dimensions overlap. This is not uncommon among *Fusicoccum* spp. Pennycook and Samuels (1985), Phillips et al (2002) and Slippers et al (2003) have all reported significant overlap in the sizes of *Fusicoccum* spp. In these cases and in the present study the species could, however, be separated when averages of conidial sizes and septation were considered. Thus, on average, conidia of *F. australis* are longer and appear more slender (higher length/width ratio) than those *F. luteum*.

Culture morphology was useful to distinguish between isolates of *B. lutea* and *B. australis*. *Botryosphaeria australis* produced a distinctly lighter and duller (more cream than yellow) yellow pigment in young cultures than *B. lutea*. In the description of *F. luteum* (Pennycook and Samuels 1985) and *B. lutea* (Phillips et al 2002), the production of a yellow pigment in culture was the easiest way to distinguish this taxon from other species. Prior to these studies, this pigment was not considered taxonomically useful (Witcher and Clayton 1963). Recent studies of other botryosphaeriaceous fungi have also recognized the value of culture morphology as a useful tool to distinguish *Botryosphaeria* spp. (Jacobs 2002, De Wet et al 2003, Slippers et al 2003).

*Botryosphaeria australis* differs in morphology and etiology from other botryosphaeriaceous fungi described from native Australian *Acacia* spp., *Banksia* spp. and *Eucalyptus* spp. Hansford (1954) described *B. banksiae* Hansford from *Banksia marginata* from Australia. The ascospores of this species are, however, one-seriate in the ascus and significantly different in size (17--20 x 13--15 µm). *Botryosphaeria acaciae* (Hansford) Dingley (= *Physalospora acaciae* Hansford) causes galls and cankers on *Acacia* spp. in Australia and New Zealand (Hansford 1954, Dingley 1970). Both of the latter studies, however, report that the teleomorph structures and galls are also associated with a *Diplodia* sp. Dingley (1970) reports 'small papilla on one end' of the ascospores, which is absent in *B. australis*. This disease was also described from Australian *Acacia* by Scurfield (1966), who identified the causal agent as a fungus in the Sphaeriaceae. Based on the differences in anamorph, the lack of an association with a gall-forming disease and differences in ascospore morphology, we are confident that *B. australis* is not the same fungus as either of those mentioned above.

*Botryosphaeria australis* appears to be native to the southern hemisphere, and most likely Australia. This hypothesis is based on the current known host and geographic distribution of this taxon. *Botryosphaeria australis* was the only *Botryosphaeria* sp. found on native species of *Acacia*, in the different areas and over the two seasons that our collections were made. Isolates from previous studies are also identified here as *B. australis*, e.g. from *E. marginata* and *Banksia caleyi* in Australia, and *Protea cynaroides* in South Africa (Smith and Stanosz 2001, Zhou and Stanosz

2001a, Denman et al 2003). Only one isolate from outside of these regions grouped with *B. australis* and this was from *Pistacia* in Italy. Smith and Stanosz (2001) identified *F. luteum* from the Australian native hosts, *Banksia*, *Jacksonia horrida*, *Isopogon tribolus*, *Dryandra tenuifolia* and a *Leucopogon* sp. These identifications were, however, based only on RAPD data, which does not separate *B. lutea* and *B. australis*. We are thus of the opinion that the exotic *S. giganteum* trees in Australia have become infected with a native pathogen.

*Botryosphaeria lutea* is common throughout the Australasian region and in Portugal, but its origin is unknown. All reports from New Zealand have been from introduced hosts. Pennycook and Samuels (1985) collected this fungus from *Malus*, *Populus* and *Actinidia* species in New Zealand. Hartill (1991) also reported it from avocado from this area. The isolates from a native Australian *Buckinghamia* sp. (Denman et al 2003), however, also group with *B. lutea*. Similarly, in Portugal *B. lutea* occurs on introduced (*Vitis* and *Sophora japonica*) and indigenous (*Fraxinus angustifolia*) hosts (Phillips et al 2002). This fungus, thus, occurs on native and exotic hosts in both Europe and Australasia, which makes it difficult to predict its natural range.

*Botryosphaeria. australis* and *B. lutea* have been moved between the northern- and southern hemispheres, most likely on germplasm of commercially valuable species. This observation is based on the pattern of distribution and host ranges revealed in this study. It is also clear from the current study that both these fungi can infect native and introduced or cultivated hosts, in both regions. These introductions can, thus, have significant implications for agricultural and forestry industries and conservation of native flora. Population level studies are needed to find the areas of greatest diversity and understand patterns of gene flow between populations of these two fungi. Such information will help to assess the current threat of these pathogens and help curtail their continued spread.

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TABLE I. Isolates considered in the phylogenetic study.

| **Culture no.**[1] | **Other no.**[1] | **Identity** | **Host** | **Location** | **Collector** |
|---|---|---|---|---|---|
| CMW7772 | | *Botryosphaeria ribis* | *Ribes* sp. | New York, USA | B. Slippers / G. Hudler |
| [2]CMW7054 | CBS121 | *B. ribis* | *R. rubrum* | New York, USA | N.E. Stevens |
| CMW9078 | ICMP7925 | *B. parva* | *Actinidia deliciosa* | New Zealand | S.R. Pennycook |
| CMW9081 | ICMP8003 | *B. parva* | *Populus nigra* | New Zealand | G.J. Samuels |
| [2]CMW10125 | BOT24 | *B. eucalyptorum* | *Eucalyptus grandis* | Mpumalanga, S Africa | H. Smith |
| CMW11705 | | *B. eucalyptorum* | *E. nitens* | S Africa | B. Slippers |
| [2]CMW992/3 | KJ93.52 | *B. lutea* | *A. deliciosa* | New Zealand | G.J. Samuels |
| CMW9076 | ICMP7818 | *B. lutea* | *Malus X domestica* | New Zealand | S.R. Pennycook |
| CMW10309 | CAP0002 | *B. lutea* | *Vitis vinifera* | Portugal | A.J.L. Phillips |
| CMW10310 | CAP037 | *B. lutea* | *V. vinifera* | Portugal | A.J.L. Phillips |
| CMW9072 | | *B. australis* | *Acacia* sp. | Melbourne, Australia | J. Roux / D. Guest |
| CMW9073 | | *B. australis* | *Acacia* sp. | Melbourne, Australia | J. Roux / D. Guest |
| CMW6837 | | *B. australis* | *Acacia* sp. | Batemans Bay, Australia | M.J. Wingfield |
| CMW6853 | | *B. australis* | *Sequoiadendron giganteum* | Canberra, Australia | M.J. Wingfield |
| CMW9075 | | *B. dothidea* | *Populus* sp. | New Zealand | G.J. Samuels |
| CMW8000 | | *B. dothidea* | *Prunus* sp. | Crocifisso, Switzerland | B. Slippers |
| CMW7060 | CBS 431 | *B. stevensii* | *Fraxinus excelsior* | Netherlands | H.A. van der Aa |
| CMW7774 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers / G. Hudler |
| CMW10130 | BOT977 | *B. rhodina* | *Vitex* sp. | Uganda | J. Roux |

[1] Designation of isolates and culture collections: CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998).

[2] ITS sequences for these isolates were obtained from GenBank (from the top down: AF241177, AF283686 and AF027745)

TABLE II. Polymorphic nucleotides (or alleles) from sequence data of three gene regions for isolates of *Botryosphaeria lutea* and *B. australis*. Positions of polymorphisms are as in the combined and aligned dataset. Alleles that are fixed in these species are indicated in upper case.

| **Species** | **Isolate number** | **β-tubulin** | | | **ITS** | | | | **EF1-α** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 40 | 98 | 270 | 584 | 873 | 878 | 958 | 1032 | 1112 | 1226 | 1246 | 1253 | 1283 | 1292 | 1323 |
| *B. lutea* | CMW9076 | T | C | G | G | T | T | C | a | C | - | C | A | - | - | C |
| | CMW992 | T | C | G | G | T | T | C | a | C | - | C | A | - | - | C |
| | CMW10309 | T | C | G | G | T | T | C | g | C | - | C | A | - | - | C |
| | CMW10310 | T | C | G | G | T | T | C | g | C | - | C | A | - | - | C |
| *B. australis* | CMW9072 | C | T | A | - | C | C | T | g | T | T | A | G | A | T | T |
| | CMW9073 | C | T | A | - | C | C | T | g | T | T | A | G | A | T | T |
| | CMW6837 | C | T | A | - | C | C | T | g | T | T | A | G | A | T | T |
| | CMW6853 | C | T | A | - | C | C | T | g | T | T | A | G | A | T | T |

TABLE III. Measurements of ascospores and conidia of *Botryosphaeria lutea* and *B. australis* and their *Fusicoccum* anamorphs.

| **Identity** | **Ascospore size (μm)** | **Conidial size[1] (μm)** | **L/W** | **Source of data** |
|---|---|---|---|---|
| *F. luteum* | Not seen | (14--)20--24(--32) x (5--)6--7(--9) [Ave. 21.7 x 6.7] – *in vitro* | 3.2 | Pennycook and Samuels 1985 |
| *B. lutea* / *F. luteum* | 18--22.5(--24) x 7.5--12 | (12--)16.5--22.5(--24) x 4.5--6(--7.5) [Ave. 17.2 x 4.5--6] – *in vivo* | 2.8 | Phillips et al 2002 |
| | | (15--)18--22.5(--24) x 4.5--6(--7.5) [Ave. 19.7 x 5.6] – *in vitro* | 3.6 | |
| *B. australis* / *F. australe* | 20--22(--23.5) x 7--8 | (17.5--)24.5(--30) x 5(--7.5) – *in vitro* | 4.8 | This study |

[1] *In vitro* (in culture) and *in vivo* (field collected samples) conidial measurements are given separately, as they differ from each other.

FIG. 1. One of the most parsimonious trees obtained through heuristic searches of the combined dataset of the ITS rDNA, β-tubulin and EF1-α regions. Support for branches and nodes are indicated as bootstrap values (1000 replicates) below and decay values above the branches, respectively. The *Botryosphaeria* spp. with *Diplodia*-like anamorphs (*B. rhodina, B. obtusa* and *B. stevensii*) are treated as outgroup taxa to which the tree is rooted. The remaining *Botryosphaeria* spp. that form the ingroup all have *Fusicoccum* anamorphs. Isolates' number, host and origin (Aust = Australia, Neth = Netherlands, NY = New York, USA, NZ = New Zealand, SA = South Africa, Swit = Switzerland, Ug = Uganda) are indicated, as well as the taxonomic identities of the clades.

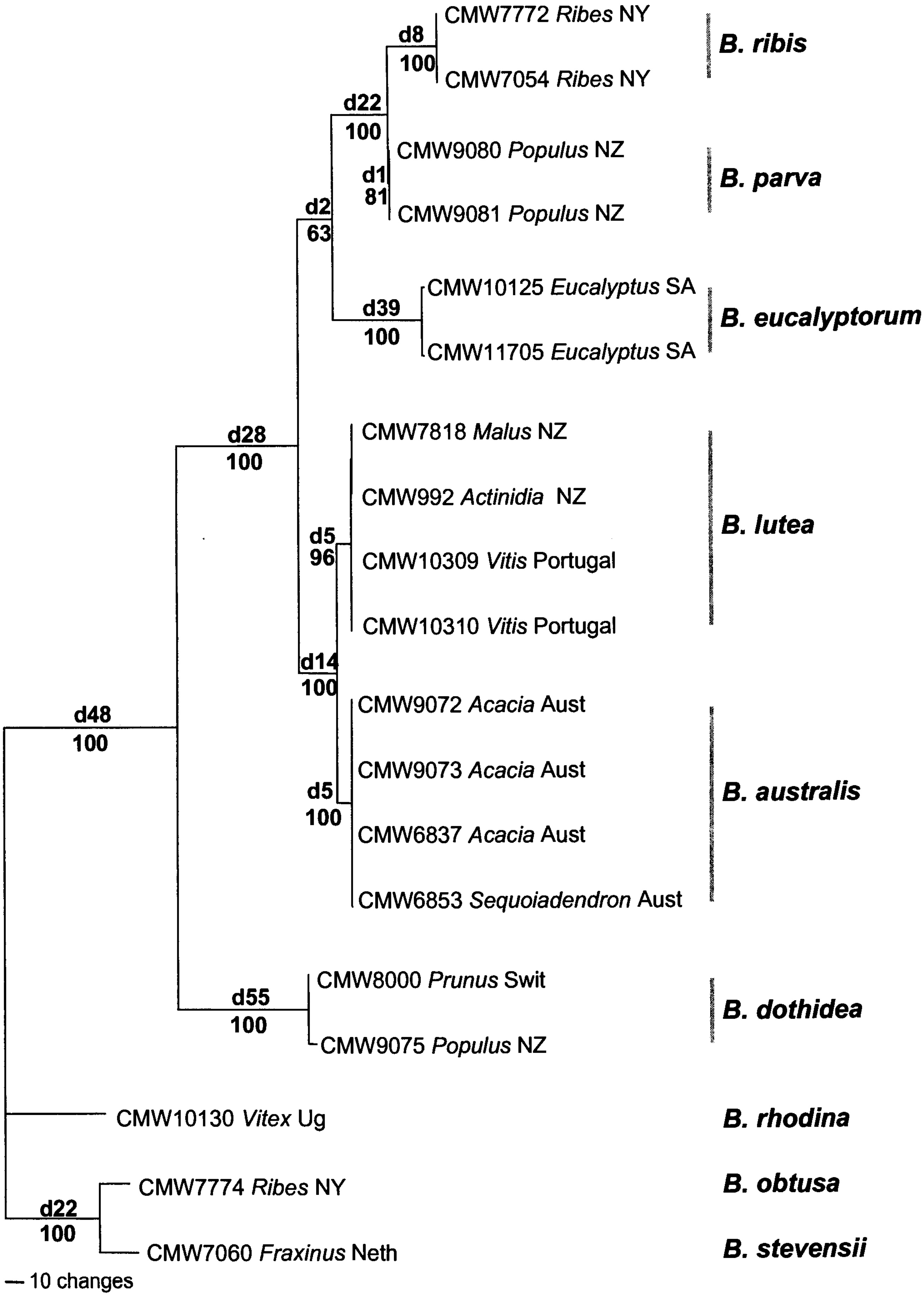

CMW7772 *Ribes* NY
CMW7054 *Ribes* NY
d8
100
d22
100
CMW9080 *Populus* NZ
CMW9081 *Populus* NZ
d1
81
d2
63
CMW10125 *Eucalyptus* SA
CMW11705 *Eucalyptus* SA
d39
100
d28
100
CMW7818 *Malus* NZ
CMW992 *Actinidia* NZ
CMW10309 *Vitis* Portugal
CMW10310 *Vitis* Portugal
d5
96
d14
100
CMW9072 *Acacia* Aust
CMW9073 *Acacia* Aust
CMW6837 *Acacia* Aust
CMW6853 *Sequoiadendron* Aust
d5
100
d48
100
CMW8000 *Prunus* Swit
CMW9075 *Populus* NZ
d55
100
CMW10130 *Vitex* Ug
CMW7774 *Ribes* NY
CMW7060 *Fraxinus* Neth
d22
100
— 10 changes
*B. ribis*
*B. parva*
*B. eucalyptorum*
*B. lutea*
*B. australis*
*B. dothidea*
*B. rhodina*
*B. obtusa*
*B. stevensii*

FIG. 2. Representative most parsimonious (MP) trees of individual analyses of (A) the ITS-rDNA, (B) *β*-tubulin and (C) EF1-α regions. To avoid repetition, only the branches carrying isolates of the species in question in this study, *Botryosphaeria lutea* and *B. australis*, are shown. The values of tree length, phylogenetic signal (g1), consistency index (CI) and retention index (RI) are given for each tree. The lengths of the branches are indicated above the branches and the bootstrap values (1000 replicates) below the branches.

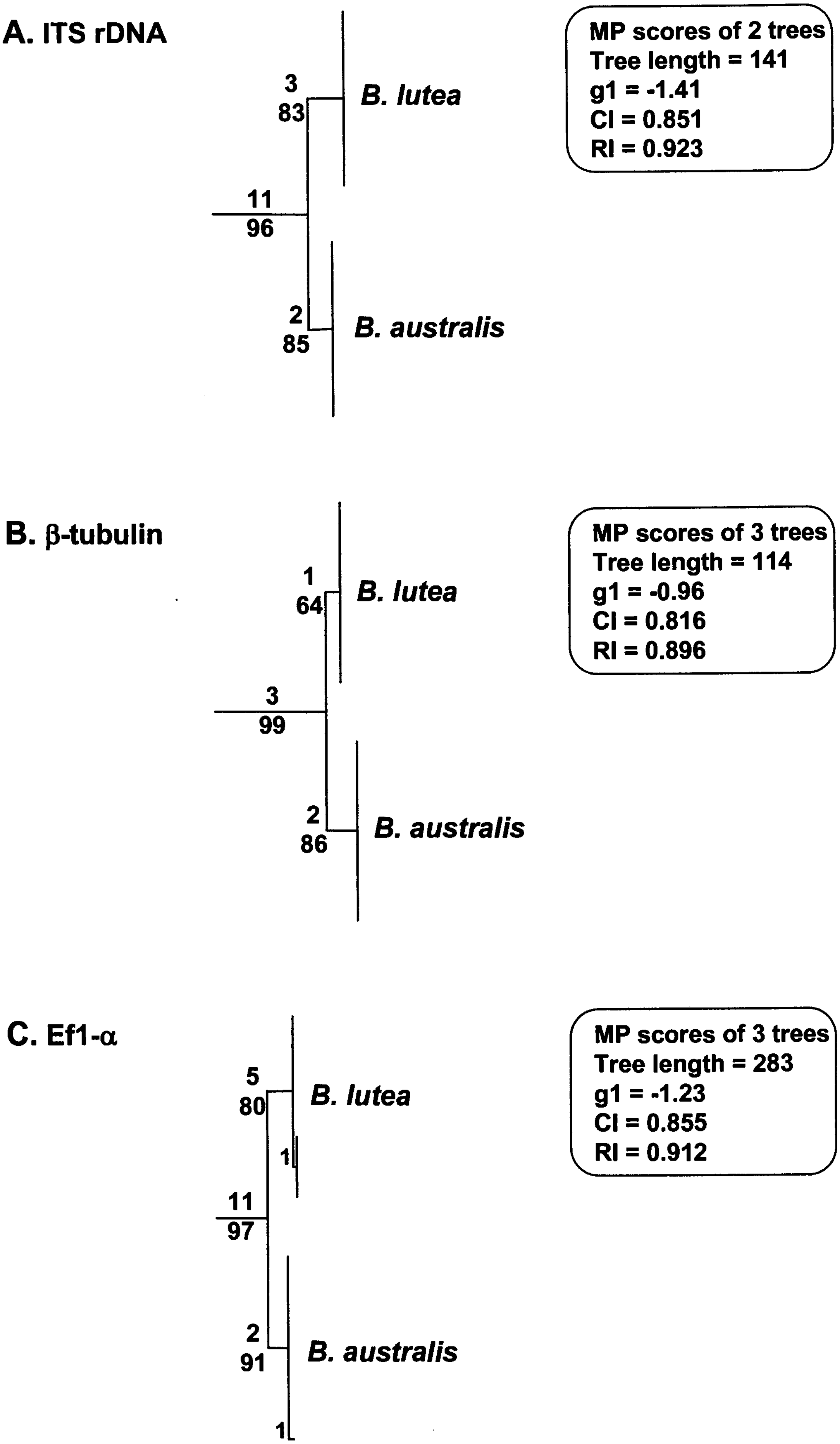

A. ITS rDNA
3
83
B. lutea
11
96
2
85
B. australis
MP scores of 2 trees
Tree length = 141
g1 = -1.41
CI = 0.851
RI = 0.923
B. β-tubulin
1
64
B. lutea
3
99
2
86
B. australis
MP scores of 3 trees
Tree length = 114
g1 = -0.96
CI = 0.816
RI = 0.896
C. Ef1-α
5
80
B. lutea
1
11
97
2
91
B. australis
1
MP scores of 3 trees
Tree length = 283
g1 = -1.23
CI = 0.855
RI = 0.912

FIG. 3. The representative branch of the *Botryosphaeria lutea* and *B. australis* clade identified using parsimony and based on ITS-rDNA sequence data. This branch contains all available sequences from this study and GenBank that group with these taxa. The tree length, phylogenetic signal (g1), consistency index (CI) and retention index (RI) are given for the tree. The lengths of the branches are indicated above the branches and the bootstrap values (1000 replicates) below the branches. GenBank sequence- and isolate numbers, host and origin (Aust = Australia, Ital = Italy, NZ = New Zealand, Port = Portugal, SA = South Africa) are indicated, as well as the taxonomic identities of the clades.

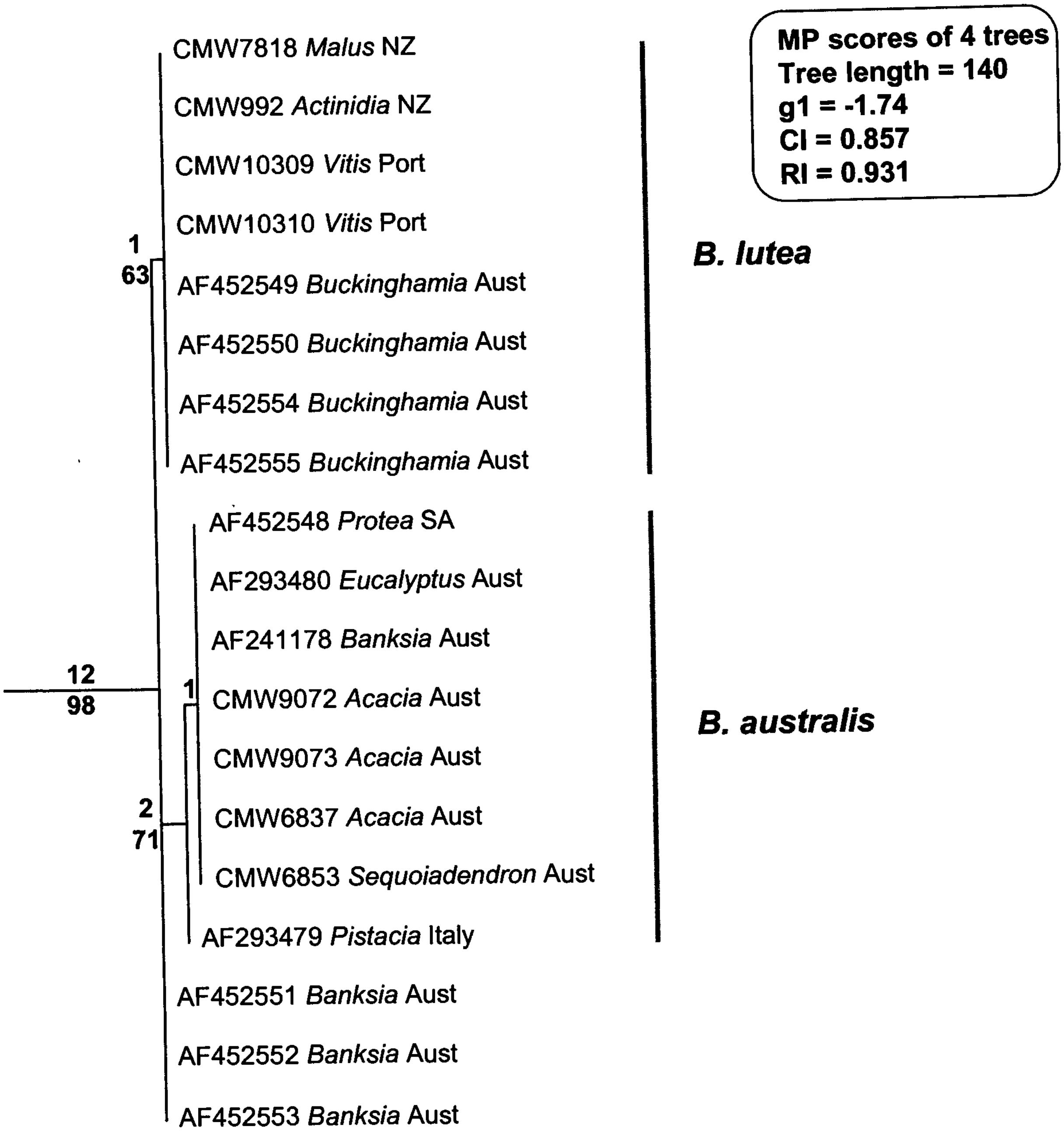

MP scores of 4 trees
Tree length = 140
g1 = -1.74
CI = 0.857
RI = 0.931
CMW7818 Malus NZ
CMW992 Actinidia NZ
CMW10309 Vitis Port
CMW10310 Vitis Port
AF452549 Buckinghamia Aust
AF452550 Buckinghamia Aust
AF452554 Buckinghamia Aust
AF452555 Buckinghamia Aust
AF452548 Protea SA
AF293480 Eucalyptus Aust
AF241178 Banksia Aust
CMW9072 Acacia Aust
CMW9073 Acacia Aust
CMW6837 Acacia Aust
CMW6853 Sequoiadendron Aust
AF293479 Pistacia Italy
AF452551 Banksia Aust
AF452552 Banksia Aust
AF452553 Banksia Aust
1
63
12
98
1
2
71
B. lutea
B. australis

FIG. 4. Asci, ascospores and pseudoparaphyses of *Botryosphaeria australis*. Bar = 10 µm. (Sketch by PW Crous).

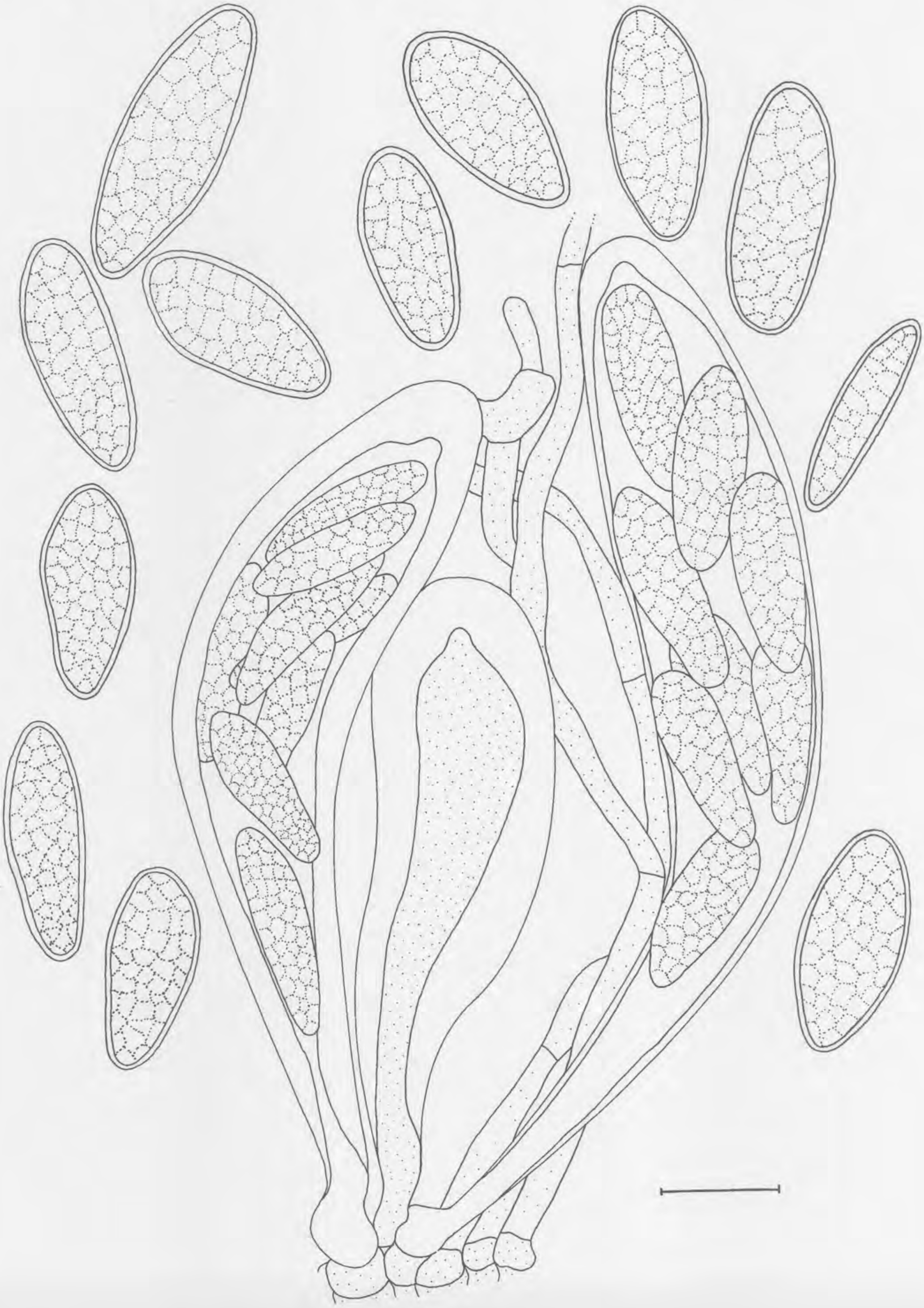
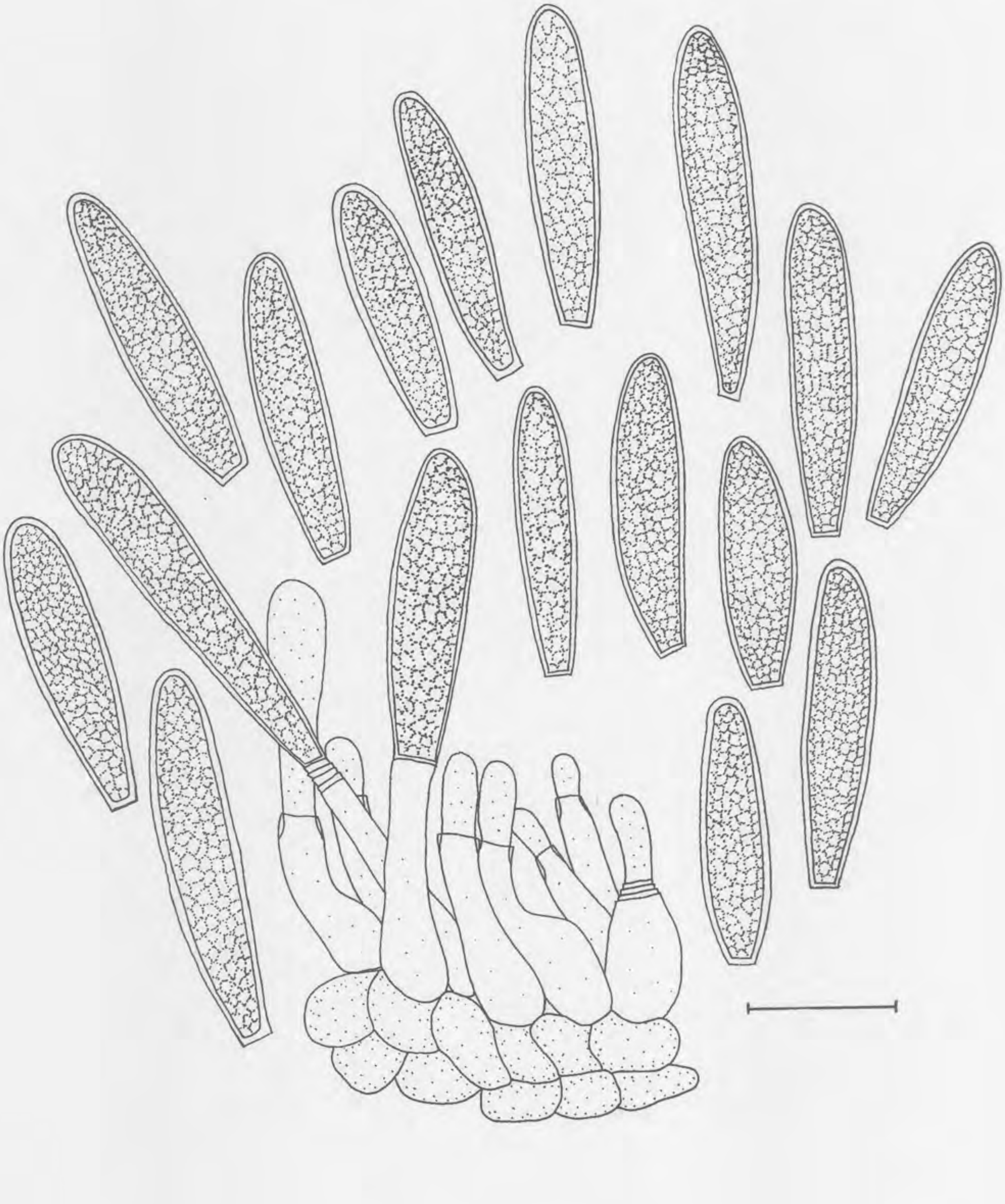
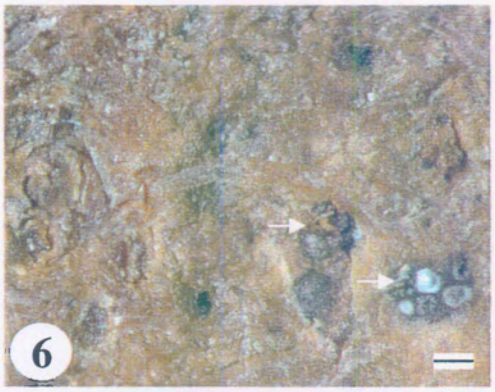


FIG. 5. Conidia and conidiogenous cells of *Fusicoccum australe.* Bar = 10 μm. (Sketch by PW Crous).



FIGS. 6-14. *Botryosphaeria australis* dissecting microscope and DIC compound-microscope micrographs. 6. Ascomata in botryose clusters that erupt through the bark. When dissected, the contents are conspicuously white (arrows). 7, 8. Sections through ascomata and ascomatal neck. Bars = 100 µm. 9. Bitunicate asci and immature ascospores. 10, 11. Mature ascospores. 12, 13. Conidiogenous cells with periclinal thickening and percurrent proliferation (arrows). 14. Fusiform conidia. Bars = 10 µm.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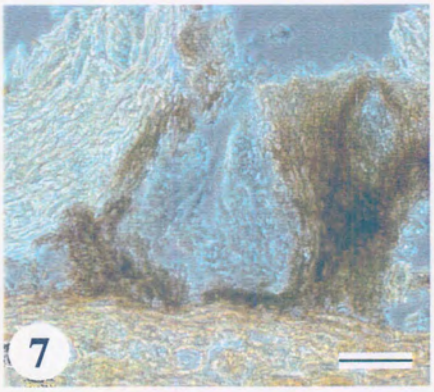
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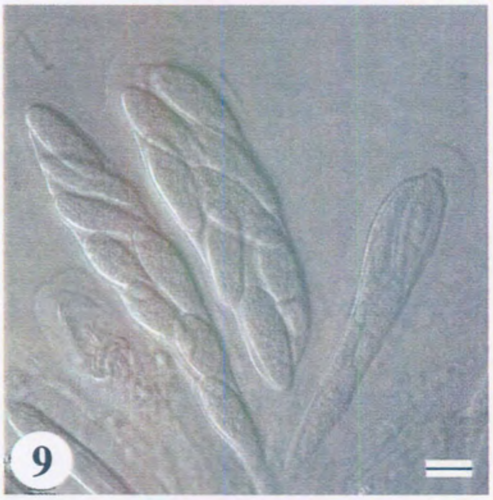
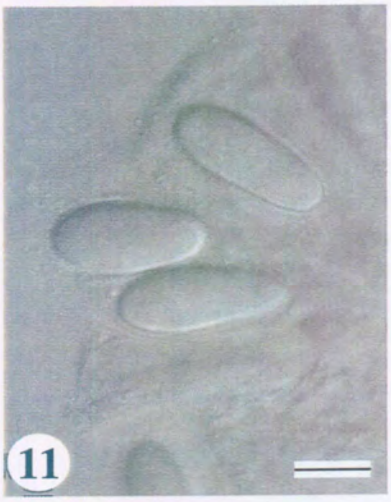
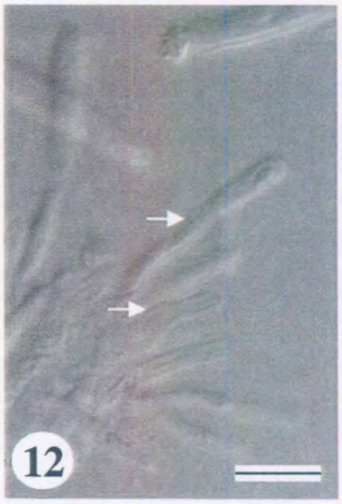
8


9


10


11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13


14

# 4

# Phylogenetic comparison of *Botryosphaeria* isolates from pome and stone fruit trees in South Africa and other regions of the world

***Abstract:*** *Botryosphaeria* spp. are recognized pathogens of pome and stone fruit trees in South Africa. The taxonomy of this group of fungi is, however, confused because morphological features are variable and overlap between species. The aim of this study was to determine the identity of the *Botryosphaeria* spp. from pome and stone fruit trees in South Africa and to develop a reliable identification technique for these fungi. Fifty-four *Botryosphaeria* isolates were collected from the various hosts in the Western Cape province of South Africa and compared with a worldwide collection of isolates from fruit trees, using DNA sequence data from the nuclear rRNA operon. Phylogenetic analyses distinguished six distinct clades for the isolates associated with fruit tree diseases. These included clades of *B. ribis*, *B. parva*, *B. australis*, *B. dothidea*, *B. stevensii* and *B. obtusa*. Isolates from pome and stone fruit trees in South Africa grouped in either the *B. australis* or *B. obtusa* clades. This represents the first report of *B. australis* from South Africa, where it occurred on apple, plum, pear and almond trees. Morphological examination of isolates from the various phylogenetic groups provided support for the identities of the fungi. PCR RFLP analysis using the restriction endonuclease (RE) *Cfo*I distinguished isolates belonging to the major clades identified in the phylogenetic analysis, except *B. stevensii* and *B. obtusa*. The latter species could, however, be distinguished using both the RE *Hae*III and morphological features. Two groups of closely related species namely, *B. ribis* and *B. parva,* and *B. australis* and *B. lutea*, also, respectively, had identical RFLP profiles. Using RFLP's, it was shown that *B. obtusa* is the dominant species (90% of isolates studied) on pome and stone fruit trees in the Western Cape province of South Africa. The results of this study and methods developed for identification of *Botryosphaeria* spp. will be useful in future epidemiological studies and in disease management.

## INTRODUCTION

Species of *Botryosphaeria* Ces. & De Not. are important pathogens of pome and stone fruit trees, causing fruit rots (e.g. black and white rot of apple), frogeye leaf spot, stem and branch cankers, gummosis, die-back and in some cases tree death (Weaver 1974, Brown and Britton 1986, Britton et al 1990, Pusey 1993, Parker and Sutton 1993a). Infection takes place either through wounds, or directly through the stomata and other openings (Brown and Hendrix 1981, Smith and Hendrix 1984, Britton and Hendrix 1989, Smith et al 1996). These fungi can then persist in healthy tissue as endophytes (Smith et al 1996). Successful infection and susceptibility of infected trees is closely linked to environmental conditions, where high temperatures, water logging and other forms of stress favor infection (Holmes and Rich 1969, McGlohon 1982, Wene and Schoeneweiss 1980, Arauz and Sutton 1989, 1990a).

Effective management of *Botryosphaeria* diseases on fruit trees is achieved through integrated control strategies, which take into account cultivar susceptibility, environmental conditions, tree management and chemical applications (Holmes and Rich 1969, Drake 1971, Starkey and Hendrix 1980, Arauz and Sutton 1990b, Parker and Sutton 1993b, Brown-Rytlewski and McManus 2000). Effective control requires knowledge regarding the taxonomy and epidemiology of the pathogen involved. Furthermore, due to enhanced quarantine requirements, correct identity of the fungal pathogens that affect these crops has become increasingly important in the export of fruit products (Palm 1999).

The *Botryosphaeria* spp. most commonly associated with diseases of pome and stone fruit are *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not., *B. obtusa* (Schwein.) Shoemaker and *B. stevensii* Shoemaker (Shoemaker 1964, Laundon 1973, Sutton 1980, Brown and Britton 1986, Proffer and Jones 1989, Britton et al 1990, Pusey 1993, Brown-Rytlewski and McManus 2000). Although the teleomorph names are preferably used, the anamorph fruiting structures of these fungi are frequently encountered and play an important role in their identification. These anamorphs are *Fusicoccum aesculi* Corda, a *Diplodia* sp. (also reported as a *Sphaeropsis* sp.) and *D. mutila* (Fr.) Mont., respectively. Despite considerable research on the *Botryosphaeria* spp. on fruit trees, the taxonomy of these fungi is incomplete and often confused (Brown and Britton 1986, Ogata et al 2000).

Some *Botryosphaeria* spp. reported on pome and stone fruit trees are less well known as pathogens of these trees. *Botryosphaeria parva* Pennycook & Samuels (anamorph = *F. parvum* Pennycook & Samuels) and *B. lutea* A.J.L. Phillips (anamorph = *F. luteum* Pennycook & Samuels) were initially described from kiwifruit, poplar and apple in New Zealand and later from vines in Portugal (Pennycook and Samuels 1985, Phillips et al 2002). There have been no other reports of their occurrence or influence on pome and stone fruit trees. *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx (anamorph = *Lasiodiplodia theobromae* (Pat.) Griffiths & Maubl.) has been associated with peach tree gummosis, but is less frequently isolated from these symptoms than *B. dothidea* and *B. obtusa* (Brown and Britton 1986, Britton et al 1990, Pusey 1993).

Problems in distinguishing *Botryosphaeria* spp. from each other arise from the overlapping morphological characteristics for many species (Von Arx and Müller 1954, Shoemaker 1964). Recent studies, using both morphological characteristics and molecular data, have, however, clearly defined species groups within the genus (Jacobs and Rehner 1998, Denman et al 2000, Smith et al 2001, Smith and Stanosz 2001, Zhou and Stanosz 2001a, b, Zhou et al 2001, Slippers et al 2003). It has for example been shown that isolates previously classified as *B. dothidea* sensu von Arx and Müller (1954) represent three taxa (Jacobs and Rehner 1998, Smith and Stanosz 2001, Slippers et al 2003). This includes *B. dothidea*, *B. parva* and *B. ribis*. Similarly, isolates previously identified as *B. obtusa*, *B. stevensii* and *B. quercuum* do not always conform to groups identified based on sequence data (Jacobs and Rehner 1998, Zhou and Stanosz 2001a).

*Botryosphaeria dothidea* and *B. obtusa* have both been recorded on pome and stone fruit and trees in South Africa (Combrink et al 1984, Crous et al 2000), but confusion regarding the overall taxonomy of *Botryosphaeria* has reduced the value of these reports. The aim of this study was to determine the identity of species of *Botryosphaeria* from pome and stone fruit trees in South Africa, using DNA based techniques and morphological characteristics. Species of *Botryosphaeria* occurring on fruit trees in other parts of the world were also compared with the South African isolates and an efficient identification protocol was devised for these fungi.

## MATERIALS AND METHODS

*Isolates and morphology.*--A total of 50 isolates, mainly from pome and stone fruit trees in South Africa, were used in this study (TABLE I). Of these, 22 isolates were from apple (*Malus* sp.), 9 from pear (*Pyrus communis*), 5 from peach (*Prunus persica*), 10 from plum (*Prunus* sp.) and 4 from other hosts in the Western Cape province. Isolates were collected between 1995 and 1999 and are representative of *Botryosphaeria*-like fungi from diseases associated with pome and stone fruit in the Western Cape province, which is the primary deciduous fruit production area of South Africa.

The conidial morphology of the isolates from South Africa was studied using a light microscope, and an Axiocam digital camera and accompanying software (Carl Zeiss, Germany). To induce sporulation, isolates were grown at 25 C on water agar (WA) (2% Biolab agar) to which sterilized pine needles had been added as a substrate. Spores were mounted in clear lactophenol and spore length, width, wall texture, shape and color of the spores recorded. Cultures were maintained on malt and yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab) at 25 C and stored on this medium at 4 C. All isolates used in this study have been 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 Raeder and Broda (1985) was used to isolate DNA from the fungi. Mycelium from actively growing cultures on MYA was used to inoculate liquid MY (2% malt extract and 0.2% yeast extract; Biolab) medium in small flasks or 1.5 mL Eppendorf tubes. These were incubated at 25 C for ca 10 days. Mycelium was harvested by centrifugation, frozen and lyophilised. The dried mycelium was ground into a fine powder and homogenised in 800 μL extraction buffer (200mM Tris-HCl pH8.0, 150mM NaCl, 25mM EDTA pH8.0, 0.5% SDS). Phenol and chloroform was added to each sample in a 5:3 ratio, vortexed and centrifuged (13000 rpm for 60 min.). The aqueous phase was transferred to a new Eppendorf and the phenol:chloroform (1:1) extraction step was repeated several times until the interface between the aqueous and upper phases was clean from contaminating proteins and cell debris. Precipitation and harvest of the nucleic acids was done using 3M NaAc pH 5.5 (0.1 v/v) and absolute ethanol (2 v/v) and centrifugation (17900 g for 30 min) at 4 C. The resulting pellet was washed with 70% ethanol, the ethanol removed and the pellet resuspended in 100 µL sterile Sabax water. Five µL RNaseA (1 mg/mL) was added to the resuspended sample and left at 37 C overnight to degrade all RNA. DNA concentrations were subsequently estimated against a λ marker standard on a 1% agarose gel.

*DNA amplification.*--The primers ITS1 (5' TTTCCGTAGGTGAACCTGC) and ITS4 (5' TCCTCCGCTTATTGATATGC) (White et al 1990) were used to amplify part of the nuclear rRNA operon in PCR reactions. The amplified region included the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene. The PCR reaction mixtures and reaction conditions were the same as those described by Slippers et al (2003). PCR products were run on 1% agarose gels, stained with ethidium bromide and visualized under UV illumination. Size estimates were made against a 100bp or λ standard size markers.

*DNA sequencing and analysis.*--Eleven of the 50 isolates from the Western Cape province of South Africa representing the different hosts and conidial types encountered, and were selected for sequencing (TABLE II). PCR products were cleaned using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, CA). Both strands of the amplicons were sequenced using the primers ITS1 and ITS4. Reactions were performed using the 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 377 Autosequencer (Perkin-Elmer Applied BioSystems, Foster City, CA).

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Phylogenetic analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford 1998). Gaps were treated as a fifth character and all characters were unordered and of equal weight, but uninformative characters were excluded. Heuristic searchers were done using stepwise (simple) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm, to find maximum parsimony trees. Branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch support was determined using 1000 bootstrap replicates (Felsenstein 1985).

In order to determine the relationships between the fungi from fruit trees in South Africa and the rest of the world, 35 sequences mainly of *Botryosphaeria* isolates occurring on fruit and fruit trees in previous studies (Jacobs and Rehner 1998, Ogata et al 2000, Zhou and Stanosz 2001a) were obtained from GenBank (TABLE II). Sequences of *Botryosphaeria* species commonly encountered on other hosts in South Africa were also included (TABLE II). Trees were rooted to *Mycosphaerella africana* Crous & M.J. Wingf. and *Guignardia bidwellii* (Ellis) Viala & Ravaz., which are closely related to *Botryosphaeria*.

*PCR RFLP analyses*.--Sequence data were used to determine polymorphisms in restriction sites of known restriction endonucleases (RE). These analyses showed that *Cfo*I would distinguish the species of *Botryosphaeria* from fruit and other hosts in South Africa. *Cfo*I was thus used to screen all 50 the isolates collected from the pome and stone fruit trees in the Western Cape region of South Africa. Hypothetical restriction maps were also determined from GenBank sequence data for species where DNA was not available. Restriction maps using the RE *Hae*III were developed for species not distinguishable with *Cfo*I.

## RESULTS

*Isolates and morphology*.--Most of 50 isolates from the Western Cape province sporulated profusely on WA supplemented with sterilized pine needles. Isolates later identified as *B. australis* produced very few fruiting structures in culture. Based on spore morphology, isolates could be separated into two distinct groups. One group had *Diplodia*-like conidia that were initially hyaline and aseptate, becoming light to dark brown and occasionally 1-septate, with age. These conidia were ovoid and 20--26 x 10--12 µm in size and are characteristic of the anamorph of *B. obtusa* (Shoemaker 1964). The second group had conidia that were typical of *Fusicoccum*, being hyaline, aseptate and fusiform and 17--25 x 5--7 µm in size. These isolates also produced a yellow pigment in culture, which is characteristic of *B. australis* Slippers, Crous & M.J. Wingf. and *B. lutea*. The conidial characteristics were within the size range reported for *F. australis* Slippers, Crous & M.J. Wingf. and *F. luteum* (Pennycook and Samuels 1985, Phillips et al 2002, Chapter 3).

*PCR and phylogenetic analyses.*--PCR products of ca 580 bp were obtained for all isolates used in this study, of which ca 520 bp were used for phylogenetic analyses. Of the total data set (after alignment) of 549 characters, 371 were parsimony-uninformative and 178 parsimony-informative. After heuristic searchers in PAUP, 343 most parsimonious trees of 328 steps were retained [consistency index (CI) = 0.81; retention index (RI) = 0.97; g1 = -0.37] (FIG. 1). The overall topology of these trees was identical as the rearrangements were only within the major clades and not between these clades.

The isolates used in the phylogenetic analyses resided in eight clades (I – VII) (FIG. 1). All the South African isolates from fruit trees used in this study grouped into either clade IV (CMW586 from apple, CMW980 from pear, CMW1133 from plum, CMW1187 from almond) with an ex-type isolate of *B. australis*; or clade VIII (CMW568 from apple, CMW918, CMW986 and CMW1050 from pear, CMW1069 from peach, CMW1159 from plum) which contains isolates of *B. obtusa*.

*PCR RFLP analyses.*--Using the RE *Cfo*I, unique banding patterns were obtained for isolates representing most of the major clades in the phylogenetic analyses. Isolates residing in Clade VII (*B. stevensii*) and clade VIII (*B. obtusa*) (FIGS. 2a, 3) could not be identified using this RE. However, isolates in these groups could be distinguished using *Hae*III (FIG. 2b). Only one profile was produced for isolates from clades *B. ribis* (Clade I) and *B. parva* (Clade II), and *B. lutea* (Clade III) and *B. australis* (Clade IV), respectively. These species are distinguished using sequence data and morphology.

All *Botryosphaeria* spp. from fruit and other hosts in South Africa were screened using this RFLP method. The identity of the isolates that belong to Clade IV and VIII based on sequence data were thus confirmed. The remainder of the 50 isolates screened, all represented *B. obtusa* (VIII).

## DISCUSSION

In this study, *Botryosphaeria* spp. from pome and stone fruit trees in South Africa as well as many other parts of the world could be separated into six distinct groups. These included clades for *B. ribis*, *B. parva*, *B. australis*, *B. dothidea*, *B. stevensii* and *B. obtusa.* Isolates from pome and stone fruit trees in South Africa were identified as representing either *B. australis* or *B. obtusa.* These identifications were supported by ITS-rDNA sequence data, PCR RFLP analysis and morphological characteristics.

This study represents the first record of *B. australis* from South Africa and it is also reported from apple, pear, plum and almond for the first time. *Botryosphaeria australis* is only known from native *Acacia* species in Australia, where it was the only *Botryosphaeria* species present (Chapter 3). For this reason, it is thought that *Acacia* in Australia might be the native host or one of the native hosts of this fungus. *Botryosphaeria australis* was not frequent in this study, with only four isolates from a collection of 50 isolates assembled over a five-year period representing the fungus. This species, therefore, appears to be a foreign pathogen on pome and stone fruit, and of minimally importance on this host in South Africa.

*Botryosphaeria lutea*, which is the sister species of *B. australis*, is known from fruit trees in New Zealand. This fungus (as *Fusicoccum luteum*) was reported from kiwifruit, apple and pear in New Zealand (Pennycook and Samuels 1985). No isolates of *B. lutea* were, however, found on pome and stone fruit trees in South Africa. These hosts are thus affected by different *Botryosphaeria* species in each area.

*Botryosphaeria obtusa* (Clade VII) was the dominant *Botryosphaeria* species isolated from diseased pome and stone fruit trees in this study. Isolates of this species represented over 90% of those collected over a five year period in South Africa. Efforts to control *Botryosphaeria* associated diseases in the area, should thus concentrate on this species. The phylogenetic clade representing this species included isolates from many parts of the world, and our findings support those of other studies (Shoemaker 1964, Laundon 1973, Sutton 1980, Brown and Britton 1986, Proffer and Jones 1989, Britton et al 1990, Pusey 1993, Brown-Rytlewski and McManus 2000, Ogata et al 2000) showing that it is one of the most important species of *Botryosphaeria* that affect pome and stone fruit trees in all these regions.

Three species, *B. dothidea*, *B. ribis* and *B. mali* Putt. (considered a synonym of *B. dothidea* by Von Arx and Müller 1954) have previously been reported from pome and stone fruit trees in South Africa (Putterill 1919, Combrink et al 1984, Crous et al 2000). Although none of these taxa were identified in the current study, these previous reports cannot be discounted, because isolates representing those species were collected in areas of South African not included in this study. It is also possible that the fungi identified in these previous reports represented either *B. australis*, *B. ribis* or *B. parva*, because the conidial features reported in these studies overlap with all these species (Putterill 1919, Combrink et al 1984, Pennycook and Samuels 1985, Phillips et al 2002).

*Botryosphaeria parva* (Clade II) and *B. dothidea* (Clade V) are common pathogens of pome and stone fruit trees worldwide. These species have previously both been treated as *B. dothidea* (Jacobs and Rehner 1998, Denman et al 2000, Smith and Stanosz 2001, Zhou and Stanosz 2001a, Slippers et al 2003). Sequences from isolates from fruit trees in Japan reside in both groups. Only sequences of *B. dothidea* (Clade V) were, however, identified from fruit trees in the USA. Based on ITS data from GenBank, one sequence from apple from the USA grouped with *B. ribis* (Clade I). The difference between *B. ribis* (Clade I) and *B. parva* (Clade II) based on ITS data is, however, inordinately small (Slippers et al 2003) and additional data will be required to confirm the identity of isolates residing in ITS Clades I and II. No isolates of *B. parva* or *B. dothidea* were, however, isolates from South African pome and stone fruit trees during this study.

The name *B. berengeriana* has recently been used for isolates from fruit trees in Asia (Sassa et al 1998, Ogata et al 2000, Al-Haq et al 2002). This name has, however, been reduced to synonymy with *B. dothidea* (Clade V) (Von Arx and Müller 1954, Slippers et al 2003). Isolates from Asia group into both Clade II and Clade V and either represent *B. parva* or *B. dothidea*.

Two sequences from *Malus* represent *B. stevensii* (Clade VII), together with an isolate from *Fraxinus*. This taxon is well known from these hosts (Shoemaker 1964, Laundon 1973, Sutton 1980). *Botryosphaeria stevensii* and *B. obtusa* have been confused with isolates of *B. quercuum* (Clade VI). Sequences in clade VI were originally identified as *B. obtusa* (KJ93.09), *B. stevensii* (KJ93.35) and *D. quercina* (KJ93.29) (Jacobs and Rehner 1998). The reason for the confusion between these taxa is mainly that the dimensions of their ascospores and conidia overlap. *Botryosphaeria quercuum* is, however, not known from *Malus* and *Fraxinus*. The easiest distinction between *B. stevensii* and *B. obtusa* is that the former species has conidial walls that are 1--2 $\mu$m thick and rarely discolor before septation, while *B. obtusa* has thinner walled conidia that commonly discolor before septation

When working with species of *Botryosphaeria*, a commonly encountered problem is that sexual structures of these fungi that are found on wood are insufficient to provide reliable identifications. Furthermore, isolates made from diseased tissue do not readily produce sexual fruiting bodies in culture and considerable effort is needed to produce asexual structures in culture. There is consequently a great need for an efficient means to identify large numbers of isolates of these fungi, reasonably rapidly. In this study, we were able to distinguish the species associated with pome and stone fruits with PCR RFLP fingerprints generated using two REs. Only one profile was produced for *Botryosphaeria ribis* and *B. parva*, and *B. australis* and *B. lutea*, respectively. Isolates from these clades must be further distinguished using sequence data and conidial morphology (Slippers et al 2003, Chapter 3). There was sequence variation among isolates from Clade II (*B. parva*), Clade V (*B. dothidea*) and Clade VIII (*B. obtusa*). This variation did not influence the RFLP patterns, except in the case of clade V for which three RFLP patterns were determined. Despite the variation in RFLP patterns of *B. dothidea*, this species could still be distinguished from the other species.

Results of this study have provided various options to resolve previously encountered problems in the identification of species of *Botryosphaeria* from pome and stone fruits. This will be important where reliable identifications are needed for quarantine purposes and were conflicts arise relating to exports and biosecurity. It should now be possible to easily distinguish species relatively rapidly using RFLP banding patterns. Where more time is available, the species have been sufficiently well characterized to be able to identify them based on conidial characteristics. In combination, these approaches will hopefully also facilitate a better understanding of diseases associated with pome and stone fruits.

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TABLE I. Isolates of *Botryosphaeria* from pome and stone fruit trees, and other hosts, from the Cape province that were used in this study.

| CMW no.[1] | BO no.[2] | Host | Location | Date isolated |
|---|---|---|---|---|
| 242 | 5 | *Malus* sp. (Apple) | Bethlehem | 9/97 |
| 258 | 27 | ‘’ | Koue Bokkeveld | ‘’ |
| 308 | 29 | ‘’ | Vyeboom | 1/97 |
| 324 | 41 | ‘’ | Vyeboom | ‘’ |
| 370 | 31 | ‘’ | Ceres | 96 |
| 388 | 35 | ‘’ | Grabouw | ‘’ |
| 427 | 43 | ‘’ | ‘’ | ‘’ |
| 432 | 45 | ‘’ | ‘’ | ‘’ |
| 442 | 152 | ‘’ | ‘’ | ‘’ |
| 443 | 53 | ‘’ | Stellenbosch | 96 |
| 447 | 65 | ‘’ | ‘’ | 11/95 |
| 474 | 69 | ‘’ | Elgin | 96 |
| 509 | 123 | ‘’ | ‘’ | ‘’ |
| 568 | 80 | ‘’ | Ceres | 1/98 |
| 586 | 83 | ‘’ | Elgin | 2/98 |
| 588 | 101 | ‘’ | Vyeboom | 9/98 |
| 612 | 137 | ‘’ | Koue Bokkeveld | 5/99 |
| 660 | 143 | ‘’ | Misgund | 85 |
| 681 | 147 | ‘’ | Ceres | 9/97 |
| 689 | 155 | ‘’ | Grabouw | 11/97 |
| 893 | 160 | ‘’ | Joubertinia | 97 |
| 913 | 227 | ‘’ | Krakeelrivier | 5/99 |
| 915 | 24 | *Pyrus communis* (Pear) | Villiersdorp | 9/96 |
| 916 | 47 | ‘’ | Grabouw | 4/97 |
| 918 | 55 | ‘’ | Villiersdorp | 9/96 |
| 933 | 71 | ‘’ | Stellenbosch | 96 |
| 980 | 76 | ‘’ | Hermanus | 1/98 |
| 986 | 85 | ‘’ | ‘’ | ‘’ |
| 1030 | 88 | ‘’ | Tulbach | 6/98 |
| 1049 | 90 | ‘’ | Hamlet | 9/98 |
| 1050 | 149 | ‘’ | Koue Bokkeveld | 11/97 |
| 1066 | 22 | *Prunus persica* (Peach) | Robertson | 9/96 |
| 1069 | 145 | ‘’ | ‘’ | ‘’ |
| 1078 | 111 | ‘’ | Joubertinia | 10/98 |
| 1084 | 134 | ‘’ | Bien Donné | 5/99 |
| 1085 | 141 | ‘’ | Ceres | 1/97 |
| 1086 | 19 | *Prunus domestica* (Plum) | Klapmuts | 9/97 |
| 1087 | 25 | ‘’ | Stellenbosch | 96 |
| 1088 | 33 | ‘’ | Villiersdorp | 10/98 |
| 1126 | 142 | ‘’ | ‘’ | ‘’ |
| 1133 | 37 | ‘’ | Klapmuts | 96 |
| 1143 | 39 | ‘’ | Franschhoek | 95 |
| 1148 | 57 | ‘’ | Grabouw | 2/97 |
| 1149 | 63 | ‘’ | Elgin | 96 |
| 1154 | 105 | ‘’ | Dennesig | 9/98 |
| 1159 | 108 | ‘’ | Swellendam | 10/98 |
| 1162 | 59 | *Prunus* sp. (Stonefruit) | Stellenbosch | 96 |
| 1175 | 73 | ‘’ | N/a | 97 |
| 1179 | 75 | *Populus* sp. | Ceres | 1/98 |
| 1187 | 86 | *Prunus dulcus* (Almond) | Hermanus | 6/98 |

[1] Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

[2] *Botryosphaeria* culture collection of AW Smit, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa.

TABLE II. Isolates considered in the phylogenetic study.

| **Culture no.**[1] | **Other no.**[1] | **Identity**[2] | **Host** | **Location** | **Collector** | **GenBank**[3] |
|---|---|---|---|---|---|---|
| CMW7772 | | *Botryosphaeria ribis* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | |
| CMW7054 | CBS121 | *B. ribis* | *Ribes rubrum* | New York, USA | N.E. Stevens | AF241177 |
| | KJ93.42 | *B. ribis / B. parva* | *Malus* sp. (apple) | Washington D.C., USA | K.A. Jacobs | AF027741 |
| CMW994 | ATCC58189 | *B. parva* | *M. sylvestris* (apple) | New Zealand | G.J. Samuels | AF243395 |
| CMW9078 | ICMP7925 | *B. parva* | *Actinidia deliciosa* (kiwifruit) | New Zealand | S.R. Pennycook | |
| BOT25 | | *B. parva* | *Eucalyptus grandis* | Swaziland | H. Smith | |
| | TO 67 | *B. parva* | *Vitis* sp. (grapevine) | Okayama Pref., Japan | H. Nasu | AB034815 |
| | TO 74 | *B. parva* | *Pyrus communis* (pear) | Tokushima Pref., Japan | H. Yamatao | AB034818 |
| | TO 76 | *B. parva* | *P. communis* (pear) | Tokushima Pref., Japan | T. Ogata | AB034819 |
| | TO 77 | *B. parva* | *A. deliciosa* (kiwifruit) | Tokushima Pref., Japan | H. Yamato | AB034820 |
| | TO 78 | *B. parva* | *Diospyrus kaki* (persimmon) | Tokushima Pref., Japan | H. Yamato | AB034821 |
| CMW992/3 | KJ93.52 | *B. lutea* | *A. deliciosa* (kiwifruit) | New Zealand | G.J. Samuels | AF027745 |
| CMW10309 | CAP002 | *B. lutea* | *Vitis vinifera* (grape) | Portugal | A.J.L. Phillips | |
| CMW9072 | | *B. australis* | *Acacia* sp. | Melbourne, Australia | J. Roux / D. Guest | |
| CMW6837 | | *B. australis* | *Acacia* sp. | Batemans Bay, Australia | M.J. Wingfield | |
| CMW586 | BO83 | *B. australis* | *Malus* sp. (apple) | Elgin, SA | W.A. Smit | |
| CMW980 | BO76 | *B. australis* | *P. communis* (pear ) | Hermanus, SA | W.A. Smit | |
| CMW1133 | BO37 | *B. australis* | *Prunus* sp. (plum) | Pickstons, Klapmuts, SA | W.A. Smit | |
| CMW1187 | BO86 | *B. australis* | *P. dulcis* (almond) | Hermanus, SA | W.A. Smit | |
| CMW9076 | 7818 | *B. lutea* | *Malus* x *domestica* (apple) | New Zealand | S.R. Pennycook | |
| | TO 1 | *B. dothidea* | *Malus* sp. (apple) | Fukusima Pref., Japan | S. Hayashi | AB034808 |
| | TO 12 | *B. dothidea* | *P. persica* (peach) | Fukusima Pref., Japan | S. Kanematsu | AB034809 |
| | TO 29 | *B. dothidea* | *P. communis* (pear) | Nagasaki Pref., Japan | T. Ogata | AB034810 |
| | TO 41 | *B. dothidea* | *P. communis* (pear) | Nagano Pref., Japan | T. Ogata | AB034813 |
| | TO 66 | *B. dothidea* | *Vitis* sp. (grapevine) | Okayama Pref., Japan | H. Nasu | AB034814 |
| | TO 72 | *B. dothidea* | *P. persica* (peach) | Fukushima Pref., Japan | T. Ogata | AB034816 |
| | TO 73 | *B. dothidea* | *P. communis* (pear) | Tokushima Pref., Japan | H. Yamato | AB034817 |
| | TO 81 | *B. dothidea* | *Malus* sp. (apple) | USA | T.B. Sutton | AB034823 |
| | TO 82 | *B. dothidea* | *Malus* sp. (apple) | USA | T.B. Sutton | AB034811 |
| | KJ94.23 | *B. dothidea* | *M. sylvestris* (apple) | Georgia, USA | P.L. Pusey | AF027747 |
| | KJ94.26 | *B. dothidea* | *Prunus persica* (peach) | Japan | P.L. Pusey | AF027749 |
| | KJ94.27 | *B. dothidea* | *P. persica* (peach) | Georgia, USA | P.L. Pusey | AF027761 |
| | ZS 97-5 | *B. dothidea* | *Malus* sp. (apple) | Wisconsin, USA | P. McManus | AF241173 |

TABLE II. Continued.

| Culture no.[1] | Other no.[1] | Identity[2] | Host | Location | Collector | GenBank[3] |
|---|---|---|---|---|---|---|
| CMW8000 | | *B. dothidea* | *Prunus* sp. | Crocifisso, Switzerland | B. Slippers | |
| | KJ93.09 | *B. quercuum*[4] | *Cercis canadensis* | District of Columbia, USA | K.A. Jacobs | AF027752 |
| | KJ93.35 | *B. quercuum*[5] | *Quercus suber* | Spain | K.A. Jacobs | AF027754 |
| | KJ93.29 | *B. quercuum* | *Quercus* sp. | California, USA | E. Hecht-Poinar | AF027753 |
| | ZS 94-6 | *B. stevensii* | *M. pumila* (apple) | New Zealand | N. Tisserat | AF243407 |
| CMW7060 | CBS 431 | *B. stevensii* | *Fraxinus excelsior* | Netherlands | H.A. van der Aa | |
| | ATCC60259 | *B. stevensii* | *M. pumila* (apple) | Unknown | H.J. Boesewinkel | AF243406 |
| | TO 79 | *B. obtusa* | *Malus* sp. (apple) | USA | T.B. Sutton | AB034822 |
| | TO 80 | *B. obtusa* | *Malus* sp. (apple) | USA | T.B. Sutton | AB034812 |
| CMW568 | BO80 | *B. obtusa* | *Malus* sp. (apple) | Ceres, SA | A. Smith | |
| CMW918 | BO55 | *B. obtusa* | *P. communis* (pear) | Villiersdorp, SA | W.A. Smit | |
| CMW986 | BO85 | *B. obtusa* | *P. communis* (pear) | Hermanus, SA | W.A. Smit | |
| CMW1050 | BO149 | *B. obtusa* | *P. communis* (pear) | Koue Bokkeveld, SA | W.A. Smit | |
| CMW1069 | BO145 | *B. obtusa* | *P. persica* (peach) | Robertson, SA | W.A. Smit | |
| CMW1159 | BO108 | *B. obtusa* | *Prunus* sp. (plum) | Swellendam, SA | W.A. Smit | |
| CMW1179 | BO75 | *B. obtusa* | *Populus* sp. (chinese poplar) | Ceres, SA | W.A. Smit | |
| CMW7774 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | |
| | ATCC60851 | *B. obtusa* | *P. persica* (peach) | Georgia, USA | K.O. Britton | AF243408 |
| | KJ93.56 | *B. obtusa* | Hardwood shrub | New York, USA | G.J. Samuels | AF027759 |
| CMW9074 | | *B. rhodina* | *Pinus* sp. | Mexico | T. Burgess | |
| CMW7063 | CBS447 | *Guignardia philoprina* | *Taxus baccata* | Netherlands | H.A. van der Aa | |

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

[2] Identities as used in this study.

[3] Sequences determined in this study has not been submitted to GenBank.

[4] Originally identified as *B. obtusa* (Jacobs and Rehner 1998).

[5] Originally identified as *B. stevensii* (Jacobs and Rehner 1998).

FIG. 1. One of 343 most parsimonious trees of 50 *Botryosphaeria* sequences, mainly from isolates from fruit trees in South Africa, New Zealand, Japan, Spain and the USA, generated with heuristic searches of 549 characters (including gaps) of the ITS1, 5.8S and ITS2 region of the nuclear rRNA operon. Bootstrap values are based on 1000 bootstrap replicates and values greater than 65% are indicated above, below or next to the branches. The trees are rooted to sequences from *Guignardia bidwellii* and *Mycosphaerella africana*. Main clades are identified as I-IX and isolate numbers are representative of that in TABLE II.

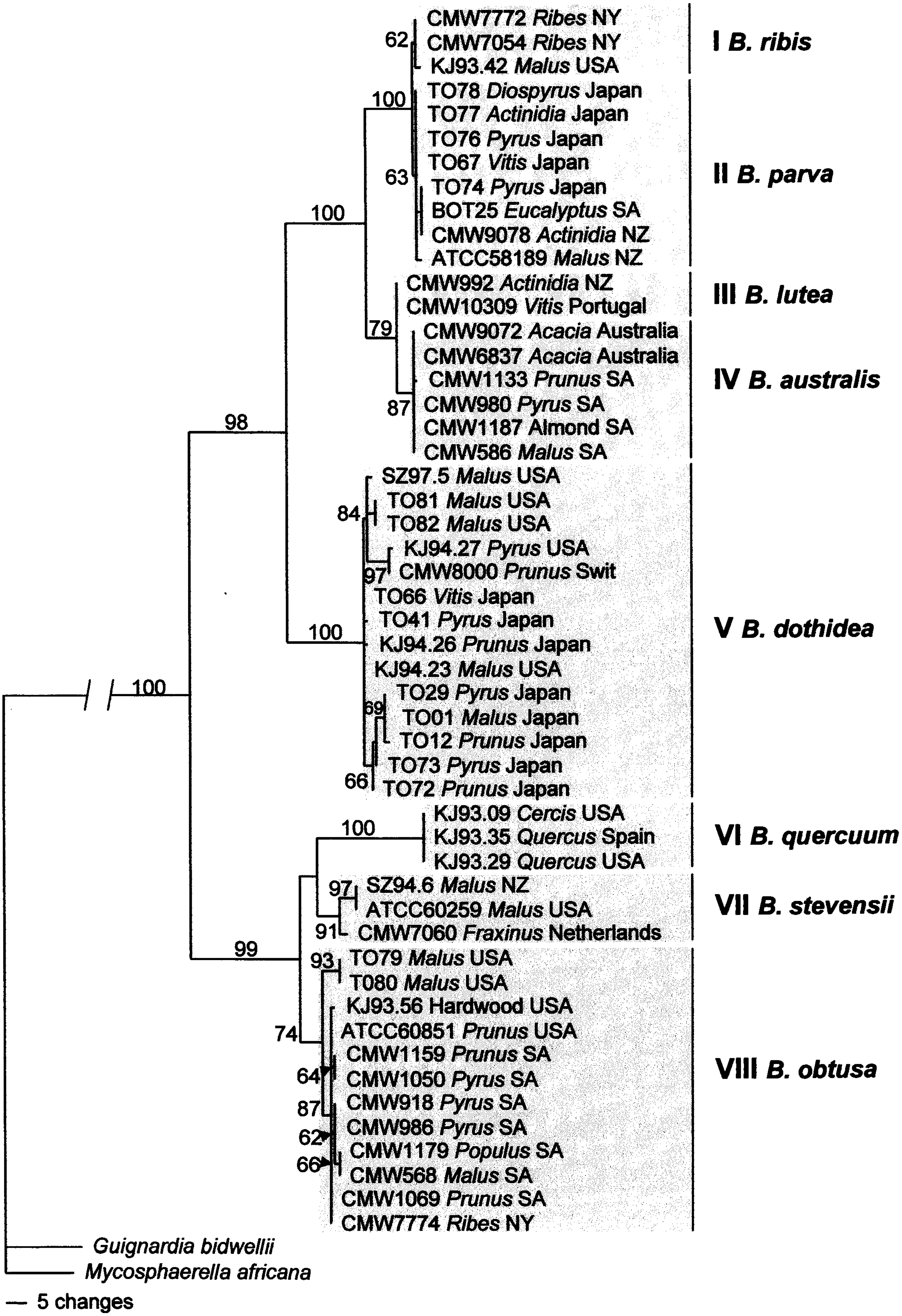

CMW7772 *Ribes* NY
CMW7054 *Ribes* NY
KJ93.42 *Malus* USA
TO78 *Diospyrus* Japan
TO77 *Actinidia* Japan
TO76 *Pyrus* Japan
TO67 *Vitis* Japan
TO74 *Pyrus* Japan
BOT25 *Eucalyptus* SA
CMW9078 *Actinidia* NZ
ATCC58189 *Malus* NZ
CMW992 *Actinidia* NZ
CMW10309 *Vitis* Portugal
CMW9072 *Acacia* Australia
CMW6837 *Acacia* Australia
CMW1133 *Prunus* SA
CMW980 *Pyrus* SA
CMW1187 Almond SA
CMW586 *Malus* SA
SZ97.5 *Malus* USA
TO81 *Malus* USA
TO82 *Malus* USA
KJ94.27 *Pyrus* USA
CMW8000 *Prunus* Swit
TO66 *Vitis* Japan
TO41 *Pyrus* Japan
KJ94.26 *Prunus* Japan
KJ94.23 *Malus* USA
TO29 *Pyrus* Japan
TO01 *Malus* Japan
TO12 *Prunus* Japan
TO73 *Pyrus* Japan
TO72 *Prunus* Japan
KJ93.09 *Cercis* USA
KJ93.35 *Quercus* Spain
KJ93.29 *Quercus* USA
SZ94.6 *Malus* NZ
ATCC60259 *Malus* USA
CMW7060 *Fraxinus* Netherlands
TO79 *Malus* USA
T080 *Malus* USA
KJ93.56 Hardwood USA
ATCC60851 *Prunus* USA
CMW1159 *Prunus* SA
CMW1050 *Pyrus* SA
CMW918 *Pyrus* SA
CMW986 *Pyrus* SA
CMW1179 *Populus* SA
CMW568 *Malus* SA
CMW1069 *Prunus* SA
CMW7774 *Ribes* NY
*Guignardia bidwellii*
*Mycosphaerella africana*
5 changes
62
100
63
100
79
87
98
84
97
100
69
66
100
100
97
91
99
93
74
64
87
62
66
I *B. ribis*
II *B. parva*
III *B. lutea*
IV *B. australis*
V *B. dothidea*
VI *B. quercuum*
VII *B. stevensii*
VIII *B. obtusa*

FIG. 2. PCR RFLP fingerprint maps of the ITS1, 5.8S and ITS2 region of isolates representative of the clades (I-VIII) in the phylogenetic analysis, for RE *Cfo*I (a) and *Hae*III (b). Arrows above the line indicate RE restriction sites, numbers below the line are DNA fragment lengths, and numbers at the end of the line represent the total fragment length.

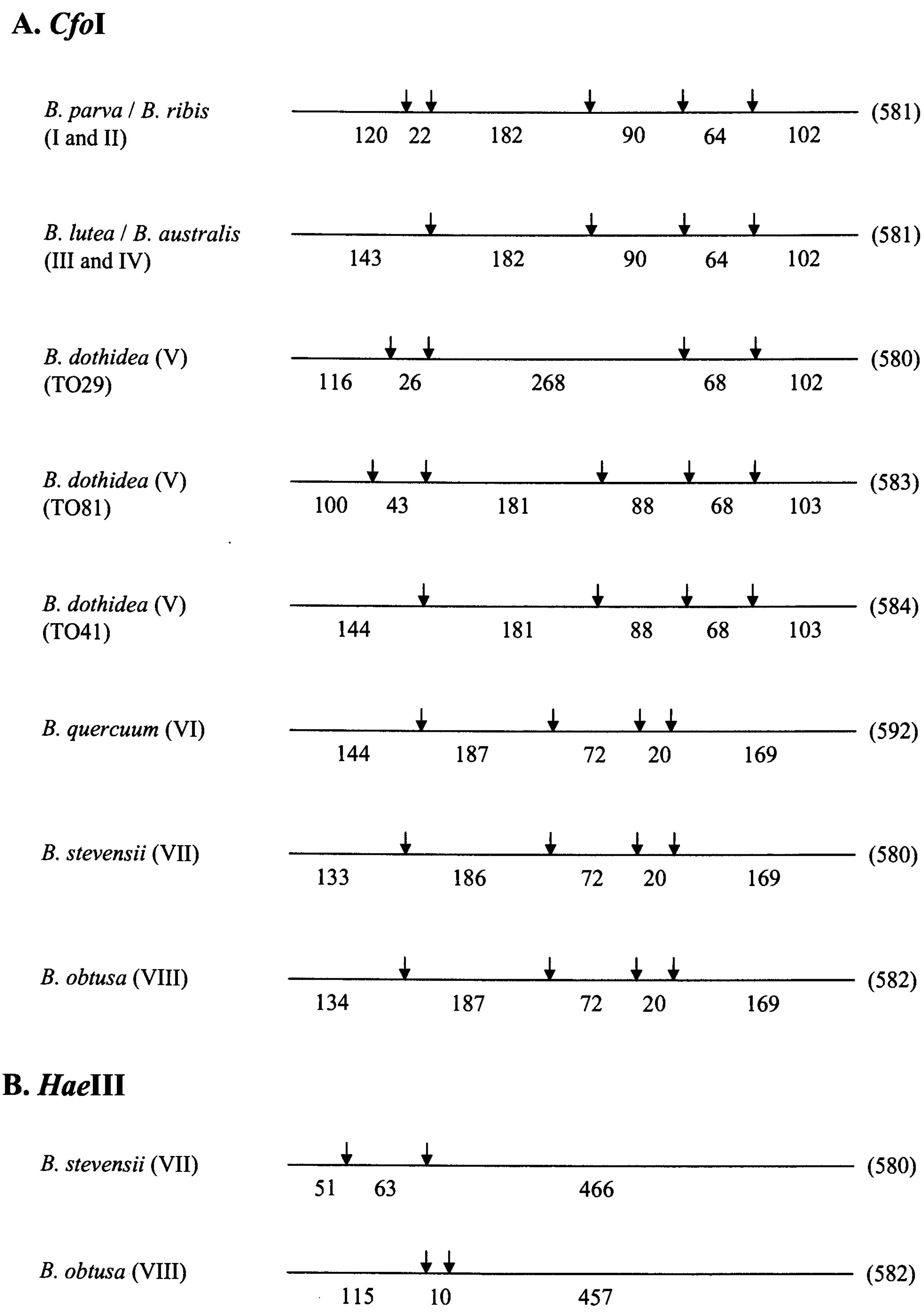
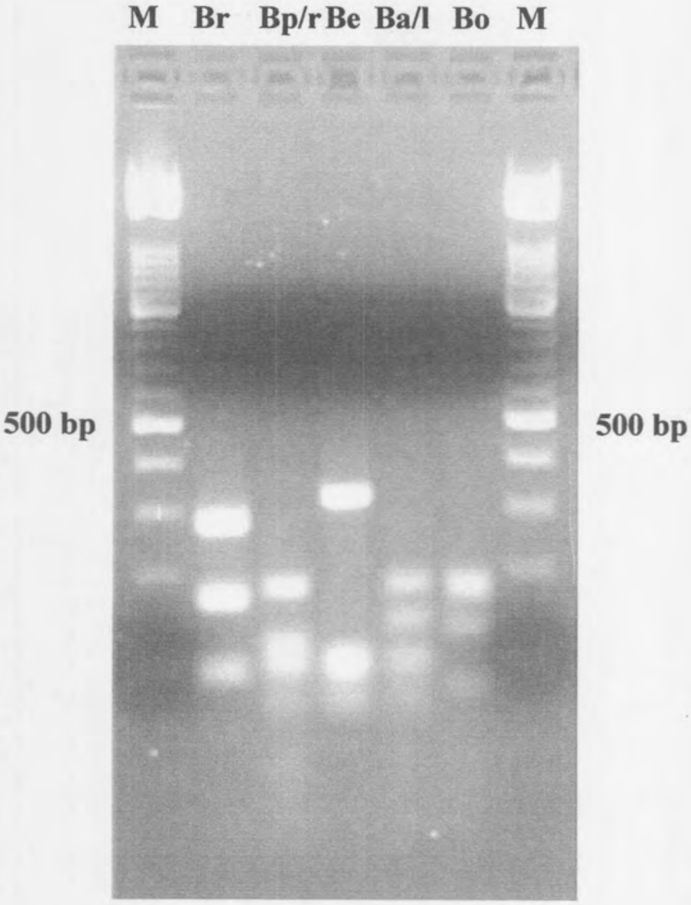

A. *Cfo*I
*B. parva* / *B. ribis* (I and II)
120 22 182 90 64 102 (581)
*B. lutea* / *B. australis* (III and IV)
143 182 90 64 102 (581)
*B. dothidea* (V) (TO29)
116 26 268 68 102 (580)
*B. dothidea* (V) (TO81)
100 43 181 88 68 103 (583)
*B. dothidea* (V) (TO41)
144 181 88 68 103 (584)
*B. quercuum* (VI)
144 187 72 20 169 (592)
*B. stevensii* (VII)
133 186 72 20 169 (580)
*B. obtusa* (VIII)
134 187 72 20 169 (582)
B. *Hae*III
*B. stevensii* (VII)
51 63 466 (580)
*B. obtusa* (VIII)
115 10 457 (582)

FIG. 3. Example of PCR RFLP fingerprints produced from the ITS1, 5.8S and ITS2 region of *Botryosphaeria* spp. that occur in South Africa, using the RE *Cfo*I. Br = *B. rhodina*; Bp = *B. ribis* / *B. parva* (I or II), Be = *B. eucalyptorum*; Ba/l = *B. lutea* / *B. australis* (III or IV); Bo = *B. obtusa* (VIII). 100 bp markers are run on either side.


M
Br
Bp/r
Be
Ba/l
Bo
M
500 bp
500 bp

# 5

## Sympatric speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees

***Abstract:*** *Eucalyptus* is one of the most dominant and important native tree genera in Australia. These trees are also widely planted as forest plantation species for the production of fibre in the southern hemisphere as well as in the tropics. *Botryosphaeria* spp. are important canker and die-back pathogens that affect *Eucalyptus* spp. They also occur endophytically in *Eucalyptus* leaves and stems. For the purpose of this study, *Botryosphaeria* strains were isolated from diseased and symptomless *Eucalyptus* material from Australia, South Africa, Hawaii and Uruguay. These isolates were induced to sporulate in culture, and compared with known species of *Botryosphaeria*. Selected isolates were also compared with authentic isolates of known *Botryosphaeria* spp. based on DNA sequence data. Three gene regions were sequenced, namely the ITS rDNA, β-tubulin and elongation factor 1-α. Five species of *Botryosphaeria* were identified from *Eucalyptus* plants. The ITS rDNA sequence data was then used to develop a PCR RFLP technique that could distinguish these five species. This technique was used to confirm the identity of the remaining isolates that were not sequenced. *Botryosphaeria eucalyptorum* and a new species, *B. irregularis*, were the most common species in eastern Australia, and are considered to be native to these plants and region. These species also occur in other countries where they have most likely been introduced along with *Eucalyptus*. *Botryosphaeria parva* was common on *Eucalyptus* in exotic environments, but rare on this host in Australia. Although *B. dothidea* was previously thought to be common on eucalypts, only one isolate each of *B. dothidea* and *B. lutea* were found in all the areas surveyed. No isolates of *B. ribis*, which was also commonly reported from *Eucalyptus*, were identified during this survey from *Eucalyptus*. Data from the present study provide the first holistic overview of the species of *Botryosphaeria* associated with *Eucalyptus*. These data will also prove invaluable in future efforts aimed at breeding for *Botryosphaeria* resistance in eucalypts.

## INTRODUCTION

*Botryosphaeria* spp. are common and widely distributed ascomycete that cause canker and die-back diseases on many woody plants (Von Arx 1987). A part of the life-cycle of these fungi is, however, spent as endophytes within healthy plant tissue (Smith et al 1996). For this reason, their introduction into new environments on germplasm could go unnoticed, e.g. *Diplodia pinea* (Desm.) J. Kickx (Burgess et al 2003). In a new environment, a specific *Botryosphaeria* sp. has the potential of infecting different hosts or to contribute to the genetic diversity and fitness of an existing population (Wingfield et al 2001a, Burgess and Wingfield 2002a). Identification and knowledge of the *Botryosphaeria* spp. that occur on plants that are moved across the world for commercial purposes are, therefore, crucially important (Palm 1999, Wingfield et al 2001b).

*Eucalyptus* species are native to Australia, but are planted world-wide as an important source of fibre, especially in the southern hemisphere and in the tropics. *Botryosphaeria* spp. are endophytes of *Eucalyptus*, but also cause severe canker and die-back diseases in exotic plantations of these plants (Wingfield et al 1991, Smith et al 1994, 1996). Species of *Botryosphaeria* are, therefore, considered to be a significant threat to the production and sustainability of *Eucalyptus* plantations.

In the past, *Botryosphaeria* spp. have been reported from native *Eucalyptus* in Australia (Davison and Tay 1983, Shearer et al 1987, Old et al 1990). The possible influence of these pathogens on tree health is currently of interest, because *Eucalyptus* plantations in Australia are increasing in extent and economic importance (Burgess and Wingfield 2002b). The risk of diseases is high in these plantations due to the increased genetic uniformity of these plants. Furthermore, planted trees are often on marginal sites and can subsequently be subjected to environmental and other stresses. Native stands adjacent to plantations might also be adversely affected by increased inoculum pressure of pathogens (Strauss 2001).

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

Previous identifications of *Botryosphaeria* spp. on *Eucalyptus* should in many cases be viewed with circumspection. This is due to the confused taxonomy of the species involved, such as *B. dothidea* and *B. ribis*. The confusion stems from the fact that these two taxa have been treated as synonyms (von Arx and Müller 1954), but that this view has not been accepted by all researchers working with *Eucalyptus* pathogens. Recent studies have shown that *B. dothidea* and *B. ribis* are distinct species (Zhou and Stanosz 2001, Slippers et al 2003). Furthermore, Slippers et al (2003) showed that isolates identified as *B. dothidea* from South Africa, represent *B. parva* Pennycook & Samuels. This taxon is morphologically similar to *B. ribis* and some reports mentioned above could also refer to this species.

Anamorph morphology and DNA sequence data have been used with substantial success to distinguish species of *Botryosphaeria*. Anamorph structures and conidia of these fungi are more commonly encountered in nature than their respective teleomorphs (Pennycook and Samuels 1985). Cultures can also readily be induced to produce the anamorph (Slippers et al 2003), and conidial morphology is more characteristic than that of the ascospores (Pennycook and Samuels 1985). Sequence data of the ribosomal DNA region have been most widely used to distinguish *Botryosphaeria* spp., often in combination with morphological characters (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001, Phillips et al 2002). In some cases, a combination of different gene regions, together with morphological characters, was necessary to delimit and describe some closely related or cryptic species (De Wet et al 2003, Slippers et al 2003, Chapter 3).

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

## MATERIALS AND METHODS

*Fungal isolates and DNA isolation.*--A total of 109 isolates were used in this study. Of these, 55 isolates were collected from the Mpumalanga and Kwazulu-Natal Provinces of South Africa between 1990 and 2001; 22 isolates from Hawaii in 2001; 29 isolates from Eastern Australia between July and December 2001; and eight isolates from Uruguay in 2002. Six isolates collected in 2001 from *Tibouchina* in Eastern Australia were also included, as pathogens of this host are known to also occur on *Eucalyptus*. The latter isolates have also been included in a study of Heath (2003), and are used here for comparative purposes. Isolates were grown on malt and yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, South Africa) at 25 C in the dark or under near UV light. Cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

A modified phenol and chloroform extraction method described by Reader and Broda (1985) was used to extract DNA from all isolates. The basic procedure is similar to that described by Slippers et al (2003). Extracted DNA was precipitated by adding 0.1 V 3 M NaAc (pH 5--5.5) and 2 V absolute EtOH at 4 C. The precipitated DNA was washed (70% EtOH), dried and resuspended to approximately 80-100 ng/µL in sterile water. DNA concentration was estimated using λ-marker standard (λ-DNA digested with *Hae*III and *Eco*RI) after electrophoresis on a 1% EtBr stained agarose gel and visualized under UV light.

*Molecular characterization.*--The extracted DNA was used as template in the amplification reactions. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) operon was amplified using the primers ITS1 (5' TCCGTAGGTGAACC TGCGG) and ITS4 (5' TCCTCCGCTTATTGATATGC) (White et al 1990). A part of the β-tubulin gene of selected isolates was amplified using Bt2a (5' GGTAACCAAAT CGGTGCTGCTTC) and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson 1995). Part of the elongation factor 1-α (EF 1-α) was amplified using the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTG AAGGAACCCTTACC) (Carbone et al 1999). The same amplification protocol was used to amplify the ITS and β-tubulin regions (using *Taq* polymerase; Roche Molecular Biochemicals, Alameda, CA) and the EF-1-α region (using Expand *Taq* Polymerase; Roche Molecular Biochemicals) as described in Slippers et al (2003). All PCR products were visualized under UV light on 1% agarose gels stained with EtBr. Sizes of fragments were estimated against a standard 100 bp marker (Roche Molecular Biochemicals).

A selected number of the *Botryosphaeria* isolates were sequenced (TABLE I). The PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Each PCR product was sequenced in both directions with the same primers used for PCR. The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) was used to perform the sequencing reactions and the sequences were run on an ABI PRISM 377/3100 Autosequencer (Perkin-Elmer Applied Biosystems).

*DNA sequence analyses.*--Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and sequences were manually aligned. In order to determine the phylogenetic relationship and the identities of the *Botryosphaeria* spp. used in this study, sequences of known *Botryosphaeria* spp. were obtained from GenBank and Slippers et al (2003), and included in the alignment (TABLE I). The *Botryosphaeria* spp. with *Fusicoccum* anamorphs from *Eucalyptus* trees were the focus of this study. The trees were thus rooted to the GenBank sequences of the sister group of *Botryosphaeria* spp. (*B. stevensii* Shoemaker, *B. obtusa* (Schwein.) Shoemaker and *B. rhodina*) with *Diplodia* or *Lasiodiplodia* anamorphs, with which it shares a common ancestor (Jacobs & Rehner 1998, Slippers et al 2003).

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

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

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

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

## RESULTS

*Molecular characterization.*--DNA fragments of approximately 600 bp (ITS-rDNA), 450 bp (β-tubulin) and 300 bp (EF1-α) were amplified in PCR reactions. A partition homogeneity test of the sequence data sets from these gene regions showed that the data could be analysed collectively (P value = 0.84). After alignment, the combined dataset consisted of 1321 characters, of which 331 were parsimony informative. Fourteen variable sites in the EF1-α were made up of two identical repeats of seven base pairs in isolates of *B. ribis*. These sites were coded as two evolutionary events by excluding twelve of the fourteen base pairs of the repeat. The combined data set contained significant phylogenetic signal compared to random sampling ($P < 0.01$; gl = -0.82) (Hillis and Huelsenbeck 1992). Two most parsimonious trees of 559 steps were retained (CI = 0.839; RI = 0.929) after heuristic searches in PAUP (FIG. 1). Both trees had the same topology and varied only within the identified clades. The topology of the MP trees and conclusions drawn from them were the same as for trees generated by distance analyses.

Seven ingroup clades (I–VII) were identified and these correspond to *B. ribis*, *B. parva*, *B. eucalyptorum*, a *Botryosphaeria* sp. (described below as a new species), *B. lutea* A.J.L. Phillips, *B. australis* Slippers, Crous & M.J. Wingf. and *B. dothidea* (FIG. 1). All isolates from *Eucalyptus* and *Tibouchina* trees grouped in clades II, III, IV, VI and VII.

Sequence variation in Clade II resulted in a separate branch with high bootstrap support (86%), although short (2 steps) and with a low decay value (d1). Analysis of polymorphisms/alleles within this group showed that there are significantly more fixed alleles that group these isolates with *B. parva*, than alleles that separate it from this species (TABLE II). Given the limited sample, and in the absence of morphological or other distinguishing characters, these data are not considered sufficient evidence to separate these isolates from *B. parva*.

Clades III and IV represent closely related, but distinct phylogenetic species. There are 19 polymorphisms between clades III and IV. Of these, 16 were fixed in both groups and in each of the three gene regions (TABLE III) (FIG. 2A–C).

It is evident from TABLE II and III that most of the polymorphisms that were not attributed to indels were transitions (3 tv, 25 ti). These transitions were mostly Cytosine to Thymine, and Guanine to Adenine. This transition bias did not influence the phylogenetic hypotheses, as was evident from the distance analyses using HKY85, as described above.

*PCR-RFLP.*--Restriction maps were determined for three restriction endonucleases (RE), *Cfo*I, *Ksp*I and *Sty*I, that would give distinct digestion patterns of ITS amplicons for all *Botryosphaeria* spp. identified by sequence data from *Eucalyptus* in this study (FIG. 3A-C). The enzymes were used separately and in a specific order. *Cfo*I produced distinctive fragment patterns for *B. parva* and *B. eucalyptorum* (FIG. 4A). From the remaining three species, *Ksp*I allows for the distinction of *B. australis*, and *Sty*I separated *B. dothidea* and the unknown *Botryosphaeria* sp. (FIG. 4B, C). The identities of 109 isolates could thus be determined using these three enzymes (TABLE IV). No *B. ribis* or *B. lutea* isolates were identified in this study using sequence data. Analysis of sequence data of these species from other hosts, however, show that these species were not distinguishable from the *B. parva* and *B. australis,* respectively, using the RFLP technique described above.

*Morphological characterization.*--Ascospores were observed from a limited number of samples, but represented all species. These ascospores were hyaline, aseptate, ovoid to elliptical, smooth with granular contents, 8-spored transversely biseriate in bi-tunicate asci, produced in spherical to papillate black ascomata, single or in botryose clusters, with a central ostiole. Conidia of all isolates included in this study were hyaline, aseptate and shapes were elliptical to fusiform, produced holoblastically on hyaline, subcylindrical conidiogenous cells, with percurrent proliferation producing periclinal thickening. These characteristics are typical of *Botryosphaeria* and its *Fusicoccum* anamorph, and represent *B. parva*, *B. australis*, *B. eucalyptorum* and *B. dothidea* and an unknown *Botryosphaeria* sp. (described below as a new species) (TABLE V; FIGS. 5-14). Morphological and cultural characters that distinguish the species from *Eucalyptus* treated here are presented in the KEY below.

# TAXONOMY

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

***Botryosphaeria irregularis*** Slippers, Crous & M.J. Wingf., sp. nov. FIGS. 5-13

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

*Etymology*. Referring to the conidia that are often irregularly shaped. Because the holomorph is often identified by the shape of the conidia, this concept is used for both the teleomorph and anamorph names.

Ascostroma indistincta. Ascomata pseudothecia, plerumque solitaria, interdum 2--3 structurae botryoide aggregatae, globosa ostiolo centrale, papillata, inclusa, 1/3 vel 2/3 emergentia, nigra, 160--340 µm; paries pseudothecii e 5--8 stratis texturae angularis, extus e cellulis atrobrunneis vel brunneis composita, intus e cellulis hyalinis revestimentum loculi facientibus. Asci bitunicati, clavati, 70--110 x 20--25 µm, octospori, inter paraphyses multas filiformes septatas raro apicem versus ramosas, 2--4 µm latas. Ascosporae fusoideae vel ovoideae, 20--22(--23.5) x 7--8 µm, unicellulares, hyalinae, laeves, contentis granularibus, in asco biseriatae. Pycnidia (in "WA" in acis pinorum sterilifactis in 7--21 diebus formata) superficialia, globosa, plerumque solitaria mycelio tectaque. Conidia in cultura facta fusiformia vel baculata, saepe flexa vel forma irregulare, basi subtruncata vel obtuse rotundata, (20--)25--27(--35) x (5--)7--9(--10) µm, hyalina, unicellularia, ante germinationem septa facientia, laeves contentis pertenue granularibus.

*Ascostroma* indistinct. *Ascomata* pseudothecia, mostly solitary, sometimes forming a botryose aggregate of 2--3 structures, globose with a central ostiole, papilate, imbedded with 1/3 to 2/3 emergent, black, 160--340 µm; pseudothecial wall comprising 5--8 layers of *textura angularis*, outer region of dark or medium brown cells, inner region of hyaline cells lining the locule. *Asci* bitunicate, clavate, 70--110 x 20--25 µm, 8-spored, between numerous filiform, septate pseudoparaphyses, rarely branched towards the tip, 2--4 µm wide. *Ascospores* fusoid to ovoid, 20--22(--23.5) x 7--8 µm (average of 50 ascospores = 21.7 x 7.6 µm, l/w 2.8), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Pycnidia* (formed on WA on sterilized pine needles within 7--21 d) superficial, globose, mostly solitary and covered by mycelium. *Conidia* produced in culture fusiform to rod-shaped, often bent or irregularly shaped, bases subtruncate to bluntly rounded, (20--)25--27(--35) x (5--)7-9(--10) µm (average of 135 conidia 26.3 x 7.2 µm, l/w 3.6), hyaline, unicellular, rarely forming septa before germination, smooth with fine granular contents. *Cultural characteristics.* Colonies white to buff (19''f) or olivaceous grey (21''''i), sometimes becoming olivaceous black (21'''d) at the centre after 7 d, with a dense mat of aerial mycelium, edges smooth to crenulate, sometimes not reaching the edge of the plate. Optimum temperature for growth 25 C, colony slow growing compared with other *Botryosphaeria* spp., reaching 34--43 mm radius on PDA after 4 d at 25 C in the dark.

*Specimens examined.* AUSTRALIA, NEW SOUTH WALES: Tidbinbilla, *Eucalyptus* sp., *M.J. Wingfield* (HOLOTYPE PREMx); *Eucalyptus* sp., *M.J. Wingfield* (PREMx); *Eucalyptus* sp., *M.J. Wingfield* (PREMx); Orbost, *Eucalyptus grandis*, *M.J. Wingfield* (PREMx). (Specimens will be submitted to PREM).

## DISCUSSION

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

*Botryosphaeria eucalyptorum* (anamorph *Fusicoccum eucalyptorum* Smith, Crous & M.J. Wingf.) was the dominant species collected from native *Eucalyptus* trees in eastern Australia. This species represented almost 50% of isolates from this host and area. *Botryosphaeria eucalyptorum* was first described from South Africa as a pathogen of plantation eucalypts (Smith et al 2001). This species appears to be restricted to *Eucalyptus* spp. and this is the first report of the species from Australia where

*Eucalyptus* are native. The abundance, wide distribution in eastern Australia, and unique host association suggest that *B. eucalyptorum* is native on *Eucalyptus* in Australia.

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

*Botryosphaeria eucalyptorum* was commonly isolated from exotic *Eucalyptus* in South Africa and Uruguay. All evidence available to us suggests that this fungus was introduced into these areas with planting stock or with seed from Australia. Such introductions of *B. eucalyptorum* would not be surprising, given its common occurrence in Australia from where *Eucalyptus* planting material and seed is commonly obtained for plantation development. This is the first report of *B. eucalyptorum* from Uruguay.

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

A relatively large number of *Botryosphaeria* isolates from *Eucalyptus* spp. in Australia grouped in a sister clade to *B. eucalyptorum*. The taxon represented by this sister clade of *B. eucalyptorum* is described in this study as *B. irregularis*. The sequence divergence between *B. eucalyptorum* and *B. irregularis* was small, but consistent across the three gene regions investigated. Each clade contained at least two synapomorphic characters per gene region, seven in total for *B. eucalyptorum* and nine for the sister clade. There is no exchange or mixing of these unique alleles despite the sympatric occurrence of these two species on the same hosts and from the areas in Australia and

South Africa. This suggests a complete sexual barrier between the groups (Taylor et al 2000, Steenkamp et al 2002). For this reason, these clades are treated here as representing sibling species. The above observations would not be possible based on a single gene phylogeny and illustrate the need for multiple gene analyses to identify species boundaries among closely related *Botryosphaeria* spp.

The distinction between *B. eucalyptorum* and *B. irregularis* was overlooked in initial identifications based solely on morphology. Subsequent to identification based on DNA sequence comparison, the value of culture morphology, differences in average conidial size (length x width) and ascospore width could be appreciated and weighted taxonomically. Morphological similarity is not uncommon between recently diverged sibling species, as has been discussed previously (Brasier 1997, Harrington and Rizzo 1999). Taylor et al (2000) thus predict that morphological species recognition will commonly aggregate two or more species that can be recognised by other methods. This is confirmed here, and in other cryptic *Botryosphaeria* spp. (De Wet et al 2003, Slippers et al 2003, Chapter 3).

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

In this study, a large number of isolates produced a RFLP profile that represents the *B. parva*-*B. ribis* complex. None of the selected isolates from this group from *Eucalyptus* could, however, be confirmed as *B. ribis* based on sequence data. Slippers et al (2003) also found that it is *B. parva*, rather than *B. ribis* or *B. dothidea*, which is associated with diseases of *Eucalyptus* in South Africa. Most of the isolates from this RFLP group are, therefore, expected to belong to *B. parva*. Clearer identification techniques are, however, needed to confirm this assumption.

The fact that no *B. ribis* isolates and only one *B. dothidea* isolate were identified from *Eucalyptus* in the four areas surveyed during this study, is contrary to many previous reports. *Botryosphaeria ribis* (anamorph *F. ribis* Slippers, Crous & M.J.

Wingf.) has been reported from *Eucalyptus* spp. in Australia, South Africa and the USA (Webb 1983, Shearer et al 1987, Crous et al 1989, Old et al 1990) and *B. dothidea* is known from this host in Britain, Hawaii, South Africa and the USA (Hodges 1983, Barnard et al 1987, Fisher et al 1993, Smith et al 1994, 2001). This enigma is most probably related to the fact that previous authors have used names based on morphological characterizations, which do not easily allow for correct identification of these fungi. The confusion between these species stems from the fact that *B. dothidea* and *B. ribis* have been treated as synonyms (von Arx and Müller 1954) and that was not accepted by all researchers working with *Botryosphaeria* spp. on *Eucalyptus*. Furthermore, the morphology characteristics of *B. ribis* and *B. parva* overlap considerably (Slippers et al 2003). *Botryosphaeria ribis* and *B. dothidea* have, therefore, possibly been previously misidentified.

Isolates from the *B. parva-B. ribis* complex represented approximately 15% of isolates from *Eucalyptus* in Australia, but almost 60% of isolates from this host in Hawaii and South Africa. All isolates from exotic *Tibouchina* in Australia were, however, represented by *Botryosphaeria parva*. It appears that fungi from this group are important pathogens of *Eucalyptus* in exotic plantations, but less common in the native environment of this host. A similar situation exists in the distribution of "morphotypes" of *D. pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. on pines. These fungal groups differ on native and introduced pines, and one type often dominates in a specific geographical area (De Wet et al 2000, Burgess et al 2003). Therefore, the species of *Botryosphaeria* affecting a specific host needs to be individually identified in every different country or environment where the host occurs.

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

One *Botryosphaeria* isolate from *E. grandis* in eastern Australia was shown in this study to represent *B. australis*. Another isolate from *E. marginata* (Smith and Stanosz 2001), has previously been shown to belong to the taxon (Chapter 3). *Botryosphaeria australis* is common on native plants such as *Acacia* spp. and *Banksia* spp. in Australia (Smith and Stanosz 2001, Denman et al 2003, Chapter 3). This fungus can thus infect *Eucalyptus* spp., although it does not seem to compete well it this niche in the areas that were surveyed.

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

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

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

## KEY TO *BOTRYOSPHAERIA* SPP. THAT OCCUR ON *EUCALYPTUS* IN AUSTRALIA, SOUTH AFRICA, HAWAII AND URUGUAY

1. Conidia in culture averaging <19 µm long, fusiform to ellipsoid, l/w ±3--3.5, becoming light brown and one to two septate with a darker brown middle cell after discharge, colony on MEA or PDA thick felt of grey aerial mycelium ............***B. parva***

1. Conidia in culture averaging ≥20 µm long, l/w 3........................................2

    2. Conidia fusiform to irregularly rod-shaped, 20--25 x 5--8 µm (average 22 x 6 µm), l/w 3--4, colony on MEA or PDA producing light yellow pigment after three days, becoming dull brown to buff with age....................................***B. australis***

    2. Conidia frequently >25 µm............................................................3

3. Conidia narrowly fusiform, 24--30 x 4--6 µm, l/w 3.5--6, colonies on MEA or PDA not producing yellow pigment and becoming gray to black with age......***B. dothidea***

3. Conidia 20--30 x 5--10 µm, clavate, l/w 3--3.5.........................................4

    4. Length x width <200.................................................***B. eucalyptorum***

    4. Length x width >200.....................................................***B. irregularis***

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TABLE I. Isolates considered in the phylogenetic study.

| **Culture no.**[1] | **Other no.**[1] | **Identity** | **Host** | **Location** | **Collector** |
|---|---|---|---|---|---|
| CMW7772 | | *Botryosphaeria ribis* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler |
| [2]CMW7054 | CBS121 | *B. ribis* | *R. rubrum* | New York, USA | N.E. Stevens |
| CMW6235 | | *B. parva* | *Tibouchina lepidota* | Melbourne, Australia | M.J. Wingfield |
| CMW6237 | | *B. parva* | *Tibouchina urvilleana* | Melbourne, Australia | M.J. Wingfield |
| CMW9071 | | *B. parva* | *Ribes* sp. | Australia | M.J. Wingfield |
| CMW9078 | ICMP7925 | *B. parva* | *Actinidia deliciosa* | New Zealand | S.R. Pennycook |
| CMW9081 | ICMP8003 | *B. parva* | *Populus nigra* | New Zealand | G.J. Samuels |
| CMW10122 | BOT21 | *B. parva* | *Eucalyptus grandis* | Mpumalanga, S Africa | H. Smith |
| CMW10123 | BOT19 | *B. parva* | *E. smithii* | Mpumalanga, S Africa | H. Smith |
| CMW6233 | | *B. eucalyptorum* | *E. nitens* | Canberra, Australia | M.J. Wingfield |
| CMW6804 | | *B. eucalyptorum* | *E. dunnii* | Towoomba, Australia | M.J. Wingfield |
| [2]CMW10125 | BOT24 | *B. eucalyptorum* | *E. grandis* | Mpumalanga, S Africa | H. Smith |
| [2]CMW10126 | BOT16 | *B. eucalyptorum* | *E. grandis* | Mpumalanga, S Africa | H. Smith |
| CMW6217 | | *B. irregularis* | *E. rossi* | Tidbinbilla, Australia | M.J. Wingfield |
| CMW6229 | | *B. irregularis* | *E. grandis* | Orbost, Australia | M.J. Wingfield |
| CMW6539 | | *B. irregularis* | *E. grandis* | Orbost, Australia | M.J. Wingfield |
| CMW6543 | | *B. irregularis* | *Eucalyptus* sp. | Orbost, Australia | M.J. Wingfield |
| [2]CMW992/3 | KJ93.52 | *B. lutea* | *A. deliciosa* | New Zealand | G.J. Samuels |
| CMW10309 | CAP002 | *B. lutea* | *Vitis vinifera* | Portugal | A.J.L. Phillips |

TABLE I. Continued.

| **Culture no.**[1] | **Other no.**[1] | **Identity** | **Host** | **Location** | **Collector** |
|---|---|---|---|---|---|
| CMW9073 | | *B. australis* | *Acacia* sp. | Melbourne, Australia | J. Roux/ D. Guest |
| CMW6837 | | *B. australis* | *Acacia* sp. | Batemans Bay, Australia | M.J. Wingfield |
| CMW9075 | | *B. dothidea* | *Populus* sp. | New Zealand | G.J. Samuels |
| CMW8000 | | *B. dothidea* | *Prunus* sp. | Crocifisso, Switzerland | B. Slippers |
| CMW7060 | CBS431 | *B. stevensii* | *Fraxinus excelsior* | Netherlands | H.A. van der Aa |
| CMW7774 | | *B. obtusa* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler |
| CMW10130 | BOT977 | *B. rhodina* | *Vitex* sp. | Uganda | J. Roux |

---

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

[2] ITS sequences for these isolates were obtained from GenBank (from the top down: AF241177, AF283686, AF283687 and AF027745).

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

| **Identity** | **Culture number** | **β-tubulin** | | | | | **ITS** | | | | **EF1-α** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 95 | 128 | 187 | 418 | 436 | 512 | 584 | 863 | 936 | 1082 | 1083 | 1094 | 1101 | 1191 | 1252 | 1310 | 1314 |
| *B. ribis* | CMW7772 | C | G | T | T | T | A | G | T | - | T | G | 1 | 1 | C | A | G | A |
| | CMW7045 | C | G | T | T | T | A | G | T | - | T | G | 1 | 1 | C | A | G | A |
| *B. parva* | CMW9080 | T | A | C | T | T | T | - | C | - | C | A | 0 | 0 | T | G | A | A |
| | CMW9081 | T | A | C | T | T | T | - | C | - | C | A | 0 | 0 | T | G | A | A |
| | CMW10123 | T | A | C | T | T | T | - | C | - | C | A | 0 | 0 | T | G | A | A |
| | CMW6235 | T | A | C | C | C | A | - | C | A | C | A | 0 | 0 | T | G | G | C |
| | CMW6237 | T | A | C | C | C | A | - | C | A | C | A | 0 | 0 | T | G | G | C |
| *B. eucalyptorum* | CMW11705 | T | A | C | C | C | T | - | C | - | - | - | 0 | 0 | T | G | G | A |
| *B. lutea* | CMW10309 | T | A | C | C | C | C | - | T | - | - | - | 0 | 0 | T | A | G | G |

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

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

| **Identity** | **Culture number** | **β-tubulin** | | | | | **ITS** | | | | | | | **EF1-α** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 80 | 98 | 275 | 331 | 367 | 566 | 567 | 570 | 599 | 654 | 843 | 969 | 1123 | 1127 | 1212 | 1228 | 1270 | 1309 | 1330 |
| *B. eucalyptorum* | CMW10125 | **t** | **T** | **A** | G | C | **T** | **-** | c | **T** | A | **T** | C | G | T | **T** | G | C | **T** | C |
| | CMW11705 | C | **T** | **A** | G | C | **T** | **-** | c | **T** | A | **T** | C | G | T | **T** | G | C | **T** | C |
| | CMW6233 | **T** | **T** | **A** | G | C | **T** | c | **-** | **T** | A | **T** | C | G | T | **T** | G | C | **T** | C |
| | CMW6804 | C | **T** | **A** | G | C | **T** | c | **-** | **T** | A | **T** | C | G | T | **T** | G | C | **T** | C |
| *B. irregularis* | CMW6543 | C | C | G | **A** | **T** | C | c | **-** | C | **C** | C | **T** | **A** | **C** | C | **A** | **T** | C | **T** |
| | CMW6539 | C | C | G | **A** | **T** | C | c | **-** | C | **C** | C | **T** | **A** | **C** | C | **A** | **T** | C | **T** |
| | CMW6229 | C | C | G | **A** | **T** | C | c | **-** | C | **C** | C | **T** | **A** | **C** | C | **A** | **T** | C | **T** |
| | CMW6217 | C | C | G | **A** | **T** | C | c | **-** | C | **C** | C | **T** | **A** | **C** | C | **A** | **T** | C | **T** |
| *B. lutea* | CMW10309 | C | C | G | G | C | C | C | G | C | A | C | C | G | T | C | G | C | C | C |
| *B. dothidea* | CMW8000 | C | C | G | G | C | C | C | C | C | A | C | C | G | T | C | G | C | C | C |

[1] Polymorphisms that are fixed in both populations are in upper case. The derived (apomorphic) characters in either of *B. eucalyptorum* or *B. irregularis* are shaded.

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

| **Identity** | **Australia** | **South Africa** | **Hawaii** | **Uruguay** | **Total** |
|---|---|---|---|---|---|
| *B. dothidea* | 1 | 0 | 0 | 0 | 1 |
| *B. parva*[1] | 4 | 26 | 18 | 1 | 49 |
| *B. australis* | 1 | 0 | 0 | 0 | 1 |
| *B. eucalyptorum* | 13 | 11 | 0 | 5 | 29 |
| *B. irregularis* | 8 | 17 | 4 | 0 | 29 |

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

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

| **Identity** | **Culture No.** | **Conidial measurements**[1] | **Host** | **Location** |
|---|---|---|---|---|
| *B. parva* | CMW6237 | (15--)17.5(--20) x 5 | *Tibouchina urvilleana* | Melbourne |
| | CMW6235 | (15--)16.3(--20) x (5--)5.5(--6.04) | *T. lepidota* | Melbourne |
| | CMW6236 | (15--)16.5(--20) x 5 | *T. lepidota* | Melbourne |
| | CMW6536 | (17.5--)18.75(--20) x 5 | *T. lepidota* | Melbourne |
| | CMW6797 | (17.5--)18.75(--22.5) x 5 | *Tibouchina* sp. | Coffs Harbour |
| | CMW6799 | (15--)17.5(--20) x (5--)5.25(--7.5) | *Eucalyptus grandis* | Kyogle |
| | CMW6802 | (17.5--)17.25(--20) x 5 | *E. grandis* | Kyogle |
| | CMW6798 | (17.5--)19.75(--20) x 5 | *E. grandis* | Hills Tuscon trail |
| | CMW6812 | (21--)24.2(--25) x (5--)5.8(--7) | *E. pillulans* | Zuills Grafton |
| *B. eucalyptorum* | CMW6550 | (22.5--)24.5(--25) x 7.5 | *E. nitens* | Uriarra |
| | CMW6551 | (20--)23(--27.5) x (5--)7(--7.5) | *E. nitens* | Uriarra |
| | CMW6804 | (22.5--)24(--27.5) x (5--)7(--7.5) | *E. dunnii* | Towoomba |
| | CMW6805 | (25--)26.5(--27.5) x (5--)6.8(--7.5) | *E. dunnii* | Towoomba |
| | CMW6810 | (22.5--)24.8(--27.5) x (7.5--)7.7(--10) | *E. grandis* | Zuills Grafton |
| | CMW6807 | (20--)23.5(--27.5) x (5--)7.3(--10) | *E. rossi* | Canberra |
| | CMW6545 | (20--)25(--30) x (7.5--)8.3(--10) | *E. rossi* | Tidbinbilla |
| | CMW6808 | (17.5--)22(--25) x (7.5--)7.3(--7.5) | *Eucalyptus* sp. | Canberra |
| | CMW6811 | (22.5--)25(--30) x (7.5--)7.3(--10) | *E. pillulans* | Zuills Grafton |
| | CMW6808 | (17.5--)22(--25) x 7.3 | *E. dunnii* | Towoomba |
| | CMW6818 | (27.5--)30.5(--35) x (7.5--)8(--10) | *E nitens* | Canberra |
| | CMW6815 | (25--)26.3(--27.5) x (5--)6.8(--7.5) | *E. dunnii* | Towoomba |

TABLE V. Continued.

| **Identity** | **Culture No.** | **Conidial measurements**[1] | **Host** | **Location** |
|---|---|---|---|---|
| *B. irregularis* | CMW6229 | (25--)25.75(--30) x (7.5--)8.25(--10) | *Eucalyptus grandis* | Orbost |
| | CMW6539 | (22.5--)26.75(--30) x (5--)7.25(--7.5) | *E. grandis* | Orbost |
| | CMW6220 | (22.5--)25.5(--27.5) x (7.5--)8.5(--10) | *Eucalyptus* sp. | Tidbinbilla |
| | CMW6543 | (25--)29.25(--35) x 7.5 | *Eucalyptus* sp. | Orbost |
| | CMW6219 | (25--)29.25(--32.5) x (7.5--)9.25(--10) | *E. rossi* | Tidbinbilla |
| | CMW6221 | (20--)24.75(--27.5) x 7.5(--10) | *Eucalyptus* sp. | Tidbinbilla |
| | CMW6545 | (20--)25(--30) x (7.5--)8.25(--10) | *Eucalyptus* sp. | Orbost |
| | CMW6222 | (22.5--)25.25(--30) x (5--)7.75(--10) | *Eucalyptus* sp. | Tidbinbilla |
| | CMW6217 | (25--)25.5(--30) x (7.5--)8.5(--10) | *E. rossi* | Tidbinbilla |
| *B. australis* | CMW6230 | (20--)23.4(--25) x (5--)5.5(--7.5) | *E. grandis* | Orbost |
| *B. dothidea* | CMW6801 | (25--)26(--27.5) x 5 | *Eucalyptus* sp. | Kyogle |

---

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

FIG. 1. One of two most parsimonious trees retained after heuristic searches of the combined dataset of ITS rDNA, β-tubulin and EF1-α sequence data. Branch supports are given as decay values above and bootstrap values (1000 replicates) below the branches. The trees are rooted to *B. obtusa, B. rhodina* and *B. stevensii*, which are all in the subsection characterized by *Diplodia*-like conidia, unlike the ingroup taxa that all have *Fusicoccum*-like conidia. Isolates' numbers, host and origin (Aust = Australia, NY = New York, USA, NZ = New Zealand, SA = South Africa), as well as the identity of the clades, are indicated.

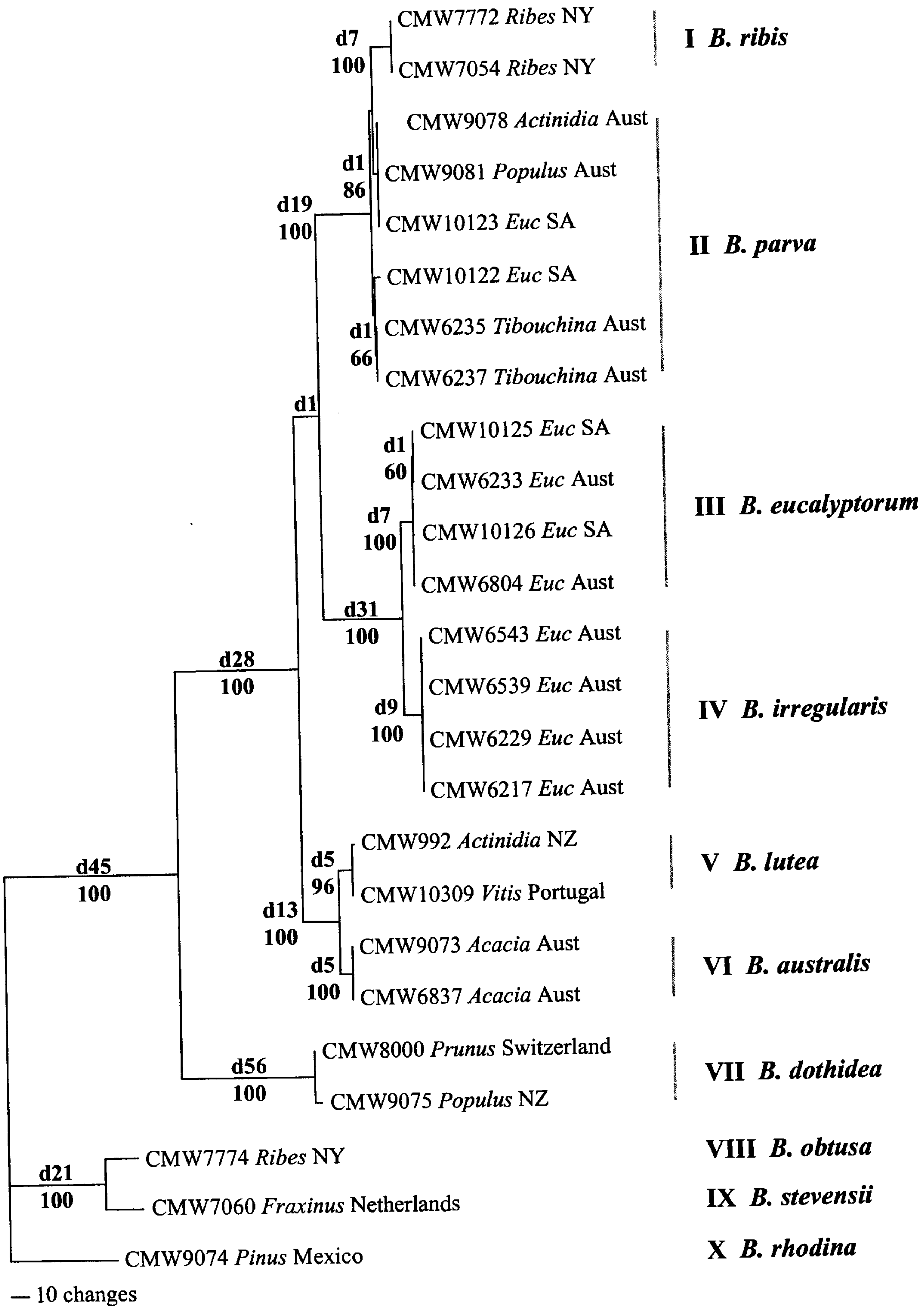

CMW7772 *Ribes* NY
d7
100
CMW7054 *Ribes* NY
I *B. ribis*
CMW9078 *Actinidia* Aust
d1
86
CMW9081 *Populus* Aust
d19
100
CMW10123 *Euc* SA
II *B. parva*
CMW10122 *Euc* SA
d1
66
CMW6235 *Tibouchina* Aust
CMW6237 *Tibouchina* Aust
d1
CMW10125 *Euc* SA
d1
60
CMW6233 *Euc* Aust
III *B. eucalyptorum*
d7
100
CMW10126 *Euc* SA
CMW6804 *Euc* Aust
d31
100
CMW6543 *Euc* Aust
CMW6539 *Euc* Aust
d28
100
d9
100
CMW6229 *Euc* Aust
IV *B. irregularis*
CMW6217 *Euc* Aust
CMW992 *Actinidia* NZ
V *B. lutea*
d45
100
d5
96
CMW10309 *Vitis* Portugal
d13
100
CMW9073 *Acacia* Aust
d5
100
CMW6837 *Acacia* Aust
VI *B. australis*
CMW8000 *Prunus* Switzerland
d56
100
CMW9075 *Populus* NZ
VII *B. dothidea*
CMW7774 *Ribes* NY
VIII *B. obtusa*
d21
100
CMW7060 *Fraxinus* Netherlands
IX *B. stevensii*
CMW9074 *Pinus* Mexico
X *B. rhodina*
— 10 changes

FIG. 2. Most parsimonious (MP) trees retained after analyzing the sequence data of three gene regions separately, showing the consistent separation of the *B. eucalyptorum* and *B. irregularis* clades. Relationships to other species considered are the same as in the tree obtained from the combined dataset (FIG. 1.) and are not shown. Trees were obtained from sequence data of the following DNA regions: (A) ITS rDNA. (B) β-tubulin. (C) EF1-α. Data of the tree length, phylogenetic signal (g1), and consistency and retention indexes (CI, RI) are given directly opposite each tree.

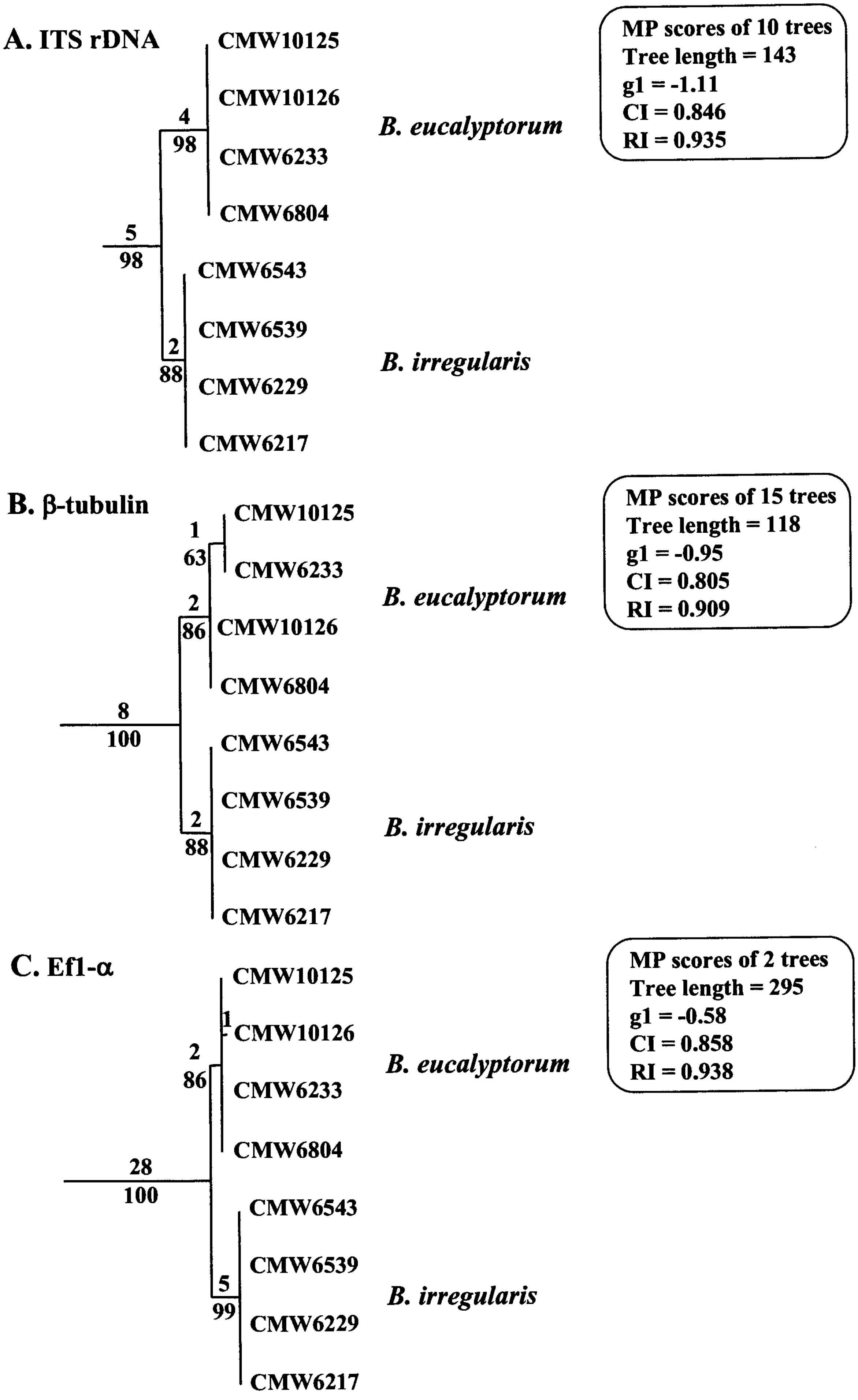

A. ITS rDNA
CMW10125
CMW10126
CMW6233
CMW6804
CMW6543
CMW6539
CMW6229
CMW6217
B. eucalyptorum
B. irregularis
4
98
5
98
2
88
MP scores of 10 trees
Tree length = 143
g1 = -1.11
CI = 0.846
RI = 0.935
B. β-tubulin
CMW10125
CMW6233
CMW10126
CMW6804
CMW6543
CMW6539
CMW6229
CMW6217
B. eucalyptorum
B. irregularis
1
63
2
86
8
100
2
88
MP scores of 15 trees
Tree length = 118
g1 = -0.95
CI = 0.805
RI = 0.909
C. Ef1-α
CMW10125
CMW10126
CMW6233
CMW6804
CMW6543
CMW6539
CMW6229
CMW6217
B. eucalyptorum
B. irregularis
1
2
86
28
100
5
99
MP scores of 2 trees
Tree length = 295
g1 = -0.58
CI = 0.858
RI = 0.938

FIG. 3. Restriction fragment length polymorphism maps of ITS rDNA PCR amplicons of five *Botryosphaeria* spp. when digested with the restriction enzymes *Cfo*I (A), *Ksp*I (B) and *Sty*I (C). The enzymes are used in succession to distinguish all five species. The total length (in base pairs) of each fragment is given in brackets, and fragment lengths are given below each line. These RFLP fragments could not distinguish *Botryosphaeria parva* from *B. ribis*, or *B. lutea* from *B. australis*.

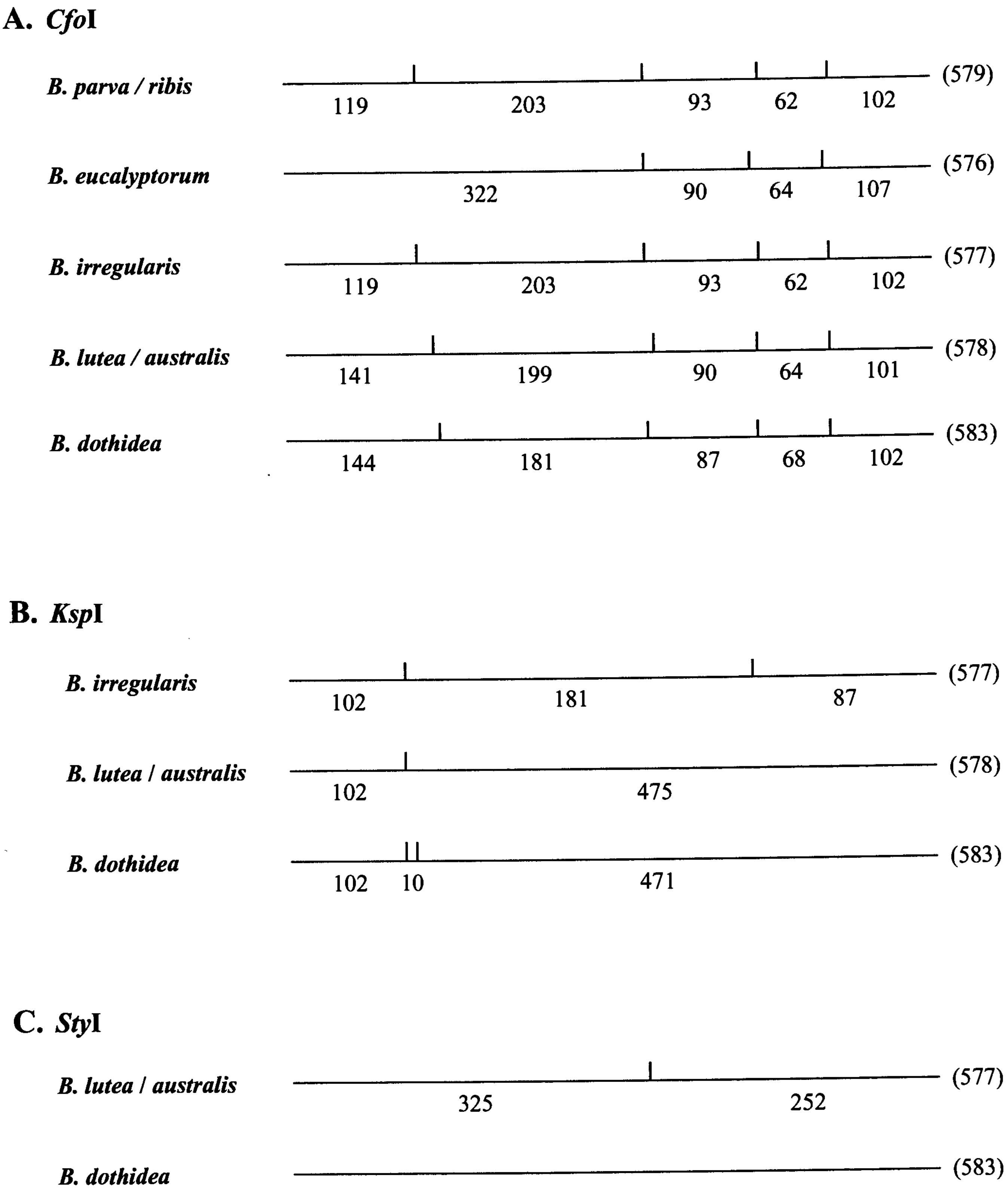
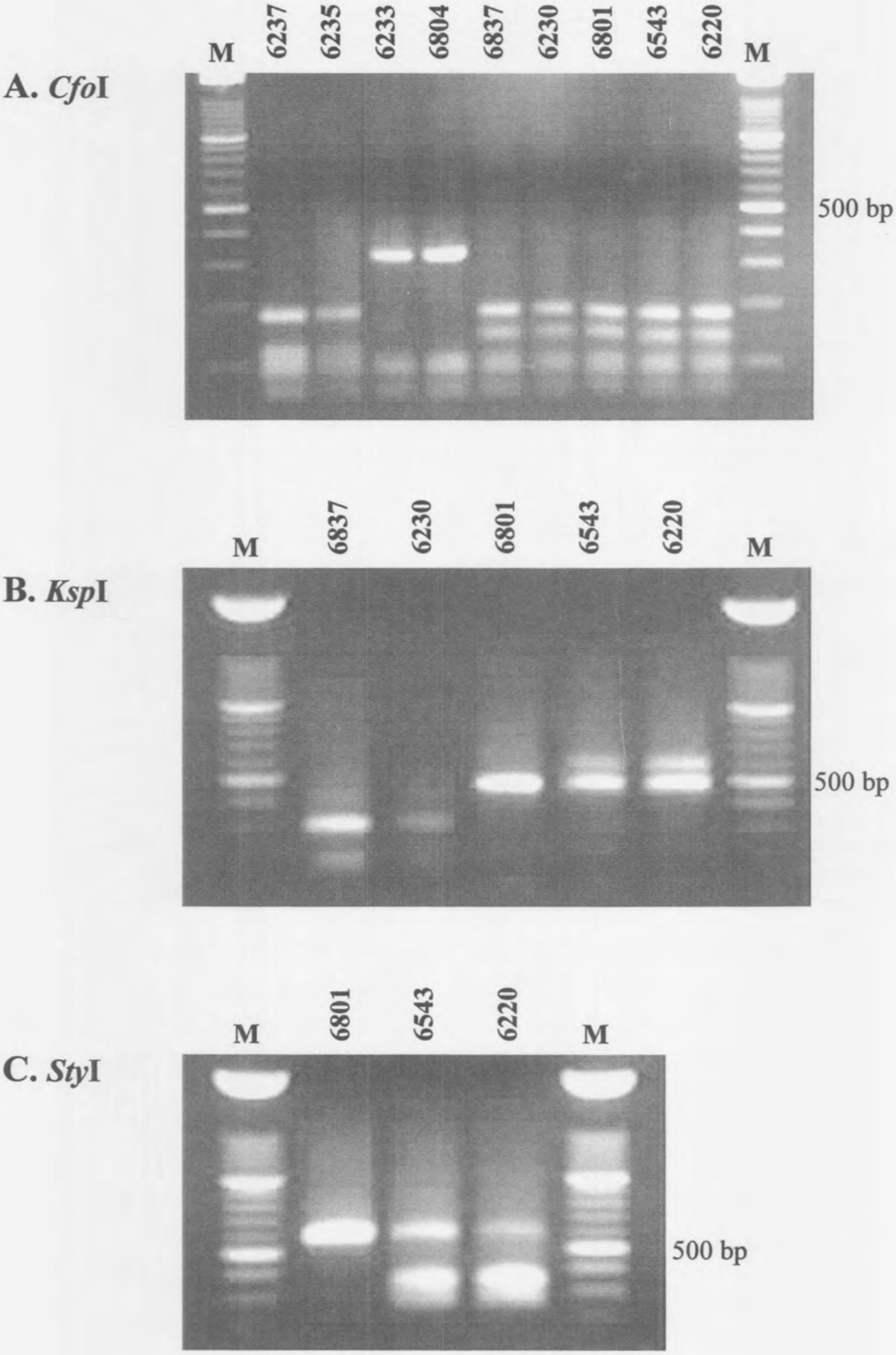
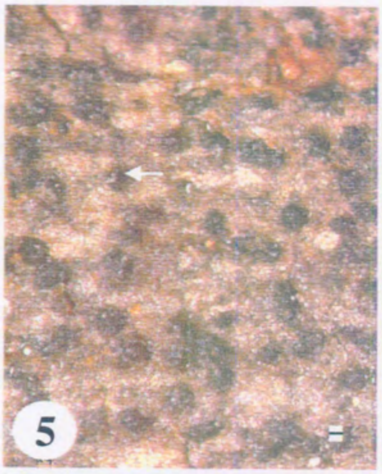

A. *Cfo*I
*B. parva / ribis*
119
203
93
62
102
(579)
*B. eucalyptorum*
322
90
64
107
(576)
*B. irregularis*
119
203
93
62
102
(577)
*B. lutea / australis*
141
199
90
64
101
(578)
*B. dothidea*
144
181
87
68
102
(583)
B. *Ksp*I
*B. irregularis*
102
181
87
(577)
*B. lutea / australis*
102
475
(578)
*B. dothidea*
102
10
471
(583)
C. *Sty*I
*B. lutea / australis*
325
252
(577)
*B. dothidea*
(583)

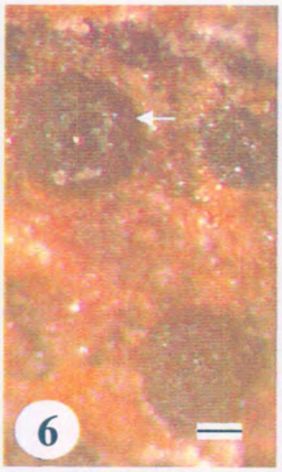
FIG. 4. Agarose gels showing fragments of ITS PCR amplicons of five *Botryosphaeria* spp., namely *B. parva* (CMW6237, CMW6235), *B. eucalyptorum* (CMW6233, CMW6804), *B. irregularis* (CMW6837, CMW6230), *B. australis* (CMW6543, CMW6220) and *B. dothidea* (CMW6801), after digestion with (A) *Cfo*I, (B) *Ksp*I and (C) *Sty*I. The numbers above the lanes refer to the CMW numbers. Lane M contains a 100 bp size marker.


A. *Cfo*I
M
6237
6235
6233
6804
6837
6230
6801
6543
6220
M
500 bp
B. *Ksp*I
M
6837
6230
6801
6543
6220
M
500 bp
C. *Sty*I
M
6801
6543
6220
M
500 bp

FIGS. 5-13. Dissecting microscope and DIC compound-microscope micrographs of *Botryosphaeria irregularis*. 5, 6. Sphaerical, singular ascomata that erupt through the bark (arrows). 7. Median, longitudinal section through an ascoma. Bars = 100 µm. 8. Bi-tunicate asci. 9. Mature ascospores. 10. Conidiogenous cells (arrows) and immature conidia. 11, 12. Fusiform to rod-shaped mature conidia that are often bent or irregularly shaped. 13. Septate germinating conidia (arrows). Bars = 10 µm.

FIG. 14. *Botryosphaeria eucalyptorum* septate germinating- (arrows) and aseptate conidia. Bars = 10 µm.


5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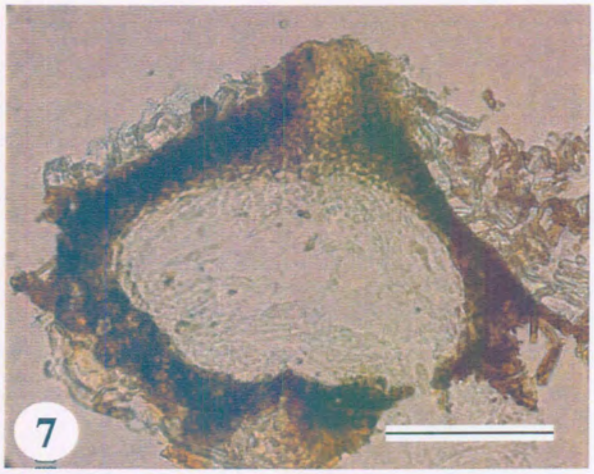
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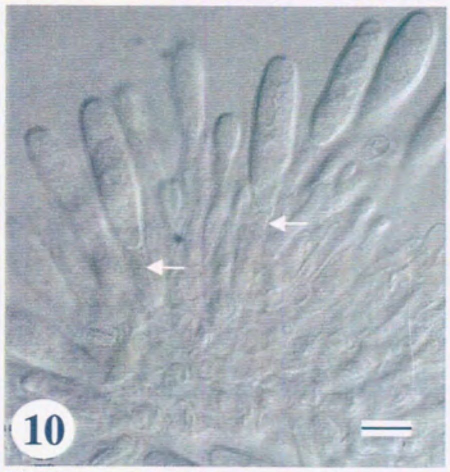
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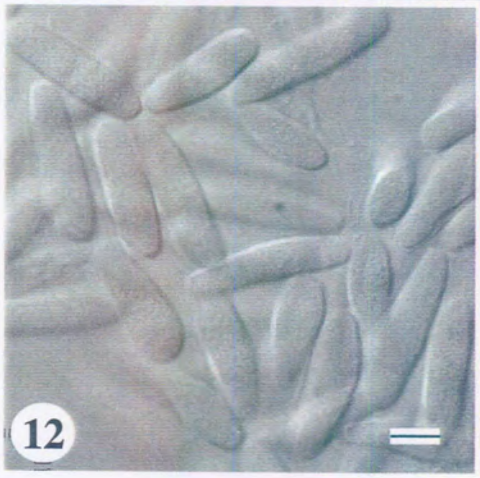
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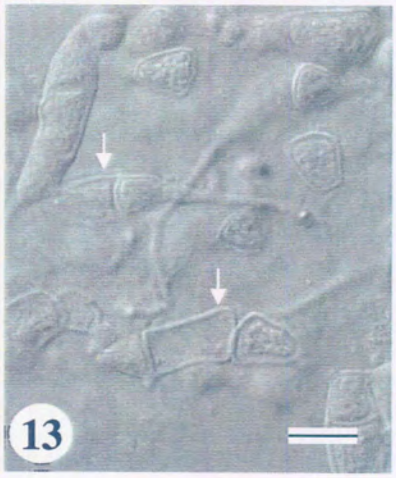
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# 6

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

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

# INTRODUCTION

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

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

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

Seedlings and cuttings have been produced in an effort to conserve *W. nobilis* (Offord 1996, Hogbin et al 2000). This material is used for two purposes. Firstly, it is being distributed to discourage poaching. Secondly, the *ex situ* population are kept in different localities to reduce the risk of diseases. The genetic diversity of this planting stock is obviously as small as that of the wild population. Diseases in the nursery, such as those already experienced due to *Botryosphaeria* die-back, thus threaten this planting stock (Hogbin et al 2000). A concern about the distribution of *W. nobilis* plants is, however, that unique pathogens that have evolved on *W. nobilis* could be spread in this way. This in turn could influence other endangered Araucariaceae.

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

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

## MATERIALS AND METHODS

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

Isolates were maintained on malt and yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, South Africa) at 25°C in the dark or under near UV light. Isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

*Molecular phylogenetic characterization.*--The eight isolates from southern hemisphere coniferous hosts that formed the basis of this study were compared to other *Botryosphaeria* spp. that are known from the area or from similar hosts in other parts of the world. These comparisons were made based on phylogenetic reconstruction using sequence data of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene, a region of the β-tubulin gene and a part of the elongation factor 1-α gene.

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

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), using heuristic searches with stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved.

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

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

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

## RESULTS

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

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

Two isolates from *Araucaria* from New Zealand (CMW10120, CMW10121) grouped with isolates of *B. parva* Pennycook & Samuels (FIG. 1, Clade III). Two isolates from *Widdringtonia* (CMW1110, CMW1112) grouped with *B. australis* Slippers, Crous & M.J. Wingf. (FIG. 1, Clade V). One isolate from *W. nobilis* grouped most closely with *B. australis*, but also distinctly separate from them (FIG. 1). Given that only one isolate was available in this case, further phylogenetic analysis could not be performed.

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

## DISCUSSION

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

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

The DNA sequences for Clade II isolates were not identical to those of *B. ribis*. Isolates residing in this clade share eight polymorphisms with *B. ribis* and four with *B. parva*. They also have four alleles that are different to those of *B ribis* and *B. parva*. All four of these unique alleles are also present in the outgroup taxa with *Diplodia* anamorphs, and are, thus, either plesiomorphic characters or homoplasies. These results suggest that Clade II isolates represent an older lineage that share a common ancestor with both *B. ribis* and *B. parva*, but have begun to accumulate unique alleles due to geographic or host separation. Alternatively, these isolates from Clade II might represent remnants of past genetic exchange between populations of *B. ribis* and *B. parva,* before these lineages were reproductively separated (Davis and Nixon 1992). The limited sample size and absence of other distinguishing characters for Clade II isolates, however, makes it impossible to test whether these isolates represent a distinct species.

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

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

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

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

One isolate from *W. nobilis* grouped sister to the *B. australis* clade. Conidia of this isolate were morphologically similar to those of *B. australis*, although they were slightly shorter on average than those of the latter taxon (Chapter 3). *Botryosphaeria australis* conidia are on average 24--25 µm long, unlike the isolate from *Wollemia,* which had conidia that were 21--22 µm long. It is, however, not possible to judge the value of these measurements based on a single isolate. This isolate might represent a unique species or simply a variant of *B. australis*, but more isolates will need to be studied to test these hypotheses. Nevertheless, this second species of *Botryosphaeria* on *Wollemia* could be important when considering conservation strategies.

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

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TABLE I. Isolates compared in the phylogenetic study.

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

TABLE I. Continued.

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

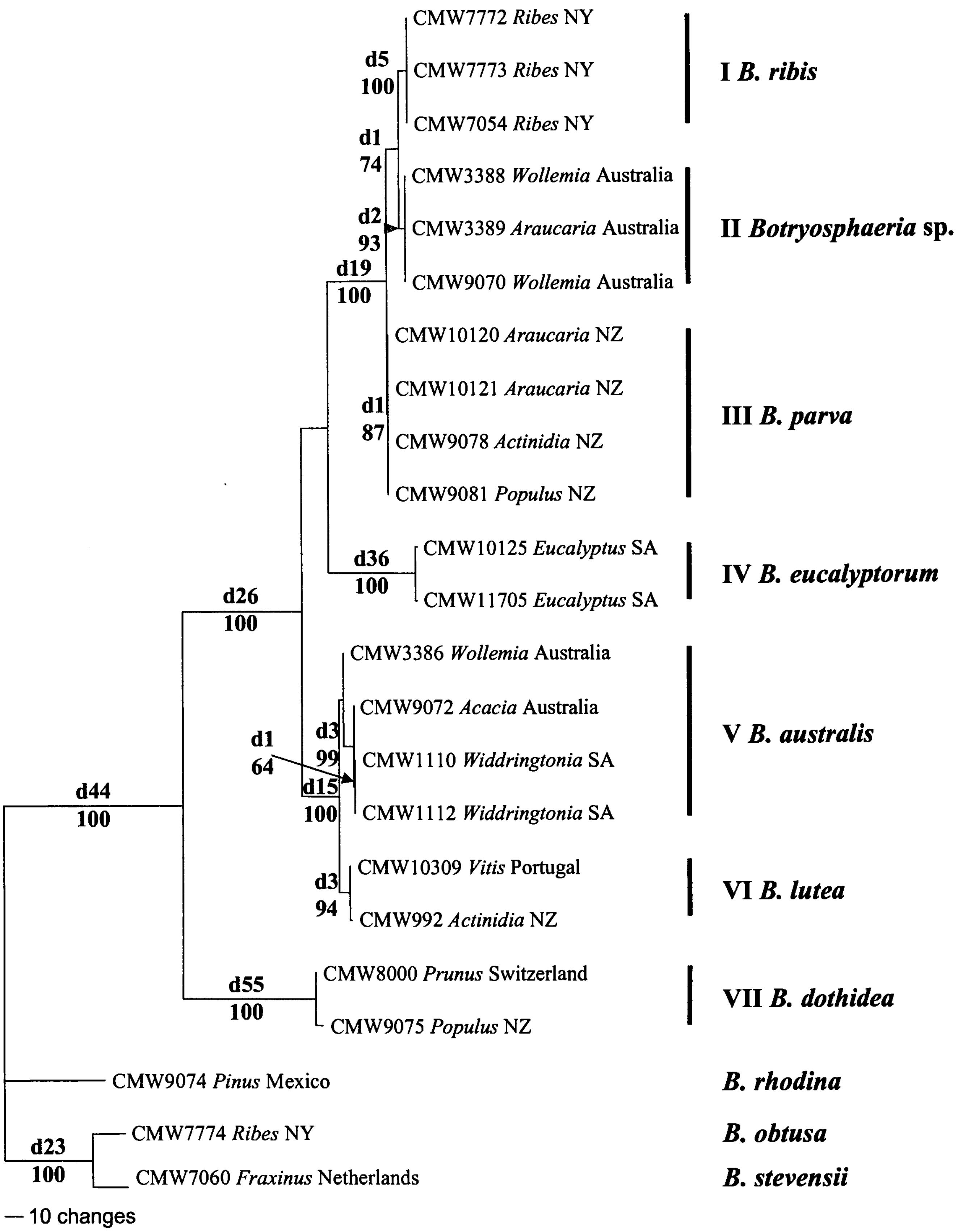
[1] Abbreviations for culture collections and isolates: CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ICMP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

[2] Identities as determined in this study.

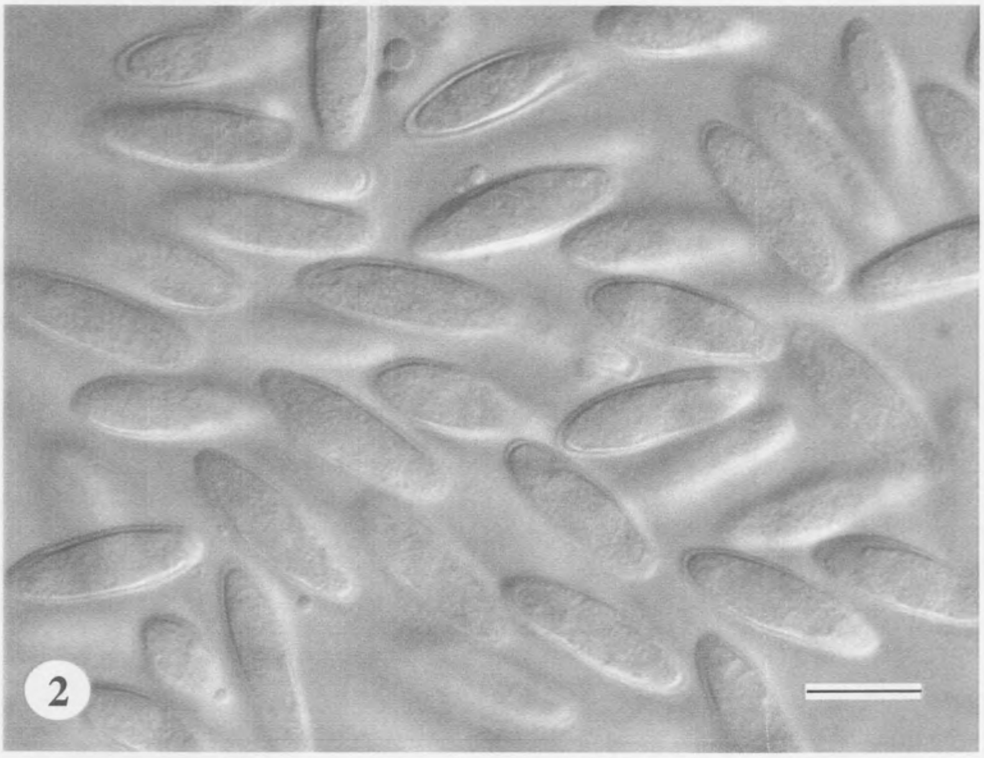
TABLE II. Polymorphic nucleotides (or alleles) from sequence data of the ITS rDNA, β-tubulin and EF-1α, that show the relationship between the unidentified *Botryosphaeria* sp. in Clade II of the phylogenetic analysis, and isolates of *B. ribis* and *B. parva*. Polymorphisms that are unique to the unidentified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

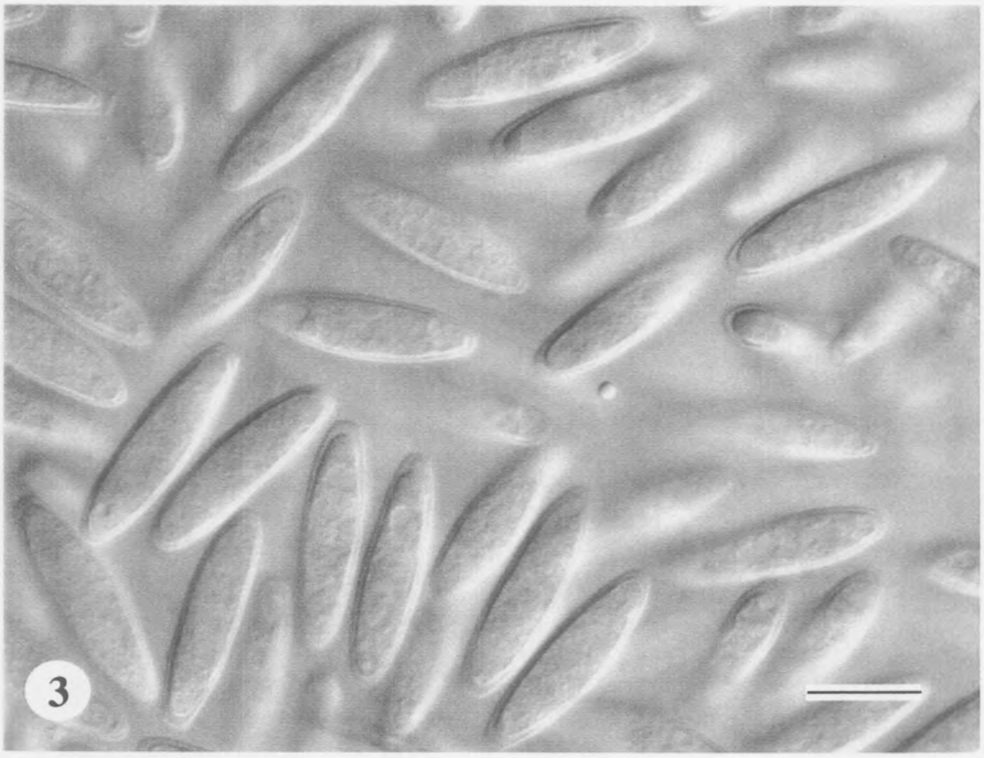
| **Identity** | **Culture no.** | **β-tubulin** | | | | | **ITS** | | | | **EF1-α** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 96 | 129 | 188 | 419 | 437 | 513 | 585 | 797 | 864 | 1081 | 1082 | 1138 | 1145 | 1190 | 1216 | 1251 | 1309 |
| *B. ribis*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

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


CMW7772 *Ribes* NY
CMW7773 *Ribes* NY
CMW7054 *Ribes* NY
CMW3388 *Wollemia* Australia
CMW3389 *Araucaria* Australia
CMW9070 *Wollemia* Australia
CMW10120 *Araucaria* NZ
CMW10121 *Araucaria* NZ
CMW9078 *Actinidia* NZ
CMW9081 *Populus* NZ
CMW10125 *Eucalyptus* SA
CMW11705 *Eucalyptus* SA
CMW3386 *Wollemia* Australia
CMW9072 *Acacia* Australia
CMW1110 *Widdringtonia* SA
CMW1112 *Widdringtonia* SA
CMW10309 *Vitis* Portugal
CMW992 *Actinidia* NZ
CMW8000 *Prunus* Switzerland
CMW9075 *Populus* NZ
CMW9074 *Pinus* Mexico
CMW7774 *Ribes* NY
CMW7060 *Fraxinus* Netherlands
d5 100
d1 74
d2 93
d19 100
d1 87
d36 100
d26 100
d1 64
d3 99
d15 100
d3 94
d55 100
d44 100
d23 100
I *B. ribis*
II *Botryosphaeria* sp.
III *B. parva*
IV *B. eucalyptorum*
V *B. australis*
VI *B. lutea*
VII *B. dothidea*
*B. rhodina*
*B. obtusa*
*B. stevensii*
10 changes

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


2


3

# 7

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

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

## INTRODUCTION

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

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

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

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

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

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

## MATERIALS AND METHODS

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

A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA, as described by Raeder and Broda (1985) and Smith et al (2001). The ITS rDNA region was amplified using the primers ITS1 and ITS4 (White et al 1990). PCR reaction mixtures, PCR conditions and visualization of amplicons were the same as those described by Slippers et al (2003). The amplicons of all three DNA regions were also purified and sequenced as described in Slippers et al (2003), using the same primers described above. BLAST searches were used to detect related sequences from GenBank. Sequences arising from these searches were downloaded from GenBank or obtained from Slippers et al (2003) and included in the analyses (TABLE I). Trees were rooted to GenBank sequence data of the related taxa, *Guignardia bidwellii* (Ellis) Viala & Ravaz. and *Mycosphaerella africana* Crous & M.J. Wingf.

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

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

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

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

# RESULTS

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

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

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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TABLE I. Isolates considered in the phylogenetic study.

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

TABLE I. Continued.

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

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

[2] Identities as used in this study.

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

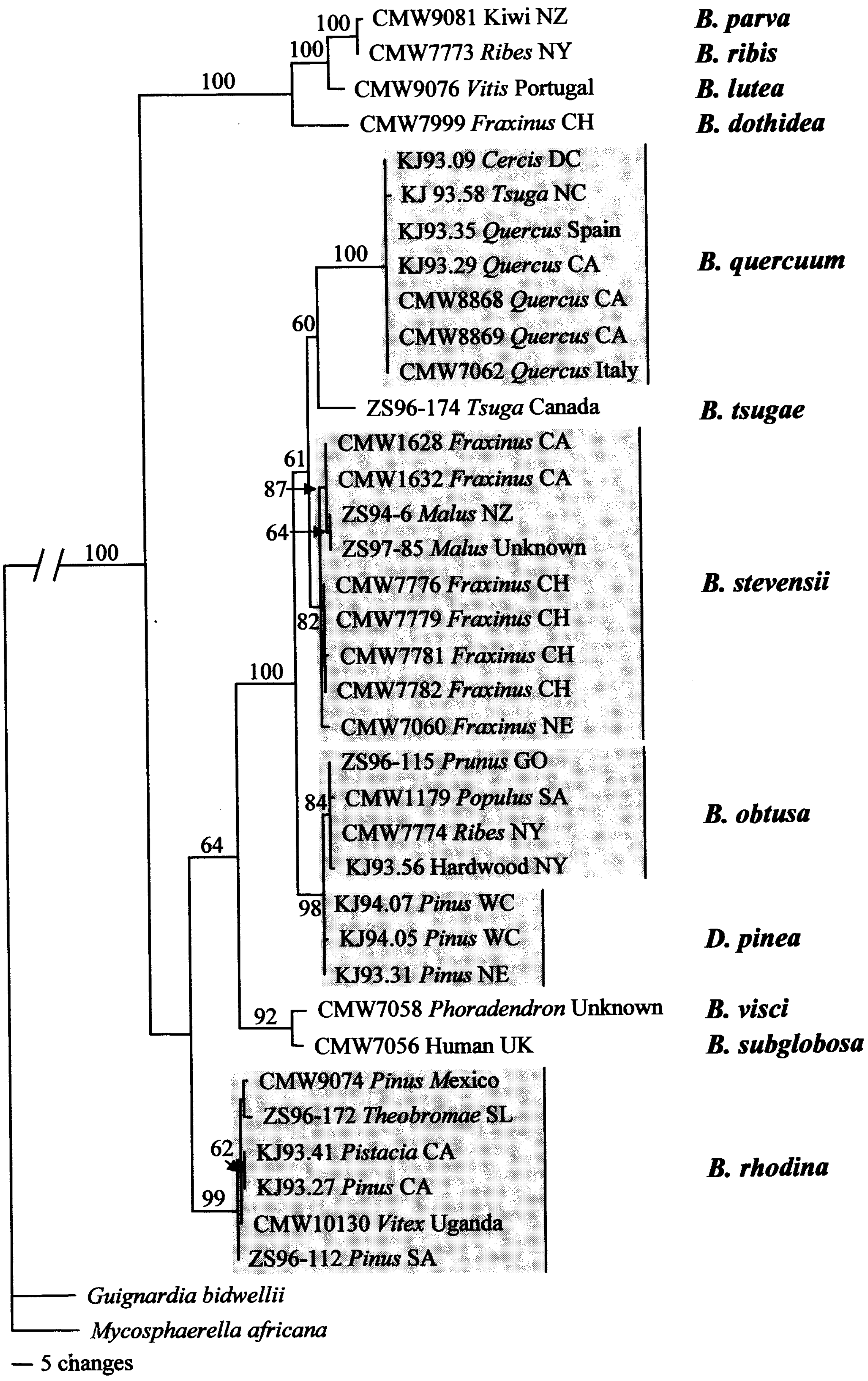
TABLE II. Conidial measurements of selected species of *Botryosphaeria* with *Diplodia* anamorphs.

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

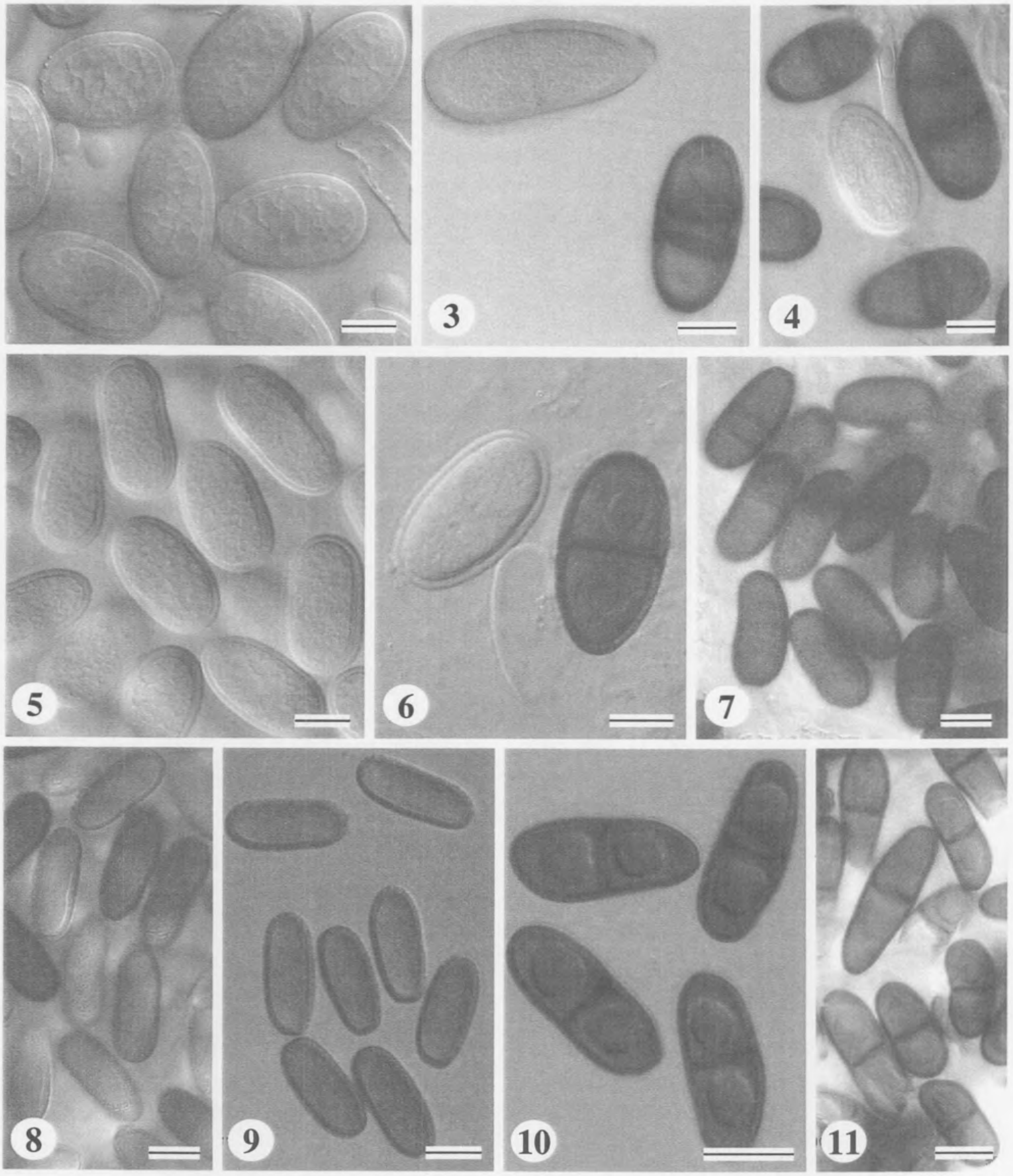
[1] Extreme measurements in brackets are actual ranges. Averages are given between extreme values, and are representative of 15-60 conidia.

[2] Conidia produced *in vitro* as described in Materials and Methods. Other isolates (CMW) are from field collected samples or from herbarium G (G). Measurements for the respective species reported by Shoemaker (1964) are also given.

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


100
CMW9081 Kiwi NZ
100
CMW7773 *Ribes* NY
100
CMW9076 *Vitis* Portugal
CMW7999 *Fraxinus* CH
KJ93.09 *Cercis* DC
KJ 93.58 *Tsuga* NC
KJ93.35 *Quercus* Spain
100
KJ93.29 *Quercus* CA
CMW8868 *Quercus* CA
60
CMW8869 *Quercus* CA
CMW7062 *Quercus* Italy
ZS96-174 *Tsuga* Canada
CMW1628 *Fraxinus* CA
61
CMW1632 *Fraxinus* CA
87
ZS94-6 *Malus* NZ
64
ZS97-85 *Malus* Unknown
100
CMW7776 *Fraxinus* CH
82
CMW7779 *Fraxinus* CH
CMW7781 *Fraxinus* CH
100
CMW7782 *Fraxinus* CH
CMW7060 *Fraxinus* NE
ZS96-115 *Prunus* GO
84
CMW1179 *Populus* SA
CMW7774 *Ribes* NY
64
KJ93.56 Hardwood NY
98
KJ94.07 *Pinus* WC
KJ94.05 *Pinus* WC
KJ93.31 *Pinus* NE
92
CMW7058 *Phoradendron* Unknown
CMW7056 Human UK
CMW9074 *Pinus* Mexico
ZS96-172 *Theobromae* SL
62
KJ93.41 *Pistacia* CA
99
KJ93.27 *Pinus* CA
CMW10130 *Vitex* Uganda
ZS96-112 *Pinus* SA
*Guignardia bidwellii*
*Mycosphaerella africana*
5 changes
**B. parva**
**B. ribis**
**B. lutea**
**B. dothidea**
**B. quercuum**
**B. tsugae**
**B. stevensii**
**B. obtusa**
**D. pinea**
**B. visci**
**B. subglobosa**
**B. rhodina**

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


3
4
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11

# 8

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

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

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

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

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

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

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

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

Twenty primer sets were designed and used to amplify the fragments from isolate CMW10122, as well as from isolates CMW1239, CMW2283, CMW7885 and CMW2387. PCR reactions were conducted using the Expand High Fidelity *Taq* polymerase enzyme and accompanying chemicals, following to the instructions of the manufacturer (Roche Molecular Biochemicals). Initial amplification was done at 54 C annealing temperature. Where multiple bands were obtained, annealing temperature was increased stepwise to 60 C until single bands were obtained. PCR amplicons were subjected to electrophoresis on 1.5 % agarose gels, stained with ethidium bromide, and visualized under UV illumination. Where size polymorphism of amplicons was not already visible on the agarose gels, the products were cleaned and sequenced, using the same primers as those used for amplification.

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

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

Species of *Botryosphaeria* belong to two groups based on their asexual states, namely those with fusicoccum-like and those with diplodia-like asexual spores (Denman et al 2000). *Botryosphaeria parva,* and most of the other species tested in this study, belongs to the *Botryosphaeria* group that has *Fusicoccum* anamorphs. When markers were tested for their ability to amplify polymorphic regions in *Diplodia pinea* (CMW2389) and *B. rhodina* (CMW2388), fragments of similar size to those found in *B. parva* (CMW10122) were amplified by only three of the eight primer pairs (TABLE II). These fragments were not sequenced.

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

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---, ---, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence. Mycologia 95:294--307.

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

| SSR Primers[1] | Primer sequence (5'-3') | Core sequence[2] | Fragment length (bp) | Annl Temp C | Melt Temp C | Comments[3] |
|---|---|---|---|---|---|---|
| BOT 1 | CGG ACA GGT CGC ATT CGC G | *(GAAG)$_2$ *GACA rich *GC rich | 187 | 54 | 64 | Monomorphic |
| BOT 2 | CTT GCC GTT GCC CTT GAG CG | *(CAA)x8(GAA)x6 interrupted | | | 66 | RFLP - *Hae*III |
| BOT 11 | CGG CAT GGT CTG CCG CTC C | *(GCAT)(GCAC)$_3$(GC)$_3$(GCAT) | 429 | 54 | 66 | Polymorphic |
| BOT 12 | GCA TCT CCG GCT ACC AAC CG | *(GA)(CA)(GT)(CT) alternating | | | 66 | RFLP - *Sau*3AI |
| BOT 15 | CTG ACT TGT GAC GCC GGC TC | *(TCTTCG(T/A)GGCGG)$_4$ | 374 | 54 | 66 | Polymorphic |
| BOT 16 | CAA CCT GCT CAG CAA GCG AC | *(CT)$_{13}$(CTT)$_8$(CTTT)$_2$(CTTTT) alternating | | | 64 | RFLP – *Cfo*I |
| B-GW2 | CAC CGA AGG CTC AAA GCA CC | *(CTT)$_{1\text{-}2}$x9(CTTT)$_{1\text{-}2}$x5(CTTTTT)$_2$ interrupted | | | 64 | |
| BOT 17 | GGC GCA ATC TCG ATT CGA GC | *(CAT)$_{12}$ | 362 | 54 | 64 | Polymorphic |
| BOT 18 | CCA CGA TGT CCG TTC ATC G | | | | 64 | |
| BOT 19 | GGC GGT CGC AGA TGC GGT C | *(GCT)$_7$T(GCT) *(GCT)$_{1\text{-}3}$ and (GC)$_{1\text{-}3}$ | 274 | 54/58 | 66 | Polymorphic |
| BOT 20 | GCC CTA TTC TGC GTG CCT CC | scattered throughout | | | 66 | |
| BOT 21 | CGC CAC CTG CCT CGC AGC AG | *(GAC(AorG))$_8$(GATA) *CT(GT)$_5$GG | 211 | 58 | 70 | Polymorphic |
| BOT 22 | GAC AGG AAC GTA ACT GCG ATC C | | | | 68 | |
| B-GW3 | GGC TGT ACG TAC CCT TCA AGC C | | | | 70 | |
| BOT 23 | CAT CGC ACA GGA GCC GAT TCT | *(T)$_{1\text{-}6}$*(A)$_{1\text{-}8}$ interrupted *(CT)$_3$*(CCT)$_2$ | 415 | 54 | 66 | Polymorphic |
| BOT 24 | CAT ACA TCG AGC TTT CTT GAG GG | | | | 68 | |
| BOT 27 | GCC GCA GCG GAA CGG TGT CGC | *(TCA)3TCG*(TC)4(CAC)2*(TC)x4(TCC)x3( | 253 | 58 | 70 | Monomorphic |
| BOT 28 | GAC GGC CTG TCG CAA CTC GG | TTC)x4 interspersed | | | 68 | RFLP – *Msp*I |
| BOT 35 | CTC CAT CCT GAT CCA GGG TCC | *CACATCT(CAT)$_4$(CAG)$_2$CGG(CAG)$_8$(CAT)$_2$ | 261 | 54 | 68 | Polymorphic |
| BOT 36 | GAC GAA TCA AGC GGG CTG CCC | CAG(CAA)$_3$ | | | 70 | |
| B-GW7 | CCG AGA CCG AAG GCT GCG CG | | | | 66 | |
| BOT 37 | GGC GTA GCG TGG GCG ACT GG | *(GCCC)$_2$GC*(GAT)$_4$C(GAT)$_4$ | 317 | 54 | 70 | Polymorphic |
| BOT 38 | CCC ATC GCC CAC TCA ACC CG | | | | 68 | |
| B-GW8 | CGT GGT GCT CCG GGC AAG GG | | | | 70 | |

[1] BOT primer numbers are those used for amplification. B-GW primers were used for genome walking.

[2] Core sequences are as observed in amplicons of CMW10122. Subscript numbers refer to the number of uninterrupted repeats, while 'x' refers to the number of times that a motive is found, interrupted, throughout the fragment.

[3] The morphism refers to the size of the fragments. Some loci that did not vary in length, however, had single point mutations that were useful for RFLP analysis.

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

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

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

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

# 9

# Differentiation and phylogeography of cryptic species in the *B. parva* - *B. ribis* complex

***Abstract:*** The identities of *B. parva* and *B. ribis* have been confused in the past because they share hosts, geographical distribution and have overlapping morphological features. A previous study using multiple gene genealogies and conidial morphology of representative ex-type and additional authentic isolates of each of these species has shown that they are distinct, but closely related. In this study, the boundaries between *B. ribis* and *B. parva* were investigated, using multiple gene sequence genealogies, together with data from highly polymorphic simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) marker data. Analyses of these data sets illustrate inter-specific boundaries and intra-specific groups. The RFLP markers provided a rapid means of distinguishing between *B. ribis* and *B. parva.* Ex-type isolates of these two species grouped within well-defined clades in sequence and SSR marker analyses and are defined as the *sensu stricto* groups of these species. However, certain groups of isolates clustered closely with, but outside the groups defining the species. These isolates are designated here as *sensu lato* groups of *B. parva* and *B. ribis.* The results show clearly that *B. parva* has been moved to new environments around the world on hosts such as *Eucalyptus.* The same genotypes of this species occurred on three continents and on up to five hosts, including indigenous and exotic hosts in countries such as Australia. Results, furthermore, suggest that recombination occurs randomly between isolates representing a world-wide population of *B. parva sensu stricto*, but that clonal reproduction also plays an important role in structuring the population of this fungus.

## INTRODUCTION

Species of *Botryosphaeria* are well known pathogens of a wide range of agricultural crops and forest trees (von Arx 1987). These fungi infect trees directly through stomata and other natural openings and can they exist as endophytes within healthy plant tissues (Fisher et al 1993, Smith et al 1996). When plants are subjected to environmental stress, endophytic infections can rapidly give rise to tissue colonization and disease symptoms. Disease symptoms include cankers and die-back, and these can lead to death of the trees (von Arx 1987, Sinclair et al 1987).

For many years, the taxonomy of *Botryosphaeria* spp. was based on spore morphology and host association. Recent studies have shown that both characters are unreliable, making the taxonomy of these fungi confused and many literature reports of species questionable or incorrect (Denman et al 2000, Slippers et al 2003). Some species of *Botryosphaeria,* identified based on spore morphology, have been found on more than one host (Punithalingam and Holliday 1973, Punithalingam and Waller 1973). Similarly, a single plant species can also be infected by several *Botryosphaeria* spp. (Denman et al 2003, Chapters 2, 5). The teleomorphs of *Botryosphaeria* spp. are rarely encountered in culture and where they are present on host tissue, ascospores of different species overlap in size ranges (Pennycook and Samuels 1985, Slippers et al 2003). Thus, identifications are commonly based on anamorph spore size and shape, but these also overlap between species (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003).

Recent studies have introduced analysis of DNA sequence data to resolve taxonomic questions pertaining to *Botryosphaeria*. These studies have contributed substantially to our understanding of the phylogenetic relationships between species (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001). They have also assisted in the identification and description of previously unknown species (Smith et al 2001, Phillips et al 2002, Denman et al 2003). Multiple gene sequence genealogies have, however, revealed that some groups identified using single gene phylogenies harbor cryptic species (De Wet et al 2003, Slippers et al 2003).

*Botryosphaeria parva* Pennycook & Samuels and *B. ribis* Grossenb. & Duggar are species that have often been confused (Smith and Stanosz 2001, Slippers et al 2003). Subsequent to the description of *B. ribis* (Grossenbacher and Duggar 1911), fungi resembling this species and its anamorph, *F. ribis* Slippers, Crous & M.J. Wingf., have been identified from many different hosts, worldwide (Punithalingam and Holliday 1973). *Botryosphaeria parva* was described for the first time in 1985 (Pennycook and Samuels 1985), and until recently was not known outside Australasia. Studies based on ITS rDNA sequence data have not been able to separate *B. ribis* and *B. parva*, but ISSR markers have shown that they reside in separate groups (Smith and Stanosz 2001, Zhou and Stanosz 2001, Zhou et al 2001). Slippers et al (2003) used sequence data of three gene regions to confirm that these are distinct species. Subsequent studies using the same multiple gene sequence approach have shown that isolates in the *B. parva* – *B. ribis* complex occur widely and on different hosts (Chapters 2, 4-6).

Multiple gene genealogies are increasingly being used to identify cryptic species in fungi (Geiser et al 1998, Taylor et al 2000, Koufopanou et al 2001, Steenkamp et al 2002). Recognition of cryptic species is, however, complicated by the difficulty involved in sampling these species prior to the availability of reliable techniques to identify them (Taylor et al 1999). Fisher et al (2001), however, showed that SSR markers, combined with multiple gene genealogies, can fulfill the dual purpose of indicating inter- and intra-specific variation in *Coccidioides immitis* G.W. Stills, thus alleviating the problem of identifying members of cryptic species, prior to further studies. Similarly, species boundaries and population level variation have been revealed by SSR markers for the cryptic species *Diplodia pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (Burgess et al 2001, 2003, De Wet et al 2003).

In this study, data from SSR markers and multiple gene DNA sequences are used to consider genetic boundaries between *B. parva* and *B. ribis*. Furthermore, PCR RFLP fingerprints are considered as a potential means of rapidly distinguishing these species. In the case of *B. parva,* where a reasonably large collection of isolates was available, the mode of reproduction in the fungus was also considered. Considering the distribution of genotypes also made it possible to determine movement of this species between native and exotic plants, as well as between different geographical regions.

## MATERIALS AND METHODS

*Origin and identity of isolates used.*--One hundred isolates from Australia, Colombia, Hawaii, New Zealand and South Africa, primarily collected by the authors or their collaborators in prior studies, were used in this study (TABLE I). All of these isolates have been characterized previously using morphological characteristics, PCR RFLP and sequence data, and thus shown to belong in the *B. parva* – *B. ribis* complex (Jacobs 2002, Rodas 2003, Slippers et al 2003, Chapters 5, 6). Isolates were obtained from diseased and asymptomatic tissue from a wide range of native and exotic hosts in five different geographic regions (TABLE I), although the majority were from *Eucalyptus* trees. Each isolate originated from a single conidium or hyphal tip to ensure that only single genotypes were considered. All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

*Phylogenetic analysis*.--ITS rDNA, $\beta$-tubulin and elongation factor 1-$\alpha$ (EF1-$\alpha$) sequence data for 22 isolates were obtained from previous studies (Slippers et al 2003, Jacobs 2002, Rodas 2003, Chapters 5, 6) (TABLE II). These data were analyzed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999). The sequence data were manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight.

Repetitive minisatellite regions in the intron of the EF1-$\alpha$ were coded to represent a single, rather than multiple evolutionary events. Data sets from the three gene regions were analyzed separately and in combination. Statistical congruence between the data sets was tested using partition homogeneity tests (PHT) (Farris et al 1995, Huelsenbeck et al 1996) in PAUP.

Most parsimonious trees were found using heuristic searches with stepwise (random) additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch supports, using 1000 bootstrap replicates (Felsenstein 1985), and estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were also determined in PAUP.

*RFLP analysis*.--Three loci with known restriction site polymorphisms, for which primers have previously been developed (Chapter 8), were used to characterize the isolates. Loci amplified with primer pairs BOT1 and BOT2, BOT11 and BOT12, BOT15 and 16 were digested with the restriction endonucleases (RE) *Hae*III, *Sau*3A and *Cfo*I, respectively. Amplicons of primer pairs BOT11 and BOT12, and BOT15 and

BOT16 also contain SSR regions and were subsequently used as size polymorphic markers. Digestions were done as previously described (Chapter 8), and fragments separated on 1.5 % agarose gels stained with ethidium bromide, and visualized under UV light.

*SSR amplification and scoring.*--Eight loci that are known to contain SSR sequences were amplified for all 109 isolates using previously developed primer pairs (Chapter 8). The primers were fluorescently labeled (TABLE III). PCR reaction mixtures and conditions were the same as those described previously (Chapter 8). PCR products were subjected to electrophoresis and visualized under UV light on agarose gels that were stained with Ethidium Bromide, and their concentrations estimated against a λ DNA standard. Amplified products were diluted 1:50 to 1:200, depending on concentration, and one $\mu$l of the dilution mixed with a LIZ$^{\text{TM}}$ internal size standard (Perkin-Elmer Applied Biosystems, Foster City, CA): formamide mix (1:14). The products were then separated on an ABI PRISM 3100 autosequencer (Perkin-Elmer Applied BioSystems). Amplicons of all loci were multiplexed in one lane, as the fluorescent labels and sizes of the fragments did not overlap in most cases. Where they did overlap in the case of BOT17&18 and BOT35&36, these amplicons were analyzed on separate gels. Results were analyzed using the Genescan® 2.1 (Perkin Elmer) and Genotyper® 3.0 (Perkin Elmer) software.

For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele size at each of the eight loci (e.g. BBFEADB) (TABLE IV). Each genotype was then assigned a unique number (TABLE IV). Missing data were treated as distinct characters when assigning numbers to genotypes. All further analyses were performed on clone corrected populations, where only one representative of each genotype was included.

*Distance analyses.*--The genetic structure among the isolates was determined using distance analysis. A clone corrected (only one representative of each genotype) data matrix was compiled from the multistate characters for SSR and RFLP markers (TABLE IV). The relationship between genotypes was determined in PAUP using Unweighted Pair-Group Method with Arithmetic mean (UPGMA) and Neighbor Joining (NJ).

A distance matrix was also calculated based on absolute distance ($D_{AD}$) using the program MICROSAT (Erich Minch; http://hpgl.stanford.edu/projects/microsat/). The total nucleotide length of alleles was used for these analyses, because the variation was due to both changes in microsatellite repeats and the flanking sequence. The distance matrix was used to construct UPGMA (Unweighted Pair-Group Method with Arithmetic mean) trees in MEGA v.2.1 (Kumar et al 2001).

*Mode of reproduction.*--Multilocus linkage disequilibrium was calculated for a world-wide group of isolates representing *B. parva sensu stricto*, using the index of association ($I_A$) (Brown et al 1980, Maynard Smith et al 1993). These isolates were identified using the various techniques described above and they represented the only population for which sufficient isolates were available to justify the analysis. The $I_A$ was calculated for the full and clone corrected (using only one representative of each genotype) dataset and with the multistate characters for each allele in the program MultiLocus (Agapow and Burt 2000). The statistical significance of observed data was determined by comparison with expected data for a 1000 randomly recombining datasets. Furthermore, the gametic linkage disequilibrium (non randomly associated polymorphic loci) was calculated in POPGENE with a probability of $P < 0.05$ (Hartl and Clark 1989). A percentage was thus calculated using the formula:

{Observed number of significant linkage disequilibria between two loci
in a group / [x(x-1/2)] where x is the number of alleles in the group}100.

## RESULTS

*Phylogenetic analyses.*--Among the individual gene sequence datasets that were analyzed, the EF1-$\alpha$ data had 8 informative sites and gave the highest level of resolution. The ITS rDNA sequence dataset had 6 informative sites (FIG. 1). The $\beta$-tubulin sequence dataset had the least variation with 5 informative sites and the clades of the tree based on these data were not well resolved (FIG. 1). The partition homogeneity test showed that DNA sequence data sets for the three regions could be combined (PHT P = 0.74) and the final combined dataset consisted of 1238 characters.

Based on the various analyses of the three DNA sequence datasets, *sensu lato* and *sensu stricto* groups were defined for *B. parva* (FIG. 2). The different analyses of the DNA sequence data indicated a consistent clade (66--74 % bootstrap support), which included the ex-type and other authentic isolates of *B. parva* (FIGS. 1, 2). This clade is considered to represent *B. parva sensu stricto.* Four isolates grouped more closely to the *B. parva s.str.* than to *B. ribis*, although their positions in the various trees were not well resolved (FIGS. 1, 2). These isolates, together with those representing *B. parva s.str.*, are considered to represent a *B. parva sensu lato* clade (FIG. 2).

Isolates believed to represent *B. ribis sensu lato* consistently grouped together and were well supported in most trees (67--94 % bootstrap value) (FIGS. 1, 2). Within *B. ribis s.l.*, the sub-clade representing ex-type and other isolates of *B. ribis* from *Ribes* in New York, the origin of the type strain, was always well supported (67--99 % bootstrap support) (FIGS. 1, 2). Isolates residing in this clade are believed to represent *B. ribis sensu stricto*. The other subgroup in the *B. ribis s.l.* clade (FIGS. 1, 2) correlated with isolates from *Wollemia* in Australia and one isolate from *Eucalyptus* in Hawaii. These are clearly different to *B. ribis s.str.*, although closely related to it.

*RFLP analysis.*--Restriction sites were scored as present (1) or absent (0), depending on whether the enzyme digested the fragment or not. Isolates of *B. ribis* and *B. parva* were not polymorphic for the loci amplified with primers pairs BOT1 and BOT2, and BOT11 and BOT12. These loci contain polymorphisms for isolates that are closely related to *B. parva* and *B. ribis* that are not treated here. Amplicons obtained using primers BOT15 and BOT16 contained a restriction site for *Cfo*I in isolates of *B. ribis s.l.*, but not in isolates of *B. parva s.l.* (TABLE IV). The *sensu stricto* and *sensu lato* groups within each of *B. ribis* and *B. parva* could not be distinguished using RFLP data.

*SSR amplification and scoring.*--The eight primer pairs amplified fragments ranging from 199 bp to 437 bp, which represented 63 alleles (TABLE III). All the loci were polymorphic within and between groups of isolates representing *B. ribis* and *B. parva.* The locus amplified by primers BOT19 and BOT20 were found to be hyper-variable (TABLE IV). There were also many missing data points for this locus (TABLES III, IV). Data from this locus were, therefore, disregarded in all further analyses. Primers BOT21 and BOT22 also did not amplify a fragment for a number of isolates. The lower variability and greater size differences between alleles obtained from these primers, however, made the data from these primers acceptable for inclusion in the analyses.

*Distance analyses*.--UPGMA and NJ distance analyses of multistate allele characters of the SSR and RFLP markers, reflected the same relationships among isolates as those based on sequence data (FIG. 3). The relationships among the isolates were the same when only SSR marker data were used, although the groups were less well resolved (data not shown).

Isolates that had been characterized as *B. parva s.str.* based on sequence data, consistently grouped together based on SSR and RFLP marker data (FIG. 3). Isolates belonging to this group originated from all the regions studied, except New York, and from all the hosts sampled, except *Wollemia* (TABLE IV). Some of these genotypes such as genotype 5 occurred on three continents or as in the case of genotype 8, on up to five hosts (TABLE IV). There was no substructure linked to geography or host for isolates in this clade and they are considered to represent a worldwide meta-population of *B. parva s.str.* (FIG. 3).

Isolates defined as *B. parva s.l.* based on sequence data, grouped close to the *B. parva s.str.* group based on SSR and RFLP data (FIG. 3). These isolates originated from *Eucalyptus* in South Africa, Colombia and Hawaii, as well as one from *Mangifera indica* in South Africa and two isolates from *Tibouchina* in Australia (TABLE IV). One isolate (CMW2475, SSR genotype 45), however, grouped with *B. parva s.str.* based on SSR data, contrary to its placement in *B. parva s.l.*, based on sequence data.

Isolates identified as *B. ribis s.l.* and *s.str.* based on sequence data grouped together based on SSR and RFLP marker data. This is possibly because *B. ribis s.str.* was represented by only three isolates from *Ribes* in New York, which contain only two genotypes. In contrast, *B. ribis s.l.* isolates were from diverse origins such as *Eucalyptus* in Colombia and Hawaii, *Mangifera* in South Africa, as well as *Araucaria* and *Wollemia* in Australia.

*Mode of reproduction*.--Only *B. parva s.str.* included sufficient isolates within a defined population to justify an investigation of mode of reproduction based on $I_A$. The observed values for the $I_A$ of these isolates fell within the range of the randomized dataset for the full and clone corrected datasets (FIG. 4A, B). This indicates that the alleles are distributed randomly among the isolates, suggesting random mating in the population. This is supported by a non-significant P-value for the clone corrected dataset ($P = 0.342$). However, the P-value for the observed $I_A$ for the full dataset was statistically significant ($P = 0.003$), indicating that the null-hypothesis of non-random mating cannot be rejected. This is most likely due to the presence of a number of isolates belonging to the same genotype in the full dataset. This would imply a high level of asexual reproduction, despite the ability of the fungus to undergo sexual reproduction. Furthermore, the value for linkage disequilibrium for alleles among isolates of *B. parva s.str.* was low (11 %), supporting the hypothesis of random mating among these isolates of *B. parva s.str.*

## DISCUSSION

In this study the separation of the closely related *B. parva* and *B. ribis* was confirmed using combined gene genealogies, SSR makers and PCR RFLP fingerprints. These data and especially the RFLP profiles have also provided a rapid and effective means to distinguish between these species. Furthermore, following characterization of *B. parva* and *B. ribis,* it was shown that these fungi, and especially *B. parva*, have been moved around the world on hosts such as *Eucalyptus*. It was clear from the SSR marker data that there is recombination among a world-wide population of *B. parva*, which was the only species for which sufficient isolates were available to consider mode of reproduction. Despite *B. parva* having the capacity to undergo sexual exchange, a number of clonal lines occurring across spatial and temporal boundaries were identified for this fungus.

Using a combination of multiple gene sequence data, SSR markers and RFLPs, is was possible to define both broad and strict boundaries for *B. parva*. Based on analysis of sequence data, the clade including the ex-type and other isolates linked to the original description of *B. parva* (Pennycook and Samuels 1985) is considered to represent this species in the strict sense. These isolates are referred to as representing *B. parva sensu stricto*. They also grouped together based on SSR marker data adding to our confidence that they reflect a closely defined group.

The SSR marker data showed no substructure among isolates of *B. parva s.str.* that could be correlated to geographic origin. In contrast isolates representing distinct genotypes were randomly distributed across Australasia, South Africa and Hawaii. Isolates having identical genotypes were found to occur between geographical regions, such as genotypes 1, 5, 8, 12 and 14 that occurred in Australia, New Zealand, Hawaii and South Africa. This significant overlap in the occurrence of discrete genotypes between the regions is most likely linked to their occurrence on *Eucalyptus*. This native

Australian tree has been introduced numerous times into South Africa and Hawaii, and germplasm continues to be exchanged, mainly but not exclusively in the form of seed. *Botryosphaeria parva* is an endophyte of *Eucalyptus* (Smith et al 1996, Slippers et al 2003) and is, therefore, easily introduced into new regions on healthy plant material and probably also on seed.

No evidence of host specialization was found for isolates of *B. parva s.str.* This fungus was found on eight of the nine hosts studied. These eight hosts are from diverse plant families including both gymnosperms and angiosperms. Furthermore, single *B. parva* genotypes were found to co-infect up to five different plants. The lack of specialization found for *B. parva* in this study implies that the importation of new genotypes of this fungus on one host could negatively affect other hosts in a region.

Certain genotypes of *B. parva s.str.* were found to co-infect native and exotic hosts in Australia. For example, genotype 9 was found on *Eucalyptus*, *Mangifera* and *Tibouchina*, and genotype 8, was found on *Eucalyptus*, *Tibouchina* and *Camphor*. Native *Eucalyptus* could thus be directly affected by genotypes that are introduced with foreign plants. The introduction of foreign plant species harboring *B. parva* genotypes to which native plants might not have resistance could seriously damage these native plants. Such movement of pathogens between introduced hosts and native *Eucalyptus* trees is seen as a one of the significant threats facing these native Australian plants (Burgess and Wingfield 2001). Likewise, introduced agricultural or ornamental plants could be negatively affected by genotypes of *B. parva* that originate on native plants.

The diversity amongst isolates of *B. parva s.str.* differed between the three regions studied. This might provide some indication of the origin of this fungus. If missing data are disregarded, only three genotypes were found on *Eucalyptus* and two on *Mangifera,* amongst 21 isolates collected in South Africa. In contrast, six genotypes were found amongst only eleven Hawaiian isolates from *Eucalyptus*. Isolates from Australasia, including Australia and New Zealand were the most diverse, with 11 genotypes from 24 isolates on seven hosts. This might indicate an Australasian origin for *B. parva*. It is unlikely that the higher diversity in Australasian isolates of this fungus reflects the more diverse range of hosts sampled from this region. This is because there is no evidence for host specialization in this taxon as previously discussed.

From the SSR allele disequilibrium and $I_A$ analyses, it is clear that the alleles are randomly associated across the global population of *B. parva s.str.* This indicates that the fungus is undergoing sexual recombination (Brown et al 1980, Maynard Smith et al 1993, Agapow and Burt 2000). Despite this apparent ability to reproduce sexually, the recovery of identical genotypes of *B. parva s.str.* across continents and during different seasons suggests that clonal reproduction also plays and important role in structuring the population. This observation is supported by analyses when the dataset was not clone-corrected. These results regarding reproduction in *B. parva s.str.* reflect those of previous studies on other fungi, where a combination of clonal and sexual processes has been shown to significantly influence population structure (Taylor et al 1999). For example, in an extensive study of the ascomycete *Sclerotinia sclerotiorum* (Carbone et al 1999), evidence for recombination was clearly shown even though the fungus primarily reproduces clonally.

The low level of diversity in *B. parva s.str.* in countries such as South Africa is inconsistent with the notion that recombination occurs freely among isolates. A possible explanation for this result would be that the fungus is primarily homothallic. This is possible because a preliminary study on *B. eucalyptorum* (Van Geuns and Slippers, unpublished data) has shown that single ascospore cultures could give rise to pseudothecia producing viable ascospores. This does not necessarily exclude the possibility of outcrossing, because many homothallic and pseudohomothallic fungi retain a certain level of outcrossing, albeit at low levels (Taylor et al 1999).

Based on DNA sequence and SSR marker data, some isolates included in this study were more closely related to *B. parva* than to *B. ribis*, but did not group within the *B. parva s.str.* clade. These isolates are designated as *B. parva sensu lato*. The isolates residing in this *B. parva s.l.* clade also shared a common RFLP marker profile with *B. parva s.str.*, where none of the REs cut in the amplified fragments. The unresolved relationships of isolates in the *B. parva s.l.* group arise from the fact that they share some polymorphisms with both *B. ribis* and *B. parva s.str.* These shared polymorphisms might be interpreted as a sign that speciation has not occurred in these isolates. This is an unlikely explanation because each group of isolates had a number of unique alleles among loci of the sequence data, SSR marker data and restriction sites. The fact that the isolates co-occur on the same hosts and in the same areas indicates that species barriers, rather than physical or other barriers, are responsible for the differentiation among these isolates.

Another possible explanation for the shared polymorphisms between isolates of *B. parva* and *B. ribis* is that they are due to hybridization or introgression between the isolates of the two species. This is a reasonable explanation as hybridization has recently been shown to occur amongst species of fungi and this process may not be uncommon (Brasier et al 1998, Brasier 2000, 2001, Newcombe et al 2000). Brasier (2000, 2001) points out that the probability for such hybridization is increased when geographically distinct pathogen populations are brought into contact, because such taxa often do not form complete reproductive barriers. As shown in this and other studies (Burgess et al 2003, Denman et al 2003, Chapter 5), *Botryosphaeria* spp. have been moved around the world on hosts such as *Eucalyptus*, *Pinus* and Proteaceae, thus making them ideal candidates for hybridization with native species.

Shared polymorphisms between isolates of *B. parva* and *B. ribis* could also be present because their lineages are not completely resolved. Taylor et al (2000) have shown that that fungi that have recently undergone speciation, will share many polymorphisms. This is due to the fact that the amount of lineage sorting is directly proportional to time after speciation has occurred. The few fixed polymorphisms between *B. parva* and *B. ribis* compared to other closely related *Botryosphaeria* spp. indicate that they have recently undergone speciation. They might, therefore, be expected to still share a number of polymorphisms.

Isolates representing the broader boundary of *B. ribis*, which is referred to as *B. ribis sensu lato*, grouped together based on DNA sequence, SSR and RFLP marker data. The fragment amplified using the primers BOT15 and BOT16 had a unique restriction site for RE *Cfo*I, which presents a simple method to identify isolates residing in this group. Multiple gene sequence phylogeny identified more than one well supported clade, amongst the isolates representing *B. ribis s.l.* For example, the ex-type culture of *F. ribis* Slippers, Crous & M.J. Wingf. and other isolates from the *Ribes* in New York, USA, were distinct within this clade, in all analyses. These isolates are regarded as representing *B. ribis sensu stricto*. Another sub-clade contained strains isolated from *Wollemia* in Australia and *Eucalyptus* in Hawaii. Representative isolates from populations will be required to determine whether these subgroups indicate species barriers or distinct populations of the same species.

The consistencies between the SSR and RFLP polymorphic marker analysis and multiple gene sequence data shows that co-dominant marker data are phylogenetically informative. The phylogenetic value of SSR marker data has been questioned because of constraints on the range of allele sizes and their high rate of mutation, resulting in high incidences of homoplasy (Nauta and Weissing 1996, Orti et al 1997, Fisher et al

2000). Other studies on fungi such as *D. pinea* and *Coccidioides immitis* have, however, also shown that that SSR data can be phylogenetically informative (Fisher et al 1997, Burgess et al 2001, De Wet et al 2003). These markers will thus be very useful to confirm speciation event between closely related groups that are less clearly resolved using other techniques.

The subgroups within *B. ribis s.l.* and *B. parva s.l.* are easily identifiable by the SSR marker data used in this study. The ability to identify isolates that represent these groups will facilitate future selection of isolates needed to determine whether these groups indicate species barriers or distinct populations of the same species. Previous authors (Davis and Nixon 1992, Taylor et al 1999) have noted the inherent difficulties in defining and identifying populations of closely related species or subspecies from which to collect samples. Thus, if populations selected to represent two closely related groups contains a mixture of isolates from both of them, then reproductive isolation might not be detected. This is especially true for fungi such as the *Botryosphaeria* spp. studied here. Because of their cosmopolitan distribution and sympatric occurrence on certain hosts, the delimitation of populations is highly problematic. Fisher et al (2000) also found that SSR data can serve the dual purpose of identifying inter- and intra specific variation among a group of isolates to enable further testing of population and species hypotheses. Results of this study provide added evidence supporting this view.

Polymorphic SSR marker data combined with multiple gene sequence data in this study have provided a substantially increased the available information about the diversity present amongst isolates in the *B. ribis* and *B. parva* complex. The combination of these datasets can now be used to determine species boundaries between subgroups within *B. parva s.l.* and *B. ribis s.l.* This study has highlighted the fact that identification of cryptic species in *Botryosphaeria* cannot rest on phenotypic or single gene phylogenies. Furthermore, the importance of representative numbers of samples is emphasized in this study. Species exist as populations that are not necessarily represented by a small number of isolates. The ability to characterize the inter-specific boundaries between *B. parva* and *B. ribis*, as illustrated in this study, can now be used to further explore the reproductive and evolutionary forces that shape populations of these species. Understanding the variation, interaction and mode of reproduction within species of *Botryosphaeria* is an important prerequisite to understanding the global distribution and pace of evolution of these important plant pathogens.

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TABLE I. Geographical origin and hosts from which isolates were obtained in this study. Isolates from native hosts are highlighted in grey.

| **Origin** | **Host** | **Culture number**[1] |
|---|---|---|
| Australia | *Araucaria* | 3388 |
| | *Cinnamomum* | 6814 |
| | *Eucalyptus* | 6798, 6799, 6802 |
| | *Mangifera* | 7799, 7025, 7026 |
| | *Ribes* | 9071 |
| | *Tibouchina* | 6235, 6236, 6536, 6795, 6797 |
| | *Wollemia* | 3389, 9070 |
| Colombia | *Eucalyptus* | 8930, 8931, 8934--8940, 8942--8948, 8952, 8953, 8955, 8957, 8958, 8964--8966 |
| Hawaii | *Eucalyptus* | 7882--7888, 7891, 7892, 7893a, b, 7894a, b, 7895, 7896--7899 |
| New Zealand | *Actinidia* | 9077--9079 |
| | *Araucaria* | 10120, 10121 |
| | *Populus* | 9080, 9081 |
| | *Tibouchina* | 10117--10119, 10127--10129 |
| New York, USA | *Ribes* | 7772, 7773, 7054 |
| South Africa | *Eucalyptus* | 937, 944, 947, 1217, 1238, 1242, 1304, 1322, 1396, 1492, 1625, 1782, BOT7, BOT19 |
| | *Mangifera* | BOT2283, BOT2292, BOT2294, BOT2298, BOT2299, BOT2324, BOT2325, BOT2330, BOT2341, BOT2347, BOT2370, BOT2390, BOT2404 |

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[1] Numbers are those of CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa. BOT numbers refer to a *Botryosphaeria* sub-collection of CMW.

TABLE II. Isolates of *Botryosphaeria parva* and *B. ribis* considered in the phylogenetic study.

| Culture no.[1,2] | SSR genotype[1] | Identity[3] | Host | Location | Collector | Reference[4] |
|---|---|---|---|---|---|---|
| CMW9071 | 11 | *Botryosphaeria parva s.str.* | *Ribes* sp. | Australia | M.J. Wingfield | Slippers et al 2003 |
| CMW9077 | 1 | *B. parva s.str.* | *Actinidia deliciosa* | New Zealand | S.R. Pennycook | " |
| CMW9078 | 12 | *B. parva s.str.* | *A. deliciosa* | New Zealand | S.R. Pennycook | " |
| CMW9079 | 13 | *B. parva s.str.* | *A. deliciosa* | New Zealand | S.R. Pennycook | " |
| CMW9080 | 8 | *B. parva s.str.* | *Populus nigra* | New Zealand | G.J. Samuels | " |
| **CMW9081** | 14 | *B. parva s.str.* | *P. nigra* | New Zealand | G.J. Samuels | " |
| CMW10120 | 8 | *B. parva s.str.* | *Araucaria heterophylla* | New Zealand | M.J. Wingfield | " |
| CMW10121 | 5 | *B. parva s.str.* | *A. heterophylla* | New Zealand | M.J. Wingfield | " |
| CMW10123 | 5 | *B. parva s.str.* | *E. smithii* | South Africa | H. Smith | " |
| CMW6235 | 28 | *B. parva s.l.* | *Tibouchina lepidota* | Australia | M.J. Wingfield | Chapter 5 |
| CMW6237 | 28 | *B. parva s.l.* | *T. urvilleana* | Australia | M.J. Wingfield | " |
| BOT2475 | 45 | *B. parva s.l.* | *Eucalyptus* sp. | Colombia | C. Rodas | Rodas 2003 |
| BOT2404 | 35 | *B. parva s.l.* | *Mangifera indica* | South Africa | R. Jacobs | Jacobs 2002 |

TABLE II. Continued.

| **Culture no.**[1,2] | **SSR genotype**[1] | **Identity**[3] | **Host** | **Location** | **Collector** | **Reference**[4] |
|---|---|---|---|---|---|---|
| **CMW7772** | 31 | *B. ribis s.str.* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | Slippers et al 2003 |
| CMW7773 | 31 | *B. ribis s.str.* | *Ribes* sp. | New York, USA | B. Slippers/G. Hudler | " |
| CMW7054 | 32 | *B. ribis s.str.* | *R. rubrum* | New York, USA | N.E. Stevens | " |
| CMW3388 | 33 | *B. ribis s.l.* | *Araucaria cunninghamii* | Australia | M. Ivory | Chapter 6 |
| CMW3389 | 34 | *B. ribis s.l.* | *Wollemia nobilis* | Australia | M. Ivory | " |
| CMW9070 | 34 | *B. ribis s.l.* | *W. nobilis* | Australia | B. Summerell | " |
| CMW7885 | 53 | *B. ribis s.l.* | *Eucalyptus* sp. | Hawaii | M.J. Wingfield | Slippers et al 2003 |

[1] Isolates in bold are ex-type.

[2] Designation of genotypes and groups as used later in the study (TABLE IV).

[3] Identities as determined in this study for *sensu lato* (*s.l.*) and *sensu stricto* (*s.str.*) groups of *B. parva* and *B. ribis*.

[4] Study where DNA sequence of the isolate was determined.

TABLE III. SSR primers, labels and size ranges for amplicons obtained in this study.

| **Primer number** | **Label** | **Size ranges (bp)** | **No. of alleles** | **No. not amplified** |
|---|---|---|---|---|
| BOT 11&12 | NED | 420-437 | 5 | 1 |
| BOT 15&16 | PET | 365-395 | 3 | 0 |
| BOT 17&18 | NED | 228-258 | 9 | 5 |
| BOT 19&20 | 6-FAM | 271-311 | 16 | 13 |
| BOT 21&22 | VIC | 199-231 | 5 | 18 |
| BOT 23&24 | 6-FAM | 415-427 | 4 | 0 |
| BOT 35&36 | NED | 221-261 | 9 | 2 |
| BOT 37&38 | VIC | 298-334 | 5 | 2 |

TABLE IV. Genotypes and RFLP profiles of isolates used in this study. Isolates are grouped according to the species groups identified using sequence data.

| **Genotype description**[1] | **SRR loci**[2] | | | | | | | **SSR** | **RFLP loci** | | | **RFLP** | **Not used** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **11&12** | **15&16** | **17&18** | **21&22** | **23&24** | **35&36** | **37&38** | **genotype** | **1&2r** | **11&12r** | **15&16r** | **genotype** | **19&20** |
| ***B. parva sensu stricto*** | | | | | | | | | | | | | |
| SA Euc 1217 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 1238 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 1242 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 1396 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | J |
| SA Euc 1625 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 937 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | L |
| SA Euc 1782 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 947 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | K |
| NZ Act 9077 | B | B | J | D | B | B | B | sr 1 | 0 | 0 | 0 | rf 1 | Q |
| SA Euc 1242 | B | B | J | ? | B | B | B | sr 2 | 0 | 0 | 0 | rf 1 | K |
| SA Euc BOT7 | B | B | J | ? | B | B | B | sr 2 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 1492 | B | B | J | D | B | C | B | sr 3 | 0 | 0 | 0 | rf 1 | K |
| SA Euc 944 | B | B | J | D | B | C | B | sr 3 | 0 | 0 | 0 | rf 1 | M |
| SA Euc 1304 | B | B | J | D | B | B | ? | sr 4 | 0 | 0 | 0 | rf 1 | K |
| SA Euc BOT19 | B | B | F | C | B | C | B | sr 5 | 0 | 0 | 0 | rf 1 | E |
| Aus Tib 6235 | B | B | F | C | B | C | B | sr 5 | 0 | 0 | 0 | rf 1 | E |
| Aus Man 7026 | B | B | F | C | B | C | B | sr 5 | 0 | 0 | 0 | rf 1 | E |
| NZ Tib 10127 | B | B | F | C | B | C | B | sr 5 | 0 | 0 | 0 | rf 1 | E |
| SA Man BOT2298 | B | B | I | C | B | A | B | sr 6 | 0 | 0 | 0 | rf 1 | O |
| SA Man BOT2347 | B | B | I | C | B | A | B | sr 6 | 0 | 0 | 0 | rf 1 | I |
| SA Man BOT2292 | B | B | I | C | B | A | B | sr 6 | 0 | 0 | 0 | rf 1 | J |
| SA Man BOT2299 | B | B | I | D | B | A | B | sr 7 | 0 | 0 | 0 | rf 1 | H |
| SA Man BOT2370 | B | B | I | D | B | A | B | sr 7 | 0 | 0 | 0 | rf 1 | H |
| SA Man BOT2283 | B | B | I | D | B | A | B | sr 7 | 0 | 0 | 0 | rf 1 | I |
| SA Man BOT2324 | B | B | I | D | B | A | B | sr 7 | 0 | 0 | 0 | rf 1 | I |

TABLE IV. Continued.

| Genotype description[1] | SRR loci[2] | | | | | | | SSR | RFLP loci | | | RFLP | Not used |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11&12 | 15&16 | 17&18 | 21&22 | 23&24 | 35&36 | 37&38 | genotype | 1&2r | 11&12r | 15&16r | genotype | 19&20 |
| ***B. parva sensu stricto*** (continued) | | | | | | | | | | | | | |
| Aus Euc 6798 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | G |
| Aus Tib 6797 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | ? |
| Aus Cam 6814 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | G |
| NZ Pop 9080 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | E |
| NZ Tib 10117 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | E |
| NZ Ara 10120 | B | B | F | C | B | A | B | sr 8 | 0 | 0 | 0 | rf 1 | ? |
| Aus Euc 6799 | B | B | F | ? | B | A | B | sr 9 | 0 | 0 | 0 | rf 1 | D |
| Aus Euc 6802 | B | B | F | ? | B | A | B | sr 9 | 0 | 0 | 0 | rf 1 | E |
| Aus Tib 6795 | B | B | F | ? | B | A | B | sr 9 | 0 | 0 | 0 | rf 1 | E |
| Aus Man 7799 | B | B | F | ? | B | A | B | sr 9 | 0 | 0 | 0 | rf 1 | D |
| Aus Man 7025 | B | B | ? | D | B | A | B | sr 10 | 0 | 0 | 0 | rf 1 | E |
| Aus Rib 9071 | B | B | K | C | B | B | B | sr 11 | 0 | 0 | 0 | rf 1 | N |
| NZ Act 9078 | B | B | F | D | B | A | B | sr 12 | 0 | 0 | 0 | rf 1 | G |
| Haw Euc 7887 | B | B | F | D | B | A | B | sr 12 | 0 | 0 | 0 | rf 1 | J |
| NZ Act 9079 | B | B | F | D | B | C | B | sr 13 | 0 | 0 | 0 | rf 1 | E |
| NZ Tib 10128 | B | B | F | D | B | C | B | sr 13 | 0 | 0 | 0 | rf 1 | E |
| NZ Pop 9081 | B | B | F | A | B | C | B | sr 14 | 0 | 0 | 0 | rf 1 | E |
| Haw Euc 7897 | B | B | F | A | B | C | B | sr 14 | 0 | 0 | 0 | rf 1 | E |
| NZ Tib 10129 | B | B | I | ? | B | A | B | sr 15 | 0 | 0 | 0 | rf 1 | E |
| NZ Tib 10118 | B | B | J | C | A | A | B | sr 16 | 0 | 0 | 0 | rf 1 | C |
| NZ Tib 10119 | B | B | G | C | B | C | B | sr 17 | 0 | 0 | 0 | rf 1 | E |
| Haw Euc 7882 | B | B | J | ? | B | A | B | sr 18 | 0 | 0 | 0 | rf 1 | ? |
| Haw Euc 7894b | B | B | J | ? | B | A | B | sr 18 | 0 | 0 | 0 | rf 1 | J |
| Haw Euc 7888 | B | B | G | ? | B | A | B | sr 19 | 0 | 0 | 0 | rf 1 | K |
| Haw Euc 7893a | B | B | K | C | B | A | B | sr 20 | 0 | 0 | 0 | rf 1 | I |
| Haw Euc 7893b | B | B | K | C | B | A | B | sr 20 | 0 | 0 | 0 | rf 1 | J |
| Haw Euc 7894a | B | B | J | C | B | A | B | sr 21 | 0 | 0 | 0 | rf 1 | I |
| Haw Euc 7896 | B | B | F | ? | B | C | B | sr 22 | 0 | 0 | 0 | rf 1 | G |
| Haw Euc 7898 | C | B | F | ? | B | B | B | sr 23 | 0 | 0 | 0 | rf 1 | I |
| Haw Euc 7899 | C | B | F | A | B | B | B | sr 24 | 0 | 0 | 0 | rf 1 | K |

TABLE IV. Continued.

| **Genotype description**[1] | **SRR loci**[2] | | | | | | | **SSR** | **RFLP loci** | | | **RFLP** | **Not used** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **11&12** | **15&16** | **17&18** | **21&22** | **23&24** | **35&36** | **37&38** | **genotype** | **1&2r** | **11&12r** | **15&16r** | **genotype** | **19&20** |
| ***B. parva sensu lato*** | | | | | | | | | | | | | |
| Aus Tib 6236 | B | C | C | C | B | A | B | sr 28 | 0 | 0 | 0 | rf 1 | L |
| Aus Tib 6536 | B | C | C | C | B | A | B | sr 28 | 0 | 0 | 0 | rf 1 | L |
| Haw Euc 7883 | A | B | F | E | D | B | B | sr 29 | 0 | 0 | 0 | rf 1 | K |
| Haw Euc 7884 | A | B | F | E | D | B | B | sr 29 | 0 | 0 | 0 | rf 1 | K |
| Haw Euc 7895 | B | B | F | E | B | D | B | sr 30 | 0 | 0 | 0 | rf 1 | P |
| SA Man BOT2404 | D | B | D | C | D | ? | B | sr 35 | 0 | 0 | 0 | rf 1 | B |
| SA Man BOT2294 | B | B | D | C | D | B | B | sr 37 | 0 | 0 | 0 | rf 1 | C |
| SA Man BOT2390 | ? | B | D | C | D | L | B | sr 38 | 0 | 0 | 0 | rf 1 | N |
| SA Man BOT2330 | D | B | D | C | D | L | B | sr 39 | 0 | 0 | 0 | rf 1 | O |
| SA Man BOT2341 | D | B | D | C | D | A | B | sr 40 | 0 | 0 | 0 | rf 1 | O |
| Col Euc 8955 | B | B | ? | C | D | G | B | sr 44 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8966 | B | B | ? | C | B | F | B | sr 45 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8958 | C | B | D | C | B | F | B | sr 46 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8948 | B | B | D | C | D | F | B | sr 47 | 0 | 0 | 0 | rf 1 | H |
| Col Euc 8930 | B | B | D | C | B | F | B | sr 48 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8931 | B | B | D | C | B | F | B | sr 48 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8935 | B | B | D | C | D | F | B | sr 47 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8936 | B | B | D | C | D | F | B | sr 47 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8937 | B | B | D | C | D | E | B | sr 49 | 0 | 0 | 0 | rf 1 | J |
| Col Euc 8938 | C | B | D | C | D | F | B | sr 50 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8940 | C | B | D | C | B | F | B | sr 46 | 0 | 0 | 0 | rf 1 | ? |
| Col Euc 8942 | B | B | D | C | D | ? | B | sr 51 | 0 | 0 | 0 | rf 1 | K |
| Col Euc 8944 | B | B | D | C | D | F | B | sr 47 | 0 | 0 | 0 | rf 1 | H |
| Col Euc 8945 | B | B | D | C | D | F | B | sr 47 | 0 | 0 | 0 | rf 1 | I |
| Col Euc 8946 | B | B | D | C | B | F | B | sr 48 | 0 | 0 | 0 | rf 1 | M |
| Col Euc 8947 | C | B | D | C | C | G | B | sr 52 | 0 | 0 | 0 | rf 1 | G |

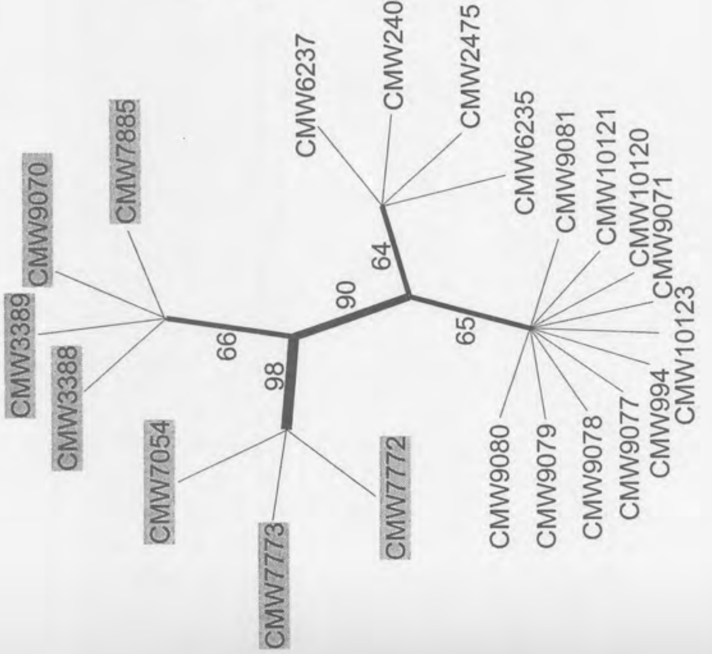
TABLE IV. Continued.

| **Genotype description**[1] | **SRR loci**[2] | | | | | | | **SSR** | **RFLP loci** | | | **RFLP** | **Not used** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **11&12** | **15&16** | **17&18** | **21&22** | **23&24** | **35&36** | **37&38** | **genotype** | **1&2r** | **11&12r** | **15&16r** | **genotype** | **19&20** |
| ***B. ribis sensu stricto*** | | | | | | | | | | | | | |
| NY Rib 7773 | D | B | B | ? | D | F | E | sr 31 | 0 | 0 | 1 | rf 2 | F |
| NY Rib 7772 | D | B | B | ? | D | F | E | sr 31 | 0 | 0 | 1 | rf 2 | G |
| NY Rib 7054 | D | B | B | C | D | F | E | sr 32 | 0 | 0 | 1 | rf 2 | G |
| ***B. ribis sensu lato*** | | | | | | | | | | | | | |
| Col Euc 8965 | C | D | B | C | B | B | C | sr 25 | 0 | 0 | 1 | rf 2 | ? |
| Col Euc 8934 | C | D | B | C | B | B | C | sr 25 | 0 | 0 | 1 | rf 2 | ? |
| Col Euc 8953 | C | D | B | ? | B | B | C | sr 26 | 0 | 0 | 1 | rf 2 | ? |
| Col Euc 8939 | C | D | ? | C | B | B | C | sr 27 | 0 | 0 | 1 | rf 2 | ? |
| Aus Ara 3388 | E | B | B | C | D | E | C | sr 33 | 0 | 0 | 1 | rf 2 | B |
| Aus Wol 3389 | E | B | B | ? | D | E | C | sr 34 | 0 | 0 | 1 | rf 2 | B |
| Aus Wol 9070 | E | B | B | ? | D | E | C | sr 34 | 0 | 0 | 1 | rf 2 | B |
| SA Man BOT2325 | D | B | B | B | B | G | B | sr 36 | 0 | 0 | 1 | rf 2 | I |
| Col Euc 8964 | B | B | ? | C | D | H | ? | sr 41 | 0 | 0 | 1 | rf 2 | F |
| Col Euc 8957 | D | B | B | C | D | F | D | sr 42 | 0 | 0 | 1 | rf 2 | J |
| Col Euc 8952 | D | B | B | C | D | H | C | sr 43 | 0 | 0 | 1 | rf 2 | C |
| Col Euc 8943 | D | B | B | C | D | H | C | sr 43 | 0 | 0 | 1 | rf 2 | D |
| Haw Euc 7885 | E | B | E | A | B | E | B | sr 53 | 0 | 0 | 1 | rf 2 | ? |
| Haw Euc 7886 | E | B | E | A | B | E | B | sr 53 | 0 | 0 | 1 | rf 2 | ? |
| Haw Euc 7891 | E | B | E | A | B | E | B | sr 53 | 0 | 0 | 1 | rf 2 | B |
| Haw Euc 7892 | E | B | E | A | B | E | B | sr 53 | 0 | 0 | 1 | rf 2 | ? |

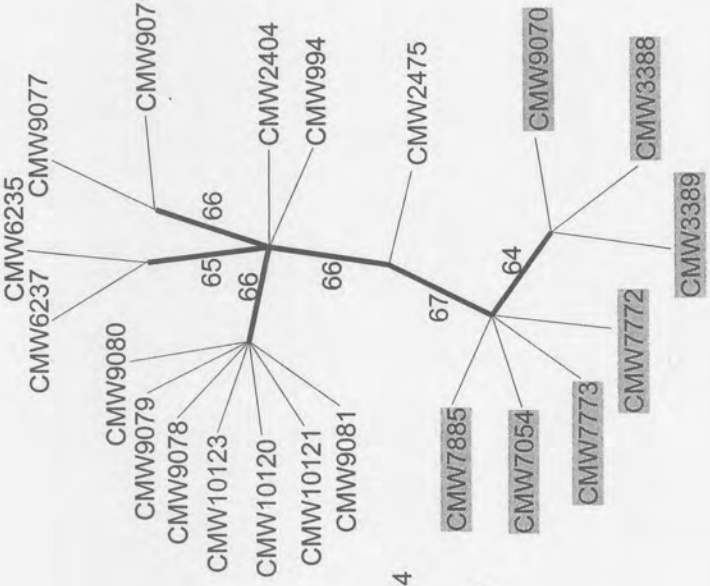
[1] First letters indicate the origin (Aus = Australia; Haw = Hawaii; Col = Colombia; NZ = New Zealand; NY = New York, USA; SA = South Africa). Second letters indicate host (Act = *Actinidia*; Ara = *Araucaria*; Cam = *Camphor*; Euc = *Eucalyptus*; Man = *Mangifera*; Pop = *Populus*; Rib = *Ribes*; Tib = *Tibouchina*; Wol = *Wollemia*). Numbers are CMW numbers (Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria), except numbers indicated as BOT, which is refers to the *Botryosphaeria* sub-collection of CMW.

[2] Missing data are indicated with a '?'. Numbers under SSR or RFLP loci (e.g. 1&2) indicate the BOT primer pair used to amplify the locus. Alleles amplified using the primer pair BOT 19&20 is also included, although it was not used in the final analyses.

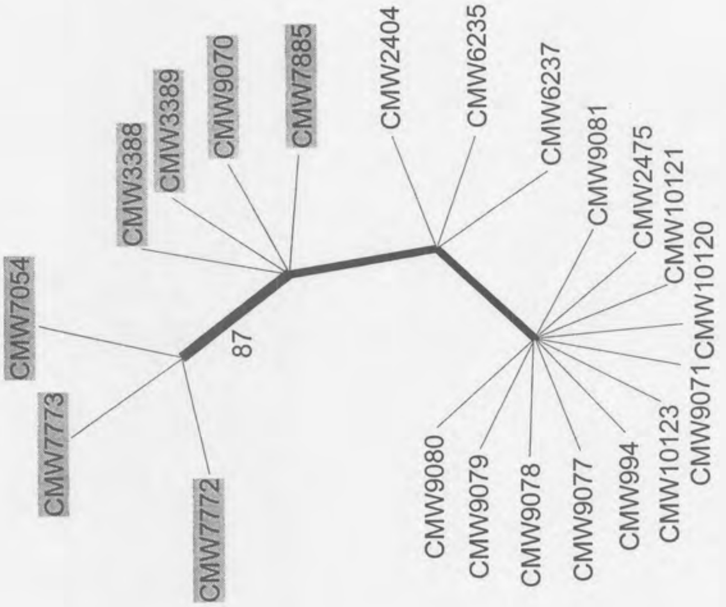
FIG. 1. Unrooted cladograms of the most parsimonious (MP) trees obtained after heuristic searches in PAUP. The thickness of the branches indicates the number of steps, and bootstrap supports (1000 replicates) for the clades are indicated next to the branches. Isolates representing *Botryosphaeria ribis* are shaded, while those representing *B. parva* are not. Trees were obtained from ITS rDNA, *β*–tubulin and EF1-*α* sequence datasets. Number of trees and tree statistics are indicated below the trees, including consistency index (CI), retention index (RI) and phylogenetic signal (g1).



EF1-*α*
1 MP tree
8 steps
CI = 1.0
RI = 1.0
g1 = - 0.85



ITS
1 MP tree
6 steps
CI = 1.0
RI = 1.0
g1 = - 0.57



*β*-tubulin
1 of 6 MP trees
7 Steps
CI = 0.71
RI = 0.92
g1 = - 0.67

FIG. 2. An unrooted cladogram indicating the relationship between *sensu stricto* and *sensu lato* groups of *Botryosphaeria parva* and *B. ribis*, indicated by differentially shaded groups. The tree was obtained from heuristic searches of combined dataset of the ITS rDNA, *β*–tubulin and EF1-*α* sequences. Ex-type isolates are underlined.

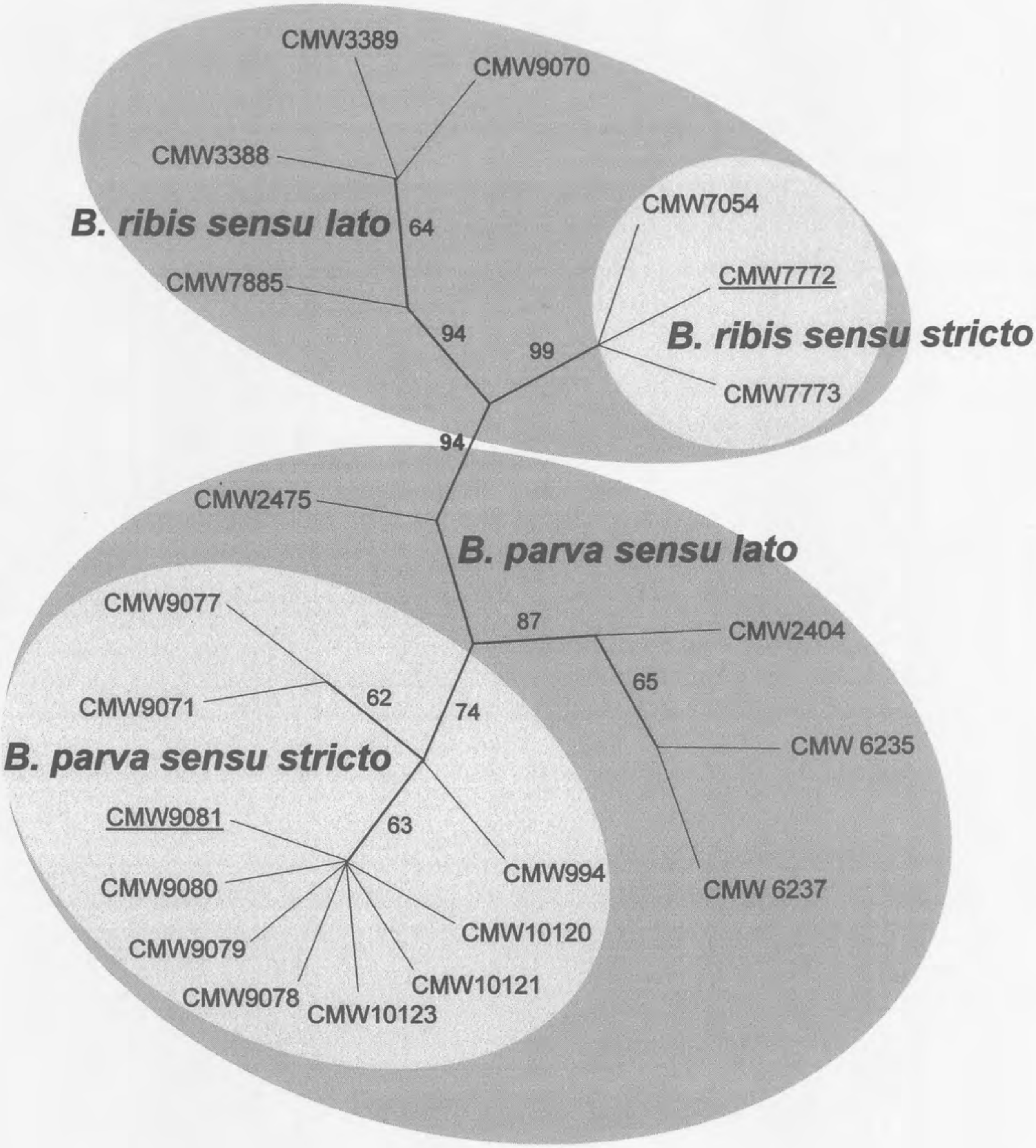
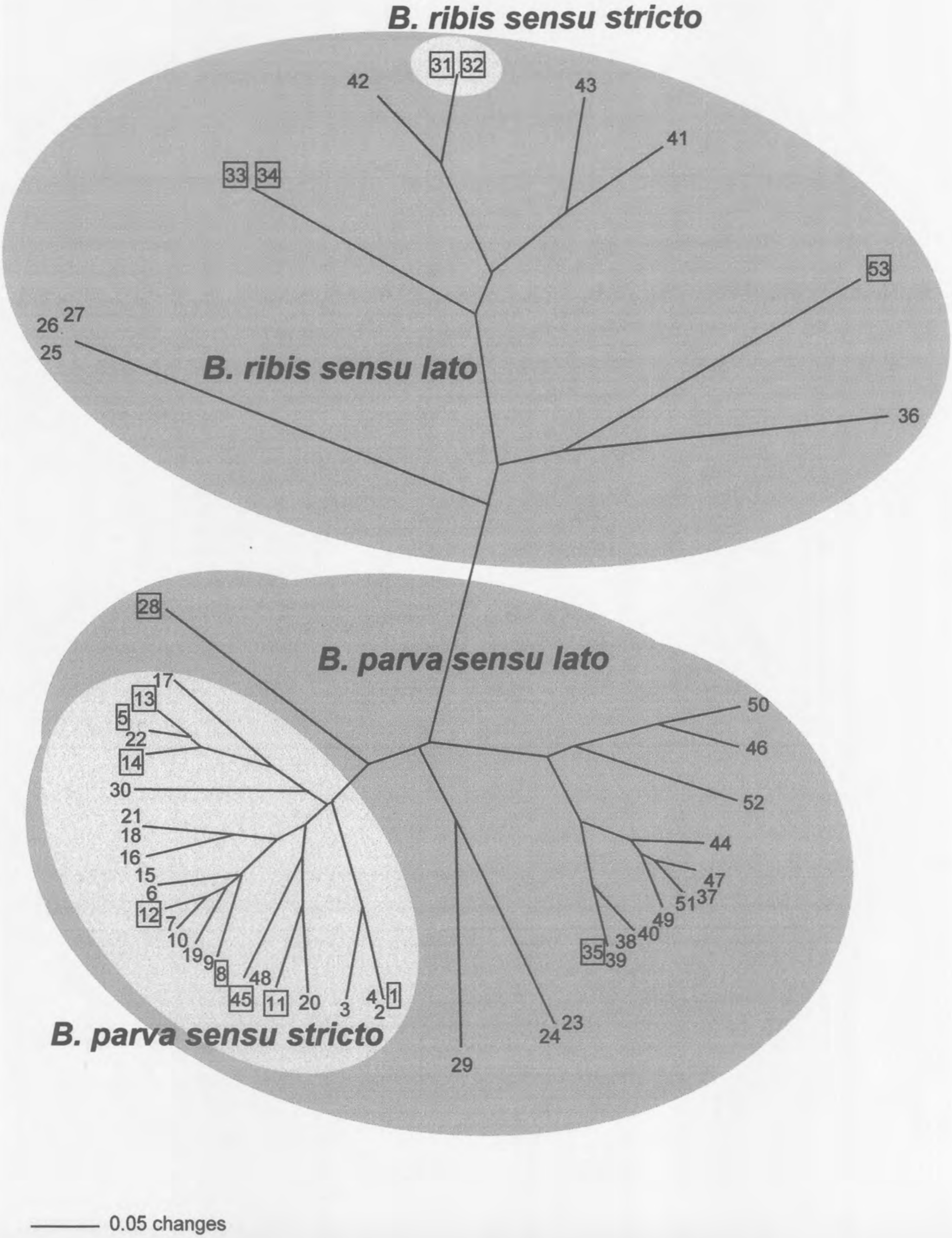
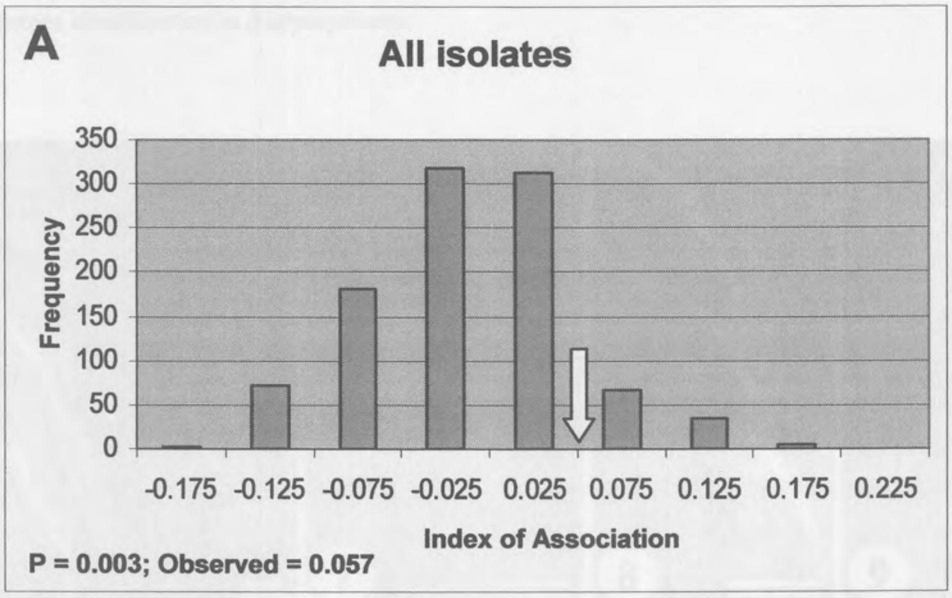

CMW3389
CMW9070
CMW3388
B. ribis sensu lato
64
CMW7054
CMW7772
B. ribis sensu stricto
CMW7885
94
99
CMW7773
94
CMW2475
B. parva sensu lato
CMW9077
87
CMW2404
65
CMW9071
62
74
CMW 6235
B. parva sensu stricto
CMW9081
63
CMW994
CMW 6237
CMW9080
CMW10120
CMW9079
CMW10121
CMW9078
CMW10123

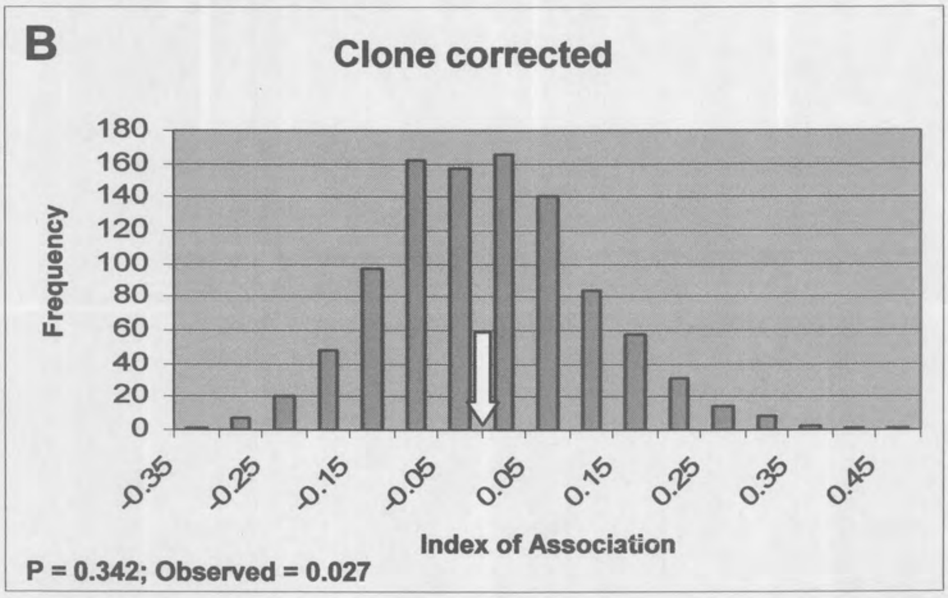
FIG. 3. An unrooted UPGMA dendrogram depicting the distance relationships between genotypes identified using polymorphic SSR markers. Genotypes that were sequenced and formed part of the phylogenetic study are in blocks. The tree was calculated from a clone corrected dataset of SSR and RFLP marker data. *Sensu lato* and *sensu stricto* groups of *Botryosphaeria parva* and *B. ribis*, as initially determined by multiple sequence data, are indicated by differentially shaded areas.



B. ribis sensu stricto
31
32
42
43
41
33
34
53
26
27
25
B. ribis sensu lato
36
28
B. parva sensu lato
17
13
5
22
14
30
21
18
16
15
6
12
7
10
19
9
8
48
45
11
20
3
4
1
2
B. parva sensu stricto
50
46
52
44
47
51
37
49
40
38
35
39
29
24
23
0.05 changes

FIG. 4. The position of the observed (arrow) Index of Association ($I_A$) of alleles for *Botryosphaeria parva sensu stricto* plotted against a randomized dataset, which represent the distribution when the population is randomly mating. The $I_A$ was calculated for (A) a dataset containing all isolates of *B. parva sensu stricto* and (B) from a clone corrected dataset where only one representative of a genotype was used. The significance (P value) of the difference between the observed value and that of the randomized dataset is indicated at the bottom of the graph.


A
All isolates
Frequency
350
300
250
200
150
100
50
0
-0.175
-0.125
-0.075
-0.025
0.025
0.075
0.125
0.175
0.225
Index of Association
P = 0.003; Observed = 0.057


B
Clone corrected
Frequency
180
160
140
120
100
80
60
40
20
0
-0.35
-0.25
-0.15
-0.05
0.05
0.15
0.25
0.35
0.45
Index of Association
P = 0.342; Observed = 0.027

# 10

# Evolving species concepts in *Botryosphaeria* over 140 years

***Abstract:*** For many groups of living and extant organisms, the identification of species is a controversial issue. These concepts are often influenced by the current knowledge or tools available to delimit the boundaries between taxa. Since the description of the genus *Botryosphaeria* 140 years ago, numerous species have been added. The concepts and techniques that have been used to identify these species have, however, changed over this time. This has led to conflicting views and considerable confusion in *Botryosphaeria* taxonomy. Recent studies employing various molecular tools have, however, helped to delimit natural boundaries between species of *Botryosphaeria.* In this review, past and present species concepts used to identify species of *Botryosphaeria* are compared. An attempt is also made to circumscribe methods to identify and describe species of *Botryosphaeria* that will prevent a recurrence of many years of confusion that has plagued the taxonomy of this genus.

## INTRODUCTION

The ascomycete genus *Botryosphaeria* Ces. & De Not. is a cosmopolitan group of fungi that has an exceptionally large host range, including gymnosperms and angiosperms (von Arx and Müller 1954, Barr 1972). These fungi can be saprophytes on dead or dying stems, branches, stalks, culms or leaves of plants. They are, however, best known as pathogens of woody plants, typically causing cankers, die-back and eventually the death of affected trees (von Arx 1987, Sinclair et al 1987). These diseases mostly follow the onset of stress due to factors other than *Botryosphaeria* infection (Christ and Schoeneweiss 1975, Wene 1979, Wene and Schoeneweiss 1980).

Infection by *Botryosphaeria* spp. has traditionally been thought to occur via wounds and this group has generally been referred to as wound pathogens (von Arx and Müller 1954) Recent discoveries have, however, shown that they can infect directly through lenticels, stomata or other openings on healthy plants without necessarily causing symptoms (Brown and Hendrix 1981, Michailides 1991, Smith et al 1996). After such infection these fungi can live endophytically in asymptomatic tissue until the host is stressed (Johnson et al 1992, Fisher et al 1993, Smith et al 1996). Given the wide host range and mode of infection, most woody plants appear have some level of *Botryosphaeria* infection.

The genus *Botryosphaeria* was erected in 1863 by Cesati and De Notaris for twelve ascomycete taxa. These include *B. dothidea* (Moug.:Fr.) Ces. & De Not., a new binomial for *Sphaeria dothidea* Moug.:Fr. described by Mougeot in Fries (1823). This species is regarded as the type of the genus. Subsequently, more than 140 species were described (Denman et al 2000).

There are few morphological distinctions between many of the *Botryosphaeria* spp. In their revision of the genus, von Arx and Müller (1954) synonymized a large number of species, particularly using the names *B. quercuum* (Schwein.) Sacc. and *B. dothidea*. *Botryosphaeria quercuum* was soon afterwards split into a number of distinct taxa (Shoemaker 1964). Although not all researchers accepted all the species synonymized under *B. dothidea*, this taxon was not revised again. *Botryosphaeria dothidea* and some of its synonyms (e.g. *B. ribis* Grossenb. & Duggar and *B. berengeriana* De Not.) have, thus been used inconsistently in the literature for many years. In terms of pathology, this has resulted in considerable confusion. Slippers et al

(2003) epitypified *B. dothidea* and characterized the taxa formerly treated under this name, including *B. ribis* and *B. parva* Pennycook & Samuels. However, the confusion of names used under these and other *Botryosphaeria* binomials in past studies remains problematic.

The most commonly used operational species concepts (OSC) for fungi include the morphological (MSC), biological (BSC), ecological (ESC) and, more recently, phylogenetic (PSC) species concepts (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 2000). In *Botryosphaeria*, like in most other fungi, the MSC has been most dominantly used to identify and describe new species. In general this species concept underestimates the true diversity, by a factor of two to three as estimated based on a growing number of recent examples (Taylor et al 2000). This order of magnitude is much greater for the morphological concepts of *B. quercuum* and *B. dothidea* sensu von Arx and Müller (1954).

The OSC in fungi has been greatly aided by the application of the BSC since the 1950's (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 2000). The BSC concept has, however, never been used for *Botryosphaeria* spp., because they rarely produce ascomata in culture. An ESC, focussed on host specialization, has been widely used to identify new taxa in *Botryosphaeria* (Cesati and De Notaris 1863, De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928, Grove 1937). Host specialization is, however, not always practical, because some *Botryosphaeria* species have very wide host ranges, e.g. *B. obtusa* (Schwein.) Shoemaker, *B. parva*, etc. (Stevens and Jenkins 1924, Punithalingam and Waller 1973, Pennycook and Samuels 1985).

In recent years, molecular data have been used increasingly to identify groups and species amongst the fungi, including *Botryosphaeria* (Palmer et al 1987, Stanosz et al 1996, Jacobs and Rehner 1998, Smith and Stanosz 2001, Zhou and Stanosz 2001a, b). Of these DNA sequence data have been most commonly applied in recent years and they have had a profound impact on the taxonomy of *Botryosphaeria* spp. Differences in DNA sequences have also been successfully combined with morphological characteristics to identify and describe *Botryosphaeria* species (Smith et al 2001, Phillips et al 2002, Denman et al 2003).

Most taxonomic studies on *Botryosphaeria* using DNA sequence differences have used ITS rDNA phylogenies. Use of this single gene can underestimate the true species diversity especially among closely related or cryptic species (Taylor et al

2000). In this regard, multiple gene sequence concordance phylogenies have been successfully applied to identify cryptic *Botryosphaeria* species, previously overlooked or of uncertain identity (De Wet et al 2003, Slippers et al 2003, Chapters 4, 6, 7).

A number of recent reviews have provided thorough discourse on species and species concepts in fungi, including contemporary techniques (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 1999, 2000). The taxonomy of *Botryosphaeria* and its anamorphs has also been adequately reviewed, including the provision of keys to important species (Denman et al, 2000, Phillips 2002 www.crem.fct.unl.pt/botryosphaeria_site). In this review, we refer to these issues only in summary. Our aim is rather to assess the influence that changing theoretical and practical ideas, and techniques, have had on the taxonomy and the understanding of the biology of *Botryosphaeria.*

## SPECIES CONCEPTS

At the outset, it is important that we define our view of *Botryosphaeria* species, as this clearly influences the views that we will present throughout this paper. We favor the definitions of Brasier (1997) and Taylor et al (2000) that consider a fungal species as an identifiable unit of biodiversity, or an identifiable unit from which other species will evolve, whether an organism has a sexual or asexual life-cycle. Furthermore, our preference is for a phylogenetic species concept implying that there is no internal phylogenetic structure within a species. The species concept defined by Nixon and Wheeler (1990), and applied to fungi (Harrington and Rizzo 1999, Taylor et al 1999, 2000), suggesting that a species is the smallest aggregate of populations identifiable by a unique character, is followed in this review.

Harrington and Rizzo (1999) and Brasier (1997) suggest that species delimiting characters should be phenotypic. Thus, multiple gene genealogies or other molecular techniques can indicate species boundaries, but in the absence of distinctive phenotypic characters, should not be used to describe taxa. If two species represent a continuum of populations, then identifying a recent barrier to recombination without further relevant phenotypic information makes the description of species obsolete. The objection that the systematic system then lacks a piece of recent evolutionary information is discarded, because this unit contains no or very little unique characters (Harrington and Rizzo 1999, Brasier 1997). This is a weakness as differentiating phenotypic characters might not yet have been discovered or their phylogenetic value could have been overlooked. This is especially true for cryptic species. An effort should thus be made to re-evaluate known phenotypic characters and to look for new distinguishing characters following molecular identification of recent species barriers (Taylor et al 2000).

## TRADITIONAL PHENOTYPIC AND ECOLOGICAL IDENTIFICATION AND CLASSIFICATION

Morphology has been the most widely used phenotypic character to distinguish between and describe species of *Botryosphaeria*. This is also true for most other fungi. There are, however, a limited number of morphological characters that are consistently useful to distinguish between these species. Furthermore, the morphological features that are most often used such as spore morphology are highly variable and sexual fruiting structures are not always present. As a consequence, these continuous characters overlap between species, making identifications very difficult. This problem is enhanced in closely related, sibling species. Other phenotypic characters such as physiology, chemical products and life-cycle have not been widely used to identify *Botryosphaeria* species. Where they have been used, they have been found to be inordinately variable within species to be of taxonomic value (Kay et al 2002).

*Teleomorph.*--For the description of the genus *Botryosphaeria* and until the 1950's, the general morphology of the ascospores (hyaline, aseptate, shape, etc,) and stromatal and ascomatal morphology was considered taxonomically and phylogenetically informative. These characters were later shown to be inordinately variable within species and of little value for distinction between species.

Cesati and De Notaris (1863) described the genus *Botryosphaeria* for a group previously named *Sphaeria*, *Gibbera* and *Dothidea* spp. Sacccardo (1877) amended the initial description to exclude species with hypocreaceous stromata or ascomata. During this time, the ascomata carried in botryose clusters in well-developed stromata and ascospores (8-spored, hyaline, ovoid to ellipsoidal, unicellular or rarely septate) were considered important characters. Between 1863 and the 1920's these characters were used to describe a large number of species (De Notaris 1863, Winter 1886, Saccardo 1877, 1882, Trotter 1928 and others).

Von Arx and Müller (1954) recognized the variation of ascomata within species of *Botryosphaeria*. In their revision of the genus they placed substantial emphasis on ascospore size to distinguish taxa. These authors studied herbarium specimens of *Botryosphaeria* spp. and other taxa and synonymized 108 species under *B. quercuum* (ascospores 24--42 x 10--18 µm) and 24 species under *B. dothidea* (ascospores 15--26 x 6--9µm). This study represented an important revision and consolidation of a diverse group of fungi. The very broad species concept used by von Arx and Müller was, however, not widely accepted. *Botryosphaeria quercuum* and *B. dothidea* were subsequently shown to include numerous distinct species (Shoemaker 1964, Slippers et al 2003).

There are a number of objections to the use of ascospore size or morphology as a primary character to delimit species of *Botryosphaeria*. While some species are easily distinguishable based on the ascospore size, in others these spores have exactly the same size and shape (Shoemaker 1964). The morphological variation that exists within some species makes it difficult to determine the relevance of a specimen such as those represented in many herbarium collections. Furthermore, in some species, the teleomorph structures are infrequently found in nature Teleomorph structures and spores are also very rarely induced in culture, which makes this character useless to identify pathogens that are isolated from disease symptoms.

*Botryosphaeria* asci and ascospores develop and mature slowly. Consequently, they are often collected in an immature state (von Arx and Müller 1954, Slippers et al 2003). The immature spores generally do not have the same dimensions as those of mature spores. They mostly lack important characters such as septa that develop with age. As previously stated, spore size was not of primary importance during the initial years when most *Botryosphaeria* species were described. Many *Botryosphaeria* species are, therefore, represented by herbarium specimens bearing immature spores. These include important species such as the type specimen of *B. dothidea*.

A possible solution to some of the problems experienced when working with early species descriptions or indistinct herbarium specimens of *Botryosphaeria* species, is to select epitype samples that better represent the specific taxon. An added advantage of these collections is that ex-epitype culture can be obtained from the epitype specimen. Such isolates are essential when assessing species concepts using contemporary DNA based tools. When selecting an epitype, effort should also be made to collect additional specimens and isolates representative of the diversity of the population from which the ex-type isolate originated.

*Botryosphaeria dothidea*, the type species of the genus, is the only species that has been epitypified in this genus (Slippers et al 2003). An ex-type isolate from this sample has been deposited in major culture collections and its DNA sequences deposited in GenBank. These data and collections now allow for easy identification of isolates thought to belong to this taxon.

Currently, *Botryosphaeria* includes species that lack or have only inconspicuous stromata that are imbedded or immersed in the host tissue and that range from uniloculate to multiloculate, with or without a papilla, (FIGS. 1-5). Consistent characters are the 8-spored, bi-tunicate, clavate asci, and the filliform pseudoparaphyses between the asci (FIGS. 6-8). The ascospores of *Botryosphaeria* are hyaline, ovoid to ellipsoid, and mostly aseptate, although some species (e.g. *B. quercuum*, *B. obtusa*) may become septate with age (FIGS. 6-8). They range from 18--50 µm x 6--18 µm in size. The ascospores of *Botryosphaeria* species vary and overlap in size, but other features are useful for species identification. Thus, the brown discoloration and up to three septa in aging ascospores of *B. quercuum* is characteristic (Shoemaker 1964). The value of teleomorph characters should thus not be completely disregarded.

The higher classification of *Botryosphaeria* is based on the morphology of the ascomata and asci. Characters such as the ontogeny of the ascomata and the interthecial tissues have also been used at the higher taxonomic placement of *Botryosphaeria*. This genus does not have true perithecial walls (pseudothecia) and has pseudoparaphyses that form in the ascomal cavities before the asci (von Arx and Müller 1954, Luttrell 1965, Eriksson 1981). Based on these characters and DNA sequence data, this genus is currently classified in the family Botryosphaeriaceae in the order Dothideales, class Loculoascomycetes (Sivanesan 1984, Kirk et al 2001, Denman et al 2000).

*Anamorph.*--Since the 1960's the value of using morphological characters of the anamorphs to delimit and identify *Botryosphaeria* spp. has been realized and widely applied (Shoemaker 1964, Pennycook and Samuels 1985, Morgan-Jones and White 1987, Denman et al 2000, Phillips et al 2002, Slippers et al 2003). There are many advantages to using anamorphs to identify the associated *Botryosphaeria* species. These forms are more frequently found in nature than teleomorphs and are easily induced in culture. Conidia are also much more diverse in shape and size than the associated ascospores. Conidial characters that can be used include wall thickness, ornamentation, maturation, color, septation, shape and size (length, width, l/w and lxw) (FIGS. 6-21).

Early taxonomy of *Botryosphaeria* anamorphs has been very confusing. Eighteen anamorphs have been linked to this teleomorph genus, the most common of which include *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Corda, *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino and *Sphaeropsis* Sacc. (Sivanesan 1984, Denman et al 2000). Recent studies (Denman et al 2000) have, however, suggested that many of these taxa need to be synonymized. For example, the type species of *Dothiorella* has been reduced to synonymy with *Diplodia*, while *Botryodiplodia* was shown to be a *nomen dubium* (Crous and Palm 1999). *Macrophoma* was reduced to synonymy with *Sphaeropsis* (Sutton 1980).

There are questions regarding the validity of separating *Sphaeropsis*, *Diplodia* and *Lasiodiplodia* (Denman et al 2000). *Sphaeropsis* and *Diplodia* differ only in percurrent proliferation in the conidiogenous cells in *Sphaeropsis* and the time of septation of ageing spores. Neither of these characters is unique to either group, or are synapomorphies, because both features are also seen in *Fusicoccum*. *Lasiodiplodia* differs from other *Diplodia* species only by prominent melanin deposits or striations in the conidial walls (Denman et al 2000). The taxonomic value of these deposits is doubtful because they also occur in other *Diplodia* spp.

Denman et al (2000) reviewed anamorph taxa of *Botryosphaeria* and concluded that *Fusicoccum* and *Diplodia* were the only two genera that can be validated. These taxa were delineated as having hyaline conidia (*Fusicoccum*) or conidia that are often pigmented when they age (*Diplodia*) (FIGS. 6-21). Phillips (2002) supports the retention of only these two anamorph genera, but has suggested that they are better defined by cell wall thickness and conidial width. *Diplodia* species have wider conidia (normally more than 10 μm) with thicker walls (0.5--2 μm), while

*Fusicoccum* has narrower conidia (normally <10 μm) with thinner walls (<0.5 μm) (Phillips 2002) (FIGS. 6-21). Von Arx and Müller (1954) note that a conglomeration of species under the *B. quercuum* has *Diplodia* (reported as *Botryodiplodia*) anamorph states. They also note that species synonymized with *B. dothidea* have *Fusicoccum* anamorph states (reported as *Dothiorella*).

Amongst *Botryosphaeria* anamorph characters, only the conidia are consistently used to distinguish taxa. The pycnidia are often indistinguishable from ascomata and of equally little value when distinguishing species boundaries (FIGS. 1-5). Conidiophores and conidial development have also not been valuable in delimiting species (FIGS. 16-17). Microconidia or spermatia have occasionally been reported (Pennycook and Samuels 1985, Denman et al 1999, 2003, Phillips et al 2002, Slippers et al 2003) (FIG. 9). They, however, appear to be less common and easily overlooked. It is not clear whether microconidia have simply been overlooked in studies that do not record their presence or whether they have any phylogenetic or taxonomic value.

Despite the value of conidial morphology, for some species considerable overlap exists when using this character. This overlap is most problematic for recently diverged species. For example, the conidia of *F. eucalyptorum* Crous, Smith & M.J. Wingf. and *F. irregulare* Slippers, Crous & M.J. Wingf. are virtually the same in shape, length and width, maturation, etc. (Chapter 5), although these species differ slightly in overall size or dimensions of the conidia. Another example is that of *B. parva* and *B. ribis*, where young conidia are indistinguishable from each other (Slippers et al 2003). *Botryosphaeria parva*, however, commonly produces two-septate conidia with a darker middle cell, unlike the single septum and consistently colored conidia in *B. ribis*. *Botryosphaeria lutea* A.J.L. Phillips and *B. australis* Slippers, Crous & M.J. Wingf. can also not be easily distinguished from each other based on conidial morphology (Chapter 3), but can be distinguished based on differences in cultural morphology. The above-mentioned differences are so slight that, in the absence of DNA based comparisons, they could easily have been considered as encompassing variation within a species.

*Cultural characteristics*.--Since the mid 1980's cultural characteristics have been commonly used to augment other characters. In general, cultures of *Botryosphaeria* spp. are easily distinguished from most other fungi by their grey to black, aerial mycelium and the grey to indigo grey or black pigment that is visible from the reverse side of Petri dishes. The appearance and color of aerial mycelium and pigments have also aided in the delimitation and rapid identification of *Botryosphaeria* taxa that are otherwise morphologically similar.

Pigments were not always recognized, e.g. Witcher and Clayton (1963) disregarded the yellow pigment in cultures of '*B. dothidea*' as variation in the species. More recently, pigments produced in culture have, however, been recognized as valuable in distinguishing certain *Botryosphaeria* species. Pennycook and Samuels (1985) found that conidial morphology overlapped between *F. luteum* Pennycook & Samuels and anamorphs of both *B. dothidea* and *B. parva*. In young cultures, *F. luteum* produces a unique bright yellow pigment. Phillips et al (2002) also noted this yellow pigment in cultures from *Vitis* and subsequently described the teleomorph, *B. lutea*. Another example is that of Jacobs (2002) who found that the only phenotypic characteristic that separates the *F. indigoticum* Jacobs, Slippers & M.J. Wingf. prov nom. from closely related taxa, is the distinctly darker (dark indigo-black) pigment in cultures of this species.

Apart from pigments, the extent and color of aerial mycelium and rate of growth of cultures has been used to characterize taxa or subspecies groups in *Botryosphaeria*. The morphotypes of the anamorph *Diplodia pinea* (Desm.) J. Kickx (= *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton) could thus be distinguished based on the mycelial texture and aerial mycelium in cultures (Palmer et al 1987). Study of greater numbers of isolates eventually showed that these characteristics are insufficiently variable to consistently distinguish all of the groups (De Wet et al 2002, Kay et al 2002). *Botryosphaeria dothidea* and *B. lutea* also have thinner mycelial mats and less aerial mycelium than *B. parva* and *B. ribis* (Pennycook and Samuels 1985, Slippers et al 2003). Pennycook and Samuels (1985) showed distinct growth rates between *B. dothidea*, *B. parva* and *F. luteum* at different temperatures. However, intra species variation, effect of different media and age of cultures, reduce the value of this character.

*Host relations*.--Ever since the first description of *Botryosphaeria*, ecology and biology have influenced the view regarding species delimitation. Two main views are prevalent. Many taxonomists have considered as important, the ability to infect a specific suite of hosts. Many *Botryosphaeria* species and their anamorphs have thus been described based on host. Examples include *B. quercuum* (from *Quercus*), *B. ribis*

*Vitis*), and many other names (Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928). This view of strict host pathogen evolution arose from a plant pathology and a co-evolutionary perspective. Grove's (1937) statement best illustrates the logical argument that led to this approach:

> 'The majority of the species of *Diplodia* and *Botryodiplodia* are extremely similar to one another, especially in regard to the spores. They can be discriminated only by host-plant…a shrub can be seen to be badly attacked by a *Diplodia*, and yet surrounding shrubs may show no sign of infection although they belong to a species which, in other localities, can be found abundantly infested with a *Diplodia* having spores apparently exactly like those of the first mentioned."

A second and strongly held view for many years has been that *Botryosphaeria* species are mainly wound infecting or saprophytic fungi (Von Arx and Müller 1954). If this were correct, they would need no host specialization. This view contributed to a very broad, 'super-species' concept for *Botryosphaeria*. Stevens and Jenkins (1924), Punithalingam and Waller (1973), Punithalingam and Holliday (1973) and others also report extremely wide host ranges for *B. ribis* and *B. obtusa* that include dicotyledenous and monocotyledenous plants.

Both the views that *Botryosphaeria* species are host specialized pathogens or that they are secondary, often saprophytic fungi are considered correct. Certain well-studied species infect a wide range of hosts, e.g. *B. obtusa*, *B. parva*, *B. dothidea, B. rhodina* (Berk. & Curtis) Arx (Punithalingam and Waller 1973, Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003). On the other hand, some well characterized species are clearly specialized on certain hosts e.g. *D. pinea* on *Pinus* and other conifers, *B. eucalyptorum* Smith, Crous & M.J. Wingf. and *B. irregularis* Slippers, Crous & M.J. Wingf. on *Eucalyptus*, *B. protea* (Wakef.) Denman & Crous and *B. protearum* Denman & Crous on Proteaceae species (Smith et al 1996, Smith et al 2001, Burgess et al 2003, Phillips et al 2002, Denman et al 2003, Slippers et al 2003). In these examples, closer examination of *Botryosphaeria* isolates from indigenous hosts has often revealed these distinct species. They have commonly been overlooked in the past, because of morphological similarities or uncertainty regarding the phylogenetic value of small morphological differences. Many of these species also occur sympatrically.

## INFLUENCE OF MOLECULAR TECHNIQUES

A number of molecular techniques have been used to study variation and relationships in the fungi. The value of all these techniques to identify species, however, varies, particularly where cryptic species are concerned. Many DNA-based and other molecular tools have been developed for studies at the population level. These tools are increasingly used to understand questions at the interface between populations and species. For this reason, we also include a discussion on some population level approaches and reflect on how they have increased our understanding of the evolution and speciation in *Botryosphaeria*.

*Isozymes.*--Isozyme patterns were used to distinguish the A and B morphotypes of *D. pinea* (Stanosz et al 1999). These morphotypes have more recently been shown to represent two distinct taxa, *D. pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf., following the production of extensive multigene phylogenies (De Wet et al 2003). Interpretation of the phylogenetic value of isozymes for *Botryosphaeria* systematics is difficult given that it provides little information. From the example discussed here, it seems that variation in these markers could indicate speciation, thus validate further investigation.

*RAPD's.*--Randomly Amplified Polymorphic DNA markers have been used to distinguish between groups within or between established morpho-species (Smith and Stanosz 1995, Stanosz et al 1999, Smith and Stanosz 2001). The advantage of using RAPD's is that they give a genome-wide perspective. A distinct disadvantage is that they are not always reproducible. This concern is diminishing with increasingly refined technology and standardized methods and reagents. They are, however, dominant markers and the level of homoplasy cannot be determined from their data (except where further analysis is done through hybridization). For this reason, RAPD data have not impacted on the identification or formal taxonomy of *Botryosphaeria*. They have, however, in some cases provided useful preliminary data used to justify further phylogenetic and taxonomic studies.

Data from RAPD's often provide support for *Botryosphaeria* groups that have been defined based on other data, such as morphology or sequence data (Smith and Stanosz 1995, 2001). This is, however, not true for comparisons between closely related taxa. In these cases RAPD's tend to under-estimate species diversity. For example, RAPD's did not distinguish between *B. ribis* and *B. parva,* nor between *B. lutea* and *B. australis* (Smith and Stanosz 2001). RAPD's can also over-estimate species diversity. Thus RAPD's identified four distinct groups in *D. pinea* (Stanosz et al 1996, De Wet et al 2000, Hausner et al 1999). These groups were later shown to represent three species, namely *D. pinea*, *D. scrobiculata* and *B. obtusa*.

Given the difficulty in interpreting the phylogenetic value of RAPD's, this technique is probably best restricted to comparisons within species and not those between taxa. In other words, it would be more useful in population studies following appropriate identification of species (Ma et al 2001).

*ISSR's.*--Inter simple sequence repeat (ISSR) PCR, which is also known as microsatellite primed PCR, has been used only once to test phylogenetic species concepts in *Botryosphaeria* (Zhou et al 2001). Unlike RAPD's the results emerging for this technique were comparable with those of other phylogenetic studies (Zhou et al 2001). ISSR markers provide a genome-wide perspective, which is similar to RAPD's. However, they are generally more reproducible, because longer primers and higher annealing temperatures are used (McDonald 1997). The data from the latter study was used to successfully distinguish between well defined species (e.g. *B. dothidea*, *B. lutea* and *B. ribis*), as well as recently diverged species (e.g. *B. ribis* and *B. parva*). In the distinction of *B. parva* and *B. ribis* ISSR data were more useful than ITS rDNA sequences, RAPD's or morphology. This technique, therefore, appears to hold promise for indicating species boundaries amongst large numbers of isolates of *Botryosphaeria.* The data obtained from ISSR markers are, however, dominant and need to be complemented by other phylogenetically informative data before taxonomic proposals can be considered. These markers have also been useful to study intra-specific variation in *B. dothidea* (Ma et al 2001).

*SSR markers.*--Simple Sequence Repeat (SSR) markers are co-dominant that are useful to study population level questions in fungi. SSR markers have recently been developed to study populations of *D. pinea* and *D. scrobiculata, B. rhodina*, and *B. parva* as well as other *Botryosphaeria* species with *Fusicoccum* anamorphs (Burgess et al 2001a, b, 2003, Chapter 8). These markers have significantly increased our understanding of the population structure, mode of reproduction and spread of these important pathogens (Burgess et al 2003, Chapter 9).

The phylogenetic value of data from SSR makers has been questioned, because of the high mutation rate and constraints on maximum size, which increase the level of homoplasy. Studies by Fisher et al 2000 (*Coccidioides immitis* G.W. Stiles) and Burgess et al 2001a (*D. pinea*) have, however, shown that these markers can reflect species boundaries, even between cryptic species. In both these cases the SSR marker data were, however, interpreted by comparing results from multiple gene genealogies, which confirmed their phylogenetic value (Kofoupanou et al 1997, Fisher et al 2000, De Wet et al 2003).

The superiority of SSR markers over other methods used to indicate species boundaries for cryptic species is well illustrated in studies of the *Botryosphaeria* anamorphs, *Diplodia pinea*. This fungus is a well known pine pathogen that has been studied extensively for many years (Punithalingam and Waterston 1970). Identification has primarily been based on the host, as well as conidial ontogeny and morphology and a large number of synonyms have emerged for it (Sutton 1980). Recent studies using RAPD markers, combined with morphology and epidemiology, have shown that there are as many as four 'morphotypes' for this taxon and these have become known as the A, B, C and I morphotypes (Palmer et al 1987, Smith and Stanosz 1995, Hausner et al 1999, de Wet et al 2000). Some studies have, however, shown considerable variation in the characters used to distinguish these morphotypes (Swart et al 1991, 1993, Kay et al 2002). Single gene phylogenies also do not separate all these morphotypes, or even clearly distinguish them from the closely related *B. obtusa*. Microsatellite markers have revealed that there is no genetic exchange between the B morphotype and the A and C morphotypes (Burgess et al 2001a). Subsequently, comparison of sequence data for some of these markers, as well as for some gene coding regions, has confirmed that the B morphotype is a distinct species, namely *D. scrobiculata* (De Wet et al 2003). These markers also showed that the 'I' morphotype represents *B. obtusa* (Burgess et al 2001a).

In one study in this thesis (Chapter 9) the separation of the closely related species *B. parva* and *B. ribis* is confirmed using combined gene genealogies, SSR makers and PCR RFLP fingerprints. These data, especially the RFLP profiles, also provided a quick and effective way to distinguish these species. Furthermore, following characterization of *B. parva* and *B. ribis* their distribution was also studied, and it was shown that these fungi, especially *B. parva*, have been moved around the world on hosts such as *Eucalyptus*. It was also clear from the SSR marker data that there is free recombination among a world-wide population of *B. parva*, which was the only species for which sufficient isolates were available to make comparisons at a population level. Despite the ability of *B. parva* to reproduce sexually a number of clonal lines were, however, identified that occurred across spatial and temporal boundaries.

SSR markers can differentiate between inter- and intra specific variation where species boundaries are unclear and this is an advantage over other techniques used to distinguish species or study populations. For example, SSR markers have not only revealed population structures in *B. parva* and *B. ribis*, but they were also used in combination with multiple gene genealogies to identify *sensu lato* and *sensu stricto* groups in these species. As a result of studies of this nature, the identified sets of isolates can be used to further characterize phenotypic distinctions between, and variation within the species. Selecting representative isolates to characterize a species, especially for cryptic species, is often difficult using other techniques (Davis and Nixon 1992, Taylor et al 1999). Fisher et al (2000) also found that SSR data can serve the dual purpose of identifying inter- and intra specific variation among a group of isolates to enable further testing of population and species hypotheses.

*Single locus sequence data.*--The Internal Transcribed Spacers and 5.8S gene of the ribosomal DNA complex (ITS rDNA) has been the most widely used in *Botryosphaeria* phylogenetics and systematics. Despite significant contributions, data from this region alone is insufficient to distinguish closely related sibling species.

The first study to test phylogenetic hypotheses for species of *Botryosphaeria* using rDNA sequence data was by Jacobs and Rehner (1998). These authors combined sequence data with morphological characters and showed that *B. dothidea* was paraphyletic. Subsequently, this paraphyletic group has been shown to represent *B. ribis* and *B. dothidea* (Smith and Stanosz 2001, Zhou and Stanosz 2001a, Zhou et al 2001, Slippers et al 2003).

A number of studies followed that of Jacobs and Rehner (1998) in using the ITS rDNA region to determine phylogenetic relationships among *Botryosphaeria* species. Data from this region was used in combination with RAPD or ISSR data (Smith and Stanosz 2001, Zhou et al 2001), morphology (Smith et al 2001, Zhou and

Stanosz 2001a, Denman et al 2003) or both (Phillips et al 2002). In most cases where these data have been combined with morphology, taxonomic proposals could be made. In some cases, however, the ITS data combined with morphology and RAPD's could not distinguish closely related species or resolve the taxonomic issues, for example in the distinction of *B. parva* and *B. ribis* (Smith and Stanosz 2001).

All the ITS-rDNA studies have shown that there is a separation between *Botryosphaeria* spp. with *Fusicoccum*-like and *Diplodia*-like anamorphs. Jacobs and Rehner (1998) first noticed this subdivision. Denman et al (2000) refers to these groups as representative of the *Fusicoccum* and *Diplodia* groups, which are the only anamorphs that they consider relevant to the genus. Zhou and Stanosz (2001a) supported these findings and refer to these groups as section Hyala and section Brunnea. However, not all *Botryosphaeria* species group in one of these two sections, e.g. *B. zeae* (G.L. Stout) Arx & E. Müll. and *B. proteae* (Denman et al 2000, FIG. 22). When a larger number of *Botryosphaeria* species representative of the true diversity of the genus are thus considered, the separation of the genus into only two groups appears to be of lower phylogenetic value.

Mitochondrial small subunit sequence data have been applied only once to phylogenetic questions in *Botryosphaeria* (Zhou and Stanosz 2001b). The phylogenies from these data were not congruent with those developed using ITS rDNA data or those data from other gene regions as discussed below. For example, the split between *Fusicoccum* and *Diplodia* was not supported by the mitochondrial small subunit DNA sequence data. *Botryosphaeria dothidea*, *B. corticis* (Demaree & Wilcox) Arx & E. Müll. and *B. mamane* D.E. Gardner, which all have *Fusicoccum* anamorphs, grouped within the *Diplodia* clade. mtSSU data, however, clearly revealed the distinction between the genus *Botryosphaeria* and *Guignardia* species that were previously described within it. The incongruence between the mitochondrial and nuclear gene sequence datasets is not unexpected given the fact that these genomic regions have independent evolutionary histories.

*Multiple gene genealogies.*--There are dangers in using single gene phylogenies to infer species phylogenies, because diversity can be either over or under estimated, depending on the evolutionary history of the genes and organisms studied (Taylor et al 2000). For this reason, a phylogenetic species concept based on concordance between unlinked gene genealogies has increasingly been promoted and applied to identify species units in fungi (Koufoponou et al 1997, Geiser et al 1998, O'Donnell et al 1998, Taylor et al 1999, 2000, Steenkamp et al 2002). Following this concept, independent gene genealogies will only be consistent in identifying a distinction between taxa after genetic isolation. Any continued genetic exchange, or even remnants of recent genetic exchange, will randomize the polymorphisms in each genealogy and thus cause discrepancies between the individual phylogenies that they indicated. Concordance and combination of multiple gene genealogies have also played an important role in defining species boundaries between closely related and cryptic *Botryosphaeria* species (De Wet et al 2003, Slippers et al 2003, Chapter 3, 5).

The inability of single gene phylogenies, co-dominant molecular data or any specific phenotypic character to confirm the distinction between the cryptic species *Diplodia pinea* and *D. scrobiculata* have been discussed above. *Botryosphaeria parva* and *B. ribis* were equally difficult to distinguish from each other. In both these species complexes, SSR markers confirmed genetic isolation between groups of isolates representing these species (Burgess et al 2001a, Chapter 9). It was, however, only through the use of combined multiple gene genealogies that phylogenetic distinction between these species could be confirmed. Following the identification of the phylogenetic species, the small phenotypic differences between these taxa could be interpreted as useful for identification.

Single gene phylogenies have indicated the distinction between *B. australis* and *B. lutea*, as well as between *B. eucalyptorum* and *B. irregularis* (Chapters 3, 5). However, these species are so closely related that the lengths of the branches defining each species are insignificant in comparison with those between more robustly defined *Botryosphaeria* species. These small differences, combined with the fact that these species are morphologically almost identical, make it difficult to justify their separation. However, when data from three independent gene regions are compared, it is clear that the polymorphisms in each genealogy group the same isolates together (Chapters 3, 5). These fixed polymorphisms in each group for the different genealogies are indicative of reproductive isolation, despite their sympatric occurrence. When these DNA sequence datasets were combined they then provided strong support for the separation of these species.

The number of genes that need to be sequenced to accurately reflect species boundaries will depend on how closely related the species are. Our experience has shown that for *Botryosphaeria*, if only two regions had to be chosen, the ITS rDNA and EF1-$\alpha$ regions would be preferable (Chapter 9). Data for the ITS rDNA region is the most widely available for *Botryosphaeria* species and is useful to place species in tentative groups. The EF1-$\alpha$ has been the most variable and thus useful to indicate distinctions between closely related species. $\beta$-tubulin sequences lacked one of the larger introns normally found in this region (B. Slippers unpublished data) and generally had little variation in most of the studies where it was used. The other gene regions (Calmodulin, Actin, rDNA Intergenic spacer) have only been used for *D. pinea* and *D. scrobiculata* and it is difficult to assess their value for other species (De Wet et al 2003).

*Species-specific primers and PCR RFLP identification.*--After the identification of a species using the methods described above, there is usually a need for a rapid and effective tool to identify the species. This is especially true where a specific host (especially cultivated and introduced hosts) is infected by a complex of *Botryosphaeria* species (Jacobs 2002, Chapters 5, 6). As discussed above, phenotypic characters are often inadequate to distinguish closely related species and sequencing large numbers of species for routine identification is impractical. In this regard species specific primers and PCR RFLP profiles have proven useful identification tools for various *Botryosphaeria* species.

Species-specific primers have been applied to identify subspecies or species groups in *Botryosphaeria* (Ma and Michailides 2002a). These primers were designed in the ITS1 region. Unfortunately, the groups for which the primers were designed, has not been identified to species level. This tool can be robust for the identification of *Botryosphaeria* species if correctly applied, and should be further explored.

There are some factors that hamper the development of species-specific primers for some *Botryosphaeria* species. The ITS rDNA region has been shown to be insufficient to distinguish closely related species (De Wet et al 2003, Slippers et al 2003). Even where ITS sequences are sufficient to distinguish closely related species, such as *B. eucalyptorum* and *B. irregularis,* and *B. lutea* and *B. eucalyptorum*, the polymorphisms are spread across the fragment (Chapter 5). Primers will, therefore, differ only by one base pair, which might not be sufficient or robust under all conditions. Species-specific primers between these closely related species will need to be developed for other regions of the genome. For example, the EF1-$\alpha$ region is consistently more variable than the ITS rDNA region (Chapter 3, 5, 9). Unfortunately, polymorphic sequences between the most closely related species, e.g. *B. ribis* and *B. parva*, and *D. pinea* and *D. scrobiculata*, are not close enough in any part of the regions sequenced thus far, to allow the development of robust primers.

Like species-specific primers, PCR RFLP fingerprints can provide effective tools to rapidly and reliably identify larger numbers of isolates that would be impractical to identify otherwise. RFLP profiles of the ITS rDNA region has been used to distinguish among distant and closely related *Botryosphaeria* species (Jacobs 2002, Chapters 4, 5). This technique overcomes the need for a continuous group of polymorphic bases, because restriction enzymes (RE) recognize single base pair changes. Unfortunately, not all polymorphisms will present restriction sites. Closely related species like *B. parva* and *B. ribis* could thus not be distinguished using this technique when ITS rDNA, $\beta$-tubulin and EF1-$\alpha$ sequence data were studied. Subsequent studies have, however, identified non-coding regions that are useful for RFLP distinction of these species (Chapter 9).

## DISCUSSION, CONCLUSIONS AND FUTURE WORK

In most DNA-based and other molecular studies, *Botryosphaeria* spp. group according to their associated anamorph species. One group (section Hyala) has anamorphs in *Fusicoccum*, while the other (section Brunea) have anamorphs in *Diplodia*. The question arises as to whether the genus should be sub-divided to reflect these groups. Our view is that it is currently not necessary or defendable to subdivide the genus. *Botryosphaeria* species form a monophyletic clade with respect to all other ascomycete genera, including the most closely related genus, *Guignardia*. The genus *Botryosphaeria*, as it has been used for the past 140 years, is well recognizable, because of the similar morphology and ecological niche and role of its species. It should be noted that the apparent split is not based on a representative sample of species in the genus. Furthermore, DNA data from species such as *B. zeae* and *B. proteae*, as well as mtSSU DNA sequence data, do not support the clear split in the genus (Denman et al 2000, Zhou and Stanosz 2001b, FIG. 22). The apparent phylogenetic substructure in the genus *Botryosphaeria* is thus not well enough characterized or understood at present to validate a division of the genus. In our view, this should await a more comprehensive study of a representative number of species.

If the teleomorph name for *Botryosphaeria* is not separated into two groups, it could be argued to synonymize the anamorph species under the older name *Fusicoccum*. This too seems unnecessary for a number of reasons. Where a holomorph concept is known, the teleomorph name will take preference. DNA based comparisons are making it easier to link the anamorph names to *Botryosphaeria* taxa. It is thus expected that the use of anamorph names will gradually diminish. Furthermore, *Diplodia* is a large genus. Changing a great number of names in this genus will take considerable effort and will also make the interpretation of past literature more complicated. A more valuable approach would be to epitypify these species and to link them to *Botryosphaeria* taxa.

The value of different characters or datasets to define species phylogenies is debatable, and might be unique for each species. Apart from multiple gene sequence and SSR markers, which will almost certainly indicate species boundaries when representative samples of populations are studied, a combination of other sets of information might indicate species boundaries. No single phenotypic character, whether from the teleomorph, anamorph or ecological data, will, however, conclusively distinguish closely related species in *Botryosphaeria*. Therefore, we would base conspecificity on the congruence between at least three of the following data sets, taking the possible variation within species into consideration as explained before: conidial spore size/shape (l/w and lxw), color, septation and discoloration pattern; cultural morphology; ITS rDNA sequence; predetermined RFLP patterns or species-specific primers; ISSR's; host and geographic origin

Apart from the value of specific characters and datasets in identifying species, there are a number of important considerations that will minimize mistakes when describing a new *Botryosphaeria* species. These include:

A. Identification of potential new species should be based on a representative number of samples. At the minimum three collections from different individual host plants, but if possible more. Unless differences between species are large, smaller sample sizes will underestimate species diversity based on phenotypic characters and overestimate them based on molecular data.

B. The most closely related species to a specific isolate can easily be determined by comparing its ITS rDNA sequence data to that in public databases. Care should, however, be taken concerning the identity of isolates in such databases. Identifications should preferably be based on ex-type or otherwise authenticated isolates where possible. The use of ad hoc identified samples is of little taxonomic value. For example, the names *B. dothidea*, *B. ribis* and *B. parva* are not correctly applied to all isolates in GenBank.

C. Physical comparisons with type and isolates should also be accompanied by a thorough investigation of possible synonyms for any new name, based on published morphological or ecological data (same host and geographical area might be a good starting point).

D. If the data is not congruent with that of known species or some consistent variation exist, and there is doubt about the phylogenetic value of such variation, multiple gene genealogies should be used to determine species boundaries.

E. Identification of new species should be followed by a description based on relevant phenotypic characters, ecological data, etc. Molecular data in isolation might identify a species, but does not circumscribe it. Morphological data alone also has little biological relevance in *Botryosphaeria.* It is the combination of molecular data with morphological characters, geographical distribution, host associations, life-cycle, etc. that gives biological identity and meaning to a *Botryosphaeria* species. Furthermore, the identification of separate groups in molecular studies that are not followed by taxonomic proposals, will lead to confusion.

F. Type material should be mature and preferably be associated with deposited anamorph material. If the anamorph is not known from nature, it can be easily induced in the laboratory. Apart from type material, it is imperative to deposit representative isolates (including ex-type) in recognized culture collections to allow future comparisons based on molecular and cultural characters. When type material or published data is insufficient to circumscribe a species, it is preferable to epitypify the taxon rather than merely rejecting the name. This will minimize an unnecessary proliferation of names, confusion and later work to amend the description.

G. An effort should be made to provide an easy identification tool (RFLP, Primers, etc.) and/or key for relevant taxa. Especially species-specific primers could be applied more widely. The intron of the EF1-α gene region seems particularly suited for this purpose, because it is more variable than any other studied thus far.

Mishler and Donoghue (1982) concluded that researchers identify species based on their own field of experience. Such an approach logically leads to conflicting views. It is hoped that the broader approach advocated here, will diminish taxonomic problems in *Botryosphaeria* and result in a more rigorous, useful and stable systematic system for this group of fungi. One prerequisite, if such a goal is to be attained, is easily accessible, updated and complete information about this group of important fungi. A first attempt at such a system has been put in place by Phillips (2002) (www.crem.fct.unl.pt/botryosphaeria_site).

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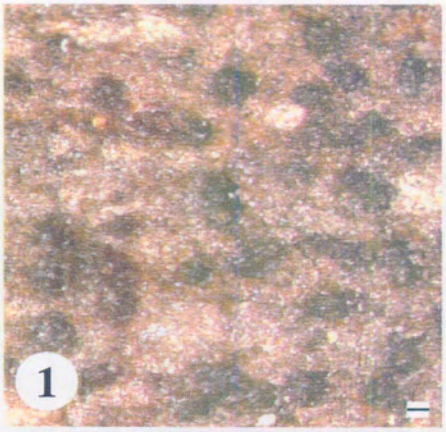
FIGS. 1-5. Dissecting microscope and phase contrast compound-microscope micrographs of ascostroma and pycnidia of *Botryosphaeria* species. 1. *B. irregularis*. Singular, semi-erumpent ascostroma. 2. *B. dothidea*. Botryose, erumpent ascomata with a central ostiole. The apices of two ascomata have been removed to show the typical white centrum contents. 3. *B. parva*. Singular pycnidium covered with grey mycelium, which was formed in culture on a pine needle. 4. *Botryosphaeria* sp. Mature pycnidia oozing conidia from an ostiole at the end of an extensive conical neck. 5. Median, longitudinal section through a mature ascoma of *B. dothidea*. Bars = 100 µm.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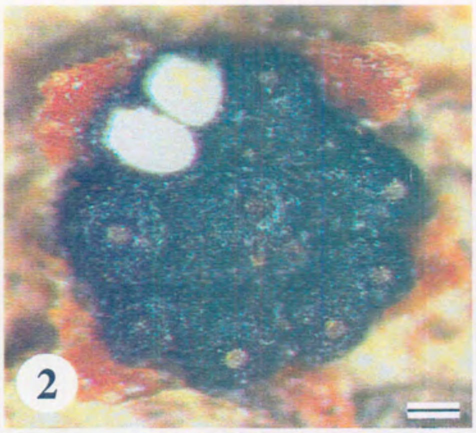
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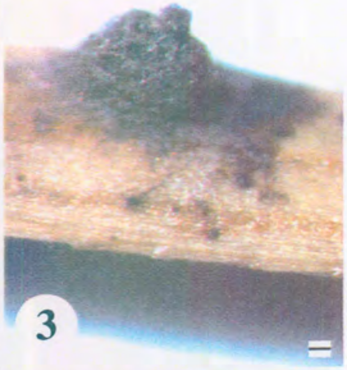
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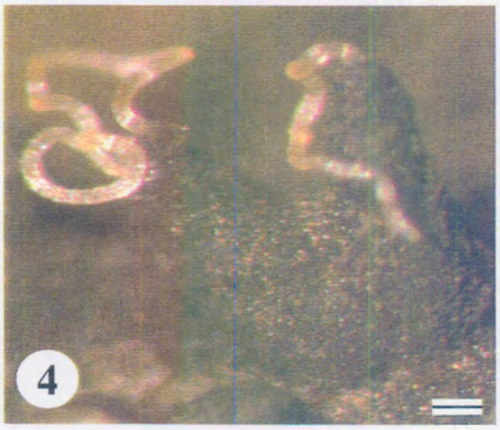
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FIGS. 6-8. Light micrographs of sexual structures of *Botryosphaeria* species. 6. *B. australis.* Immature (right) and mature (left) asci. 7. *B. australis* and 8. *B. irregularis.* Mature ascospores can be ellipsoidal to spindle shaped, smooth or with granular contents. Bars = 10 µm.

FIGS. 9-16. Light micrographs some morphological characteristics of *Fusicoccum* anamorphs of *Botryosphaeria* species. Conidia of these species are normally hyaline, thin walled and narrower than those of *Diplodia* species. 9. *B. dothidea.* Spermatiophores and spermatia. 10. *B. parva.* Fusiform conidia of medium length (~17--20 µm x 5--6 µm). 11. *B. dothidea.* Narrow fusiform and long conidia (22--25 µm x 5--6 µm). 12. *F. mangiferum.* Short, fusiform conidia (>15 µm x 5 µm). 13. *Fusicoccum* sp. (previously *Dothiorella* 'long'). Long and broad bacilliform or cylindrical conidia (24--30 x 6 µm). 14. *B. eucalyptorum.* Hyaline, septate, germinating conidia. 15. *B. parva.* Septate, mature conidia with a pigmented middle cell. 16. *B. dothidea.* Immature conidia and conidiogenous cells. Bars = 10 µm.

FIGS. 17-21. Light micrographs some morphological characteristics of *Diplodia* anamorphs of *Botryosphaeria* species. Conidia of these species are normally thick walled, broader than those of *Fusicoccum* species, regularly septate and pigmented with age. 17. *B. stevensii.* Immature conidia and conidiogenous cells. 18. *B. quercuum.* Young hyaline conidia with thick glassy walls. 19. *B. obtusa.* Aseptate, pigmented conidia with rough walls. 20. Immature hyaline and mature conidia that are pigmented and septate. 21. *B. quercuum.* Three septate and pigmented conidium. Bars = 10 µm.


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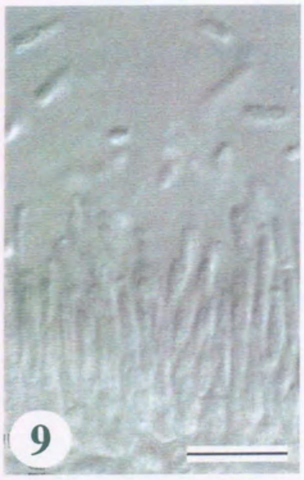
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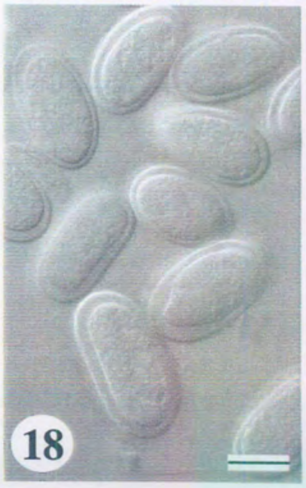
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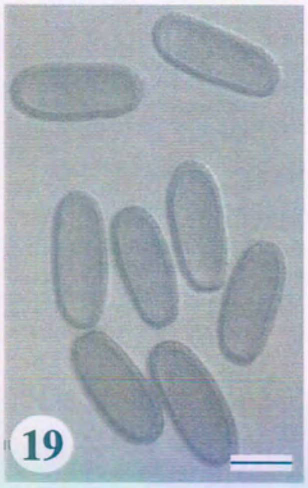
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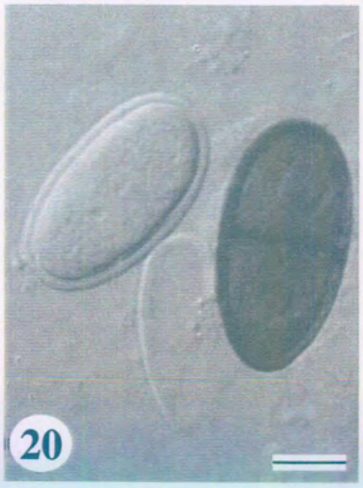
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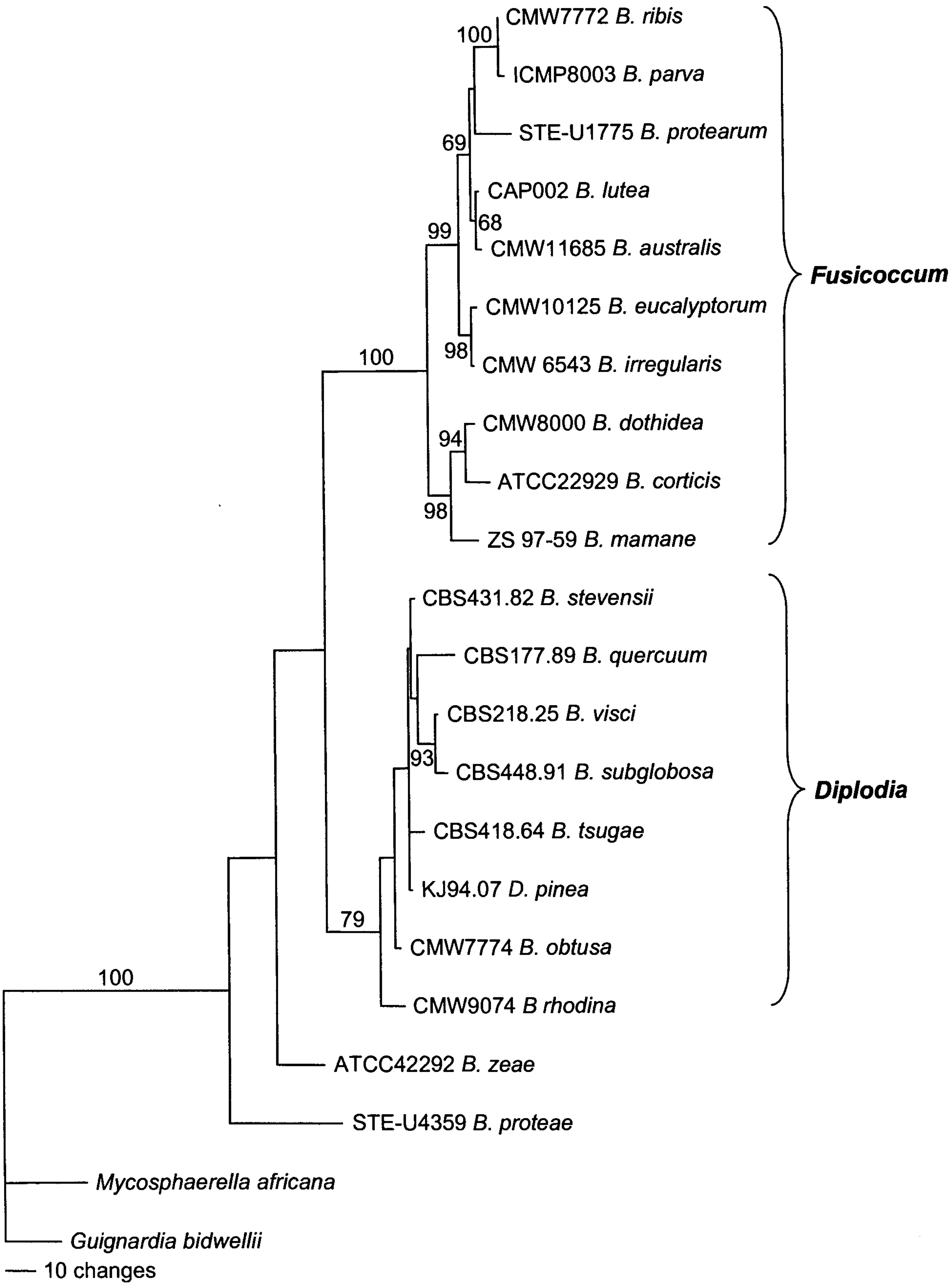
FIG. 22. One of two most parsimonious trees of 401 steps (gl = -0.43, CI = 0.71, RI = 0.83) obtained from heuristic searches of DNA sequence data of the ITS rDNA region indicating the phylogenetic relationships between various *Botryosphaeria* species and *Diplodia pinea*. Fifty characters from the hypervariable ITS1 region were excluded, because of ambiguous alignments. Bootstrap values (1000 replicates) are indicated above or below the branches. The tree is rooted to the outgroup taxa, *Mycosphaerella africana* and *Guignardia bidwellii*. Isolate numbers refer to: ATCC = American Type Culture Collection, Manassas, VA; CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998); STE = Department of Plant Pathology, University of Stellenbosch, South Africa; ZS = Zhou and Stanosz (2001a).


CMW7772 B. ribis
ICMP8003 B. parva
STE-U1775 B. protearum
CAP002 B. lutea
CMW11685 B. australis
CMW10125 B. eucalyptorum
CMW 6543 B. irregularis
CMW8000 B. dothidea
ATCC22929 B. corticis
ZS 97-59 B. mamane
CBS431.82 B. stevensii
CBS177.89 B. quercuum
CBS218.25 B. visci
CBS448.91 B. subglobosa
CBS418.64 B. tsugae
KJ94.07 D. pinea
CMW7774 B. obtusa
CMW9074 B rhodina
ATCC42292 B. zeae
STE-U4359 B. proteae
Mycosphaerella africana
Guignardia bidwellii
Fusicoccum
Diplodia
100
69
68
99
98
100
94
98
93
79
100
10 changes