Characterization of latent Botryosphaeriaceae on diverse *Eucalyptus* species

by

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MAGISTER SCIENTIAE

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Declaration

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

Happy Mamodise Maleme

December 2008

I dedicate this thesis to my late father Matthews, my mother Florence and my sisters Modiehi, Dimakatso and Nthabeleng

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SUMMARY

The Koala bears housed and maintained by the Pretoria Zoo are relying on 20 different *Eucalyptus* species in the *Eucalyptus* arboretum in Pretoria for food. Species of the Botryosphaeriaceae are well known endophytes and pathogens of *Eucalyptus* in South Africa and elsewhere. This study was aimed at characterizing the Botryosphaeriaceae infecting these trees planted for the Koala bears. The experimental set-up also provided the opportunity to broaden our knowledge regarding the *Eucalyptus* species preferences of the Botryosphaeriaceae, as well as to reveal their potential movement to and from surrounding trees.

In Chapter 1, the literature is reviewed regarding the biology of fungal endophytes, with a specific focus on Botryosphaeriaceae on *Eucalyptus*. Such a review clearly shows that, despite much previous work, we still only have a limited understanding of the biology and ecology of most fungal endophytes. This is particularly true for tree endophytes. Botryosphaeriaceae are clearly important and widespread canker and dieback pathogens of *Eucalyptus*. The taxonomic confusion that plagued the group, however, hindered a clear understanding of their diversity and biology. The use of molecular tools together with morphological characteristics has improved the ability to separate these fungi at species level. These tools are important for future work to better understand the true diversity. This would be a prerequisite if we are to gain a better understanding of the biology of these fungi and design with management strategies to control them.

In Chapter 2, five species of Botryosphaeriaceae were identified from *Eucalyptus* species in the Pretoria arboretum, South Africa. Two species were described here as new species of Botryosphaeriaceae, within *Neofusicoccum*, namely *N. ursorum* prov. nom. and *N. crypto-australe* prov. nom. This is the first report of the latter species on *Eucalyptus* in South Africa. The other species included *N. parvum*, *N. eucalypti* comb. nov. (previously known as *Dichomera eucalypti*) and *B. dothidea*. *Neofusicoccum parvum* was the most common species isolated, followed by *N. ursorum* and *N. eucalypti*. The identifications were all based on the morphological characteristics, including cultural and conidial morphology, and DNA sequence data of the internal transcribed spacer region (ITS 1 and 4), and the translation elongation factor 1-α. The closely related species *N. parvum* and *N. ribis* were distinguished using a previously designed PCR-RFLP technique. As for the distinction between the cryptic species *N. crypto-australe* and *N.*

australe, it was necessary to analyze a third gene region (β- tubulin) to confirm their separation using the phylogenetic species concept.

All isolates obtained from this study produced lesions on stems of *Eucalyptus camaldulensis* (clone ZG-14) in the pathogenicity trial conducted under green house conditions. Of all species, *N. eucalypti* and *N. crypto-australe* were found to be the most pathogenic and *B. dothidea* the least pathogenic. There was, however, also significant variation in virulence between isolates of the same species. The results clearly show the potential threat of species of Botryosphaeriaceae to *Eucalyptus*. Field trials should be conducted in future studies to validate the findings in the greenhouse trials.

Isolates representing different species identified in Chapter 2, were used for designing molecular tools for the *in vitro* and *in vivo* identification of Botryosphaeriaceae (Chapter 3). Sequences of the translation elongation factor 1-α were compared, and unique polymorphisms identified. Species specific primers were designed around these polymorphisms. All the primers designed were proven to be specific enough to distinguish the five different species from each other. The sensitivity of all primers were shown to detect fungal DNA concentration between 50 ng/µL and 0.01ng/µL. Preliminary tests of these primers on *Eucalyptus* leaves were done, and latent infections of *N. parvum* (the most common species) could be identified. More samples are likely to reveal the latent infections of other species using these tools. Future studies could now use these tools for the rapid identification of the fungi on *Eucalyptus*. It could be expanded to other hosts and more species of the Botryosphaeriaceae as well.

The results presented in this study provided detailed information on species of the Botryosphaeriaceae from the 20 different species of *Eucalyptus* in a Pretoria arboretum. It yielded unique species, as well as well known pathogens of this host. All species isolated in this study were found to be pathogenic on *Eucalyptus*. The knowledge foundation, data and tools provided by this study can now be applied to characterize the infection biology, fine scale distribution and population diversity of these fungi. It is likely to bring new insights into the ecology of these organisms, their potential origin and movement between hosts. The presence of these fungi in other parts of South Africa should also be considered.

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PREFACE

Since the description of *Botryosphaeria dothidea* by Cesati and De Notaris in 1863, Botryosphaeriaceous fungi have become well known for some serious diseases of agricultural crops, fruit trees, and trees in plantation forests. More recently, this group of fungi have also become known as endophytes causing latent infections on their hosts. These latent infections normally change to active disease when conditions turn unfavourable for the host, causing stress on the plant. Amongst the woody hosts affected by these fungi are members belonging to the Myrtaceae Family, including *Eucalyptus*. *Eucalyptus* diseases caused by Botryosphaeriaceae have been documented in various parts of the world, with canker and dieback being the most commonly reported symptoms.

Eucalyptus have been established as exotics in plantation forests in South Africa for more than a century. Recently, a variety of *Eucalyptus* species were established for feeding Koala bears housed by the Pretoria Zoo. For this project an *Eucalyptus* arboretum was established, including 20 different species. A preliminary investigation indicated that species of Botryosphaeriaceae were present and causing diseases in the arboretum. This provided an opportunity to study the Botryosphaeriaceae diversity in this defined location, investigate potential host preference of different species of the Botryosphaeriaceae and their ability to cause latent infection, and lastly to compare their relation to Botryosphaeriaceae infecting naturally regenerated *Eucalyptus* spp. surrounding the arboretum. Such information would provide a foundation for dealing with these diseases in future.

As background to the thesis, the literature was reviewed pertaining to endophytic fungi (Chapter 1). The focus was on both grasses and trees, with a particularly focus on Botryosphaeriaceae infecting *Eucalyptus* spp. It also highlights the poor definition of the term ‘endophyte’ in the literature regarding the ecological role of these fungi. Endophytes are able to adopt different ecological roles depending on a number of factors. The literature on Botryosphaeriaceae as endophytes and/ or latent pathogens on *Eucalyptus* is assessed in the light of recent taxonomic changes, their mode of infection, transmission and dispersal, stress as an inducer for disease expression, the symptoms they cause, as well as the need for prevention of their spread.

The first and main aim of the project was to identify species of the Botryosphaeriaceae from 20 different *Eucalyptus* species from the *Eucalyptus* arboretum

in Pretoria (Chapter 2). This was achieved by sampling branches and leaves from trees from each row representing the different species. Both asymptomatic and diseased material were sampled. Corresponding samples were also randomly taken from naturally regenerated, wild *Eucalyptus* surrounding the arboretum. All isolates obtained that were typical of the Botryosphaeriaceae in culture morphology were identified to the species level. DNA based tools, together with morphology, were used to characterize different species of Botryosphaeriaceae. Apart from known *Neofusicoccum* and *Botryosphaeria* species, two undescribed *Neofusicoccum* spp. were identified and are described here for the first time. A taxonomic revision is also made for a *Neofusicoccum* sp. which was previously described under *Dichomera*. The pathogenicity of all the species to eucalypts was tested. Two-year old trees of *Eucalyptus camaldulensis* (clone ZG-14) were used for this purpose in a trial conducted under green house conditions.

Most Botryosphaeriaceae were obtained from asymptomatic leaves. There is a need for tools to quickly and accurately identify these latent infections *in vivo*. A second aim of the thesis was therefore to develop molecular tools for the *in vivo* identification of the five different species of Botryosphaeriaceae identified in Chapter 2. These markers were tested on DNA extracted from both purified cultures and *Eucalyptus* leaves. The development and testing of these markers are presented and discussed in Chapter 3.

The thesis is finalised by a summary discussion of the data obtained and conclusions made in this study. Insights from this work that could guide future studies to better understand the Botryosphaeriaceous fungi, their mode of transmission, ecological role and prevention of spread is also included in this section.

Chapter 1

The biology of fungal endophytes, with specific reference to the Botryosphaeriaceae on *Eucalyptus* trees

1.0. Introduction

Fungal endophytes form an integral part of microbial communities that are commonly associated with plants. They have many different ecological roles that include mutualism, commensalism and parasitism (Carroll 1988; Arnold 2007; Saikkonen 2007; Sieber 2007). They also occur on a variety of hosts that include trees, shrubs, grasses, mosses, ferns and lichens (Stone *et al*. 2000; Zhang *et al*. 2006). Although there is abundant evidence for the positive effects that endophytes confer on some hosts such as grasses, there is little information on the ecological role that endophytes play on other hosts such as trees.

Most work on fungal endophytes of plants has been done on grasses, most notably tall fescue grasses and with a focus on either *Acremonium* or *Epichloë* species. These fungi are important for host survival and abundant evidence supports this view (Siegel *et al*. 1987, 1993; Clay 1988, 1990; Funk *et al*. 1993; Saikkonen *et al*. 1998). However, it has been proposed that this association depends on environmental conditions and nutritional availability (Müller and Krauss 2005). If nutritional resources are limited, endophyte-host association may change from mutualism to commensalism and even to antagonism or parasitism. For example, improved performance was observed on endophyte-infected *Festuca pratensis* as compared to uninfected plants on highly fertilized and watered soil. On the contrary, under low nutrient and water conditions, few tillers and lower root and total biomass were observed in *F. pratensis* infected with endophytes. In this case, an endophyte shifted from being a mutualist to an antagonist under limited nutritional resources (Ahlholm *et al*. 2002*b*). Under conditions of stress, the endophytes are hypothesized to act as commensalists or antagonists (Stanosz *et al*. 2001; Desprez-Loustau *et al*. 2006; Slippers and Wingfield 2007).

The host genotype plays an important role in the endophyte-host association. An endophyte might thus act as a mutualist, antagonist or commensalist based on the host genotype. For example, Redman *et al*. (2001) demonstrated that well-known plant pathogens in the genus *Colletotrichum*, i.e. *C. magna*, *C. coccodes*, *C. orbiculare*, *C. musae, C. lindemuthianum, C. gaminicola*, *C. glioesporiodes* and *C. acutatum*, were observed in a non-pathogenic/mutualistic lifestyle in tomato and pepper cultivars, which were known not to be susceptible to these pathogens.

The existence of endophytes in trees was first reported in *Picea canadiensis* in the Pacific North West (Lewis 1924). Since that time, there have been many reports focusing on fungal endophytes on trees (e.g. Petrini 1986; Rodrigues 1994; Azevedo *et al*. 2000; Frohlich *et al*. 2000; Stone *et al*. 2000; Arnold *et al*. 2003). A predominant view is that these fungi are highly diverse in most hosts. For example, Gamboa and Bayman (2001) found 38 morphospecies of endophytes from leaf segments of two populations of the tree species *Guarea guodonia*. These included *Phomopsis*, *Colletotrichum*, *Xylaria* and *Rhizoctonia* like fungi. These fungi were found in 95 % of leaf segments examined. More recently nine fungal genera were identified in both young and mature leaves of teak (*Tectona grandis* L.) and the rain tree (*Samanea saman* Merr.) in the study of foliar endophytes on these trees (Chareprasert *et al*. 2006). A diverse assemblage of endophytes has also been reported from *Lucuala* sp. (palm) in Brunei Darassalam and from *L. ramsayi* palm in Australia (Frohlich *et al*. 2000). Furthermore, 418 endophyte morphospecies were obtained from 83 leaves segments of two tree species *Heisteria concinna* (Olacaceae) and *Ouratea lucens* (Onchnaceae) (Arnold *et al*. 2000). Unfortunately only a few of these studies have considered the infection biology and transmission of these endophytes in trees (Deckert and Peterson 2000; Arnold and Herre 2003; Kaneko and Kaneko 2004). This will be essential if we are to better understand the role of these fungi and how they are spread in the environment.

Members of the Botryosphaeriaceae are well-known endophytes in a variety of tree hosts. Some of the better studied hosts include species in the families Myrtaceae (*Eucalyptus* spp.), Proteaceae (*Protea* spp.), Leguminoceae (*Acacia* spp.) and Pinaceae (*Pinus* spp.) (Fisher *et al*. 1993; Smith *et al*. 1996*a*; Stanosz *et al*. 1997; Burgess *et al*. 2001; Denman *et al*. 2003; Mohali *et al*. 2006). The Botryosphaeriaceae do not usually cause disease symptoms on hosts with which they are associated, unless the hosts are predisposed through environmental stress, in which case they act as latent pathogens (Schoeneweiss 1981; Swart and Wingfield 1991; Blodgett and Stanosz 1997; Desprez-Loustau *et al*. 2006).

Some species of Botryosphaeriaceae are known to be serious latent pathogens on a wide range of agricultural crops of economic importance including fruit trees such as apples, peaches, pears, avocado, mango, macadamia and pome fruits (Hartill 1991; Johnson *et al*. 1991, 1992; Biggs 1995; Ogata *et al*. 2000; Hartill and Everett 2002; Slippers *et al*. 2005;

Slippers *et al*. 2007). To give just two examples, there is a high frequency of *B*. *dothidea* as a latent pathogen on fruit clusters of Pistachio, as well as some latent infection of leaves (Ahimera *et al*. 2003). *Botryosphaeria dothidea* is also known to quiescently infect mangoes causing stem end rot post-harvest disease (Johnson *et al*. 1992). The species, however, also occurs on numerous other fruits and forest trees (Slippers and Wingfield 2007). Even though some species of the Botryosphaeriaceae are known as latent pathogens on many different hosts, the ecological role of most species as endophytes, especially in native ecosystems, is far from fully understood.

Given the ecological and economic importance of endophytes of trees, a clearer understanding of their lifestyle is needed. This review, therefore, aims to examine the different ecological roles of endophytes and thus to provide a basis for their further study. A specific focus is on the Botryosphaeriaceae as a key endophyte group in tree hosts.

2.0. Defining fungal endophytes

The term endophyte is loosely used in the literature to refer to fungi that occur inside plant tissue. The association is sometimes mutualistic where both partners benefit (Clay 1991) or commensalistic where one partner benefits and the other remains unaffected (Seiber 2007). Some endophytes may spend part or all their life cycle in the host without causing disease (Carroll 1988; Clay 1988, 1991; Petrini 1996). Others, typically referred to as latent pathogens, may exist as endophytes for part of their life cycle, but they can cause disease symptoms under certain conditions (Sinclair and Certauskas 1996). Disease symptoms often occur after the onset of environmental stress such as drought, hail damage, frost, hot or cold winds (Schoeneweiss 1981; Pusey 1989; Old *et al*. 1990). The term endophyte thus often refers to fungi with vastly different ecological roles (see Fig. 1).

Hawksworth *et al*. (2001) noted that “no agreement exists as to whether the term endophyte should be restricted only to completely asymptomatic and / or mutualistic fungi within aerial plant parts or to fungi that grow endophytically regardless of their symptoms or disease effects”. Some authors have argued that endophytes are often closely related or have evolved from plant pathogenic fungi, and that makes their differences more difficult to interpret (Carroll 1988). The life cycle of endophytes, which at some point becomes parasitic, also makes the distinction between the terms mutualism and parasitism difficult as

it relates to them (Carroll 1988). On the other hand, some case studies on specific hosts have shown that there is no close relatedness between their endophytes and known pathogens with a latent phase. For example, endophytes of *Pinus monticola* that were compared to parasites based on sequence homology, were found not to be closely related (Ganley *et al*. 2004).

Rodriguez and Redman (1997) propose that there is an evolutionary change between parasites, mutualists, and / or saprophytes (commensalists). This evolutionary change is multi-directional, i.e. the fungi can evolve from mutualists to saprophytes and to pathogens or from pathogens to mutualists and to saprophytes. Schulz and Boyle (2005) refer to this evolution as a "developmental and evolutionary continuum". A mutualism-parasitism continuum for example exists in the symbiosis between *Epichloë* sp. and grasses, where the fungus possess both the mutualistic and parasitic nature in its life cycle (Müller and Krauss 2005; Schardl *et al*. 2004). The assumption is that the change from mutualism to parasitism might be due to some balanced antagonism or equilibrium in endophyte-host interaction. If the balance is disturbed, disease development occurs (Kogel *et al*. 2006).

3.0. Endophytes of grasses

3.1. Fungal endophytes as mutualists on grasses

Much research has been undertaken to understand the interaction of endophytes and grasses. Among the best studied of the fungal endophytes are those residing in the tribe *Balansieae*, which are Ascomycetes fungi belonging to the family Clavicipitaceae (Clay 1988, 1990; Bacon 1995). These include *Epichloë* and *Neotyphodium* species, previously known as *Acremonium*. These endophytes have been reported to persist asymptomatically in grasses with some mutualistic effect. Dingle and Mcgee (2003) also observed the reduction of leaf rust disease in wheat infected with *Chaetomium* and *Phoma* species as fungal endophytes. Due to the abundant indications of the positive effect that these endophytes confer on their hosts, effort has been made to also understand the mutualistic role they play in other grasses, specifically wild grasses or grasses in natural stands (Saikkonen *et al*. 2000; Brem and Leuchtmann 2002).

Mutualism is attributed to the ability of fungal endophytes to produce one or more compounds or secondary metabolites that enhance the host's vigour, photosynthetic rate and increases the host's resistance against abiotic and biotic stress. Insect herbivory, pathogens

and drought are some of the factors that are most likely to cause stress and these are affected by endophytes (Siegel *et al*. 1987; Clay 1988, 1990, 1993; Funk *et al*. 1993; Saikkonen *et al*. 1998). For example, *Lolium multiflorum* produced significantly more vegetative tillers and allocated more biomass to roots and seed when infected with *Neotyphodium* spp. as compared to uninfected individuals (Vila-Aiub *et al*. 2005). Larvae of the fall armyworm (*Spodoptera frugiperda*) showed low pupal mass, as well as low rate of survival and mass gain, when fed on grass infected with endophytes (Clay 1988). Likewise, Tunali and Marshall (1995) demonstrated that culture filtrates of some *Acremonium* spp. and *Neotyphodium* spp. caused abnormal elongation of the hypha, lysis of the conidia and abnormal germ tubes of some pathogens. In turn, the fungi acquire nutrition, long lasting protection and dissemination via seed or vegetative material from the host (Latch 1993; Siegel 1993). Such mutualistic roles of endophytic fungi imply that they can be used as biological control organisms against pests and pathogens.

3.2. Fungal endophytes parasitic on grasses

Grass endophytes can be parasitic, where pathogens have a latent phase in their life cycle such as the smut fungi (Petrini 1986; Latch 1998). One example showing endophyte-parasitism is that of *Epichloë* endophytes, where the sexual state is able to sterilize hosts such as *Brachypodium sylvaticum* by preventing flowering and seed formation (Siegel 1993; Meijer 2001). Faeth and Sullivan (2003) also demonstrated that a *Neotyphodium starrii* endophyte on *Arizona fescue* plants reduced host growth and reproduction, and fewer germinated seeds were also observed. Fungal pathogens with a latent phase normally referred to as endophytes have been reported on a number of grass hosts including rice (*Oryza sativa*) and maize (*Zea mays*) and their negative effects have been well documented (Fisher and Petrini 1992; Pinto *et al*. 2000). These parasitic fungi fall into class three (3) based on a fungal lifestyles classification and ecosystem dynamics defined by Rodriguez and Redman (1997). This classification states that “fungi that are quickly ‘walled off’ or inhibited from colonization by plant defence responses or metabolic inhibitors, remain quiescent until the host becomes senescent”.

Environmental factors such as nutritional deficiencies and other potential pathogens that bring about stress on host plants play an important role in either sustaining an

endophyte-host association as mutualistic or turning it into a parasitic relationship. If the host is stressed or where there are insufficient nutrients available for growth, the mutualistic association often becomes parasitic (Müller and Krauss 2005). Plant defence response or metabolic inhibitors with minimal effect prevent these fungi from causing disease symptoms and the endophytes can consequently live in a latent form within a plant host until it dies. Therefore, the early assumptions that endophytes do not cause disease have definitively been shown to be false and the fungi are clearly able to adopt a parasitic lifestyle under suitable conditions (Stanosz *et al*. 2001; Faeth 2002; Faeth and Fagan 2002).

3.3. Infection and transmission of grass endophytes

Grass endophytes reproduce both sexually and asexually in their life cycle (Schardl *et al*. 2004). For example, *Epichloë sylvatica* that infects *Brachypodium sylvaticum* was reported to reproduce both sexually and asexually (Meijer 2001). In such cases, *Epichloë* spp. (sexual form) has an antagonistic effect because flowering and seed formation is suppressed. This is due to the formation of the stroma followed by the fruiting structure around the developing inflorescence. *Neotyphodium* spp. (asexual form) has a mutualistic effect and the fungi grow vegetatively into the seed from one plant to the other.

Grass endophytes are commonly vertically transmitted and the hyphae grow internally and intercellularly in the host tissue (Wilson 1993; Saikkonen *et al*. 2004). The infection is thus systemic throughout the whole plant. Saikkonen *et al*. (2004) proposed that the morphological arrangement of grasses allows easy systemic growth of the fungi throughout the plant tissue. Vertical transmission to seed progeny in most cases promotes single species dominance where a single fungal genotype is transmitted from one generation to the next.

Studies demonstrating vertical transmission of endophytes have mostly been done on cultivated or controlled tall fescue and perennial grasses (Clay 1998; Saikkonen *et al*. 2004). However, recent work on wild grasses has also revealed the possibility of horizontal transmission. For example, no evidence of vertical infection was observed when perennial ryegrass plants were infected with *Epichloë typhina*. All seeds were found not to be infected with this endophyte (Chung and Schardl 1997). Therefore, a possibility of horizontal

transmission cannot be excluded especially when dealing with endophytes on natural grasses.

4.0. Endophytes of trees

4.1. Fungal endophytes as mutualists on trees

Considerably less is known regarding fungal endophytes of trees than those of grass endophytes with regard to their protective mutualistic role (Carroll 1995). Recently, evidence suggesting potentially mutualistic effects in tree-fungal interactions has been reported (Arnold *et al*. 2003). The presence of endophytes (common genera *Colletotrichum*, *Xylaria* and *Fusarium*) on *Theobroma cacao* L. seedlings reduced the incidence of leaf necrosis and mortality when challenged with pathogenic *Phytophthora* species. Furthermore, Rubini *et al*. (2005) demonstrated the antagonistic effect of *Gliocladium catenulatum* against *Crinipellis perniciosa*, the cause of Witches' Broom Disease of *T. cacao* under greenhouse conditions. The presence of endophytic fungi on *T. cacao* was also shown to increase the host defence system against *Phytophthora palmivora* (Herre *et al*. 2007). Clearly not much research has been conducted on the protection to trees conferred by endophytes. Future research should also focus on other mutualistic roles that an endophyte might play, other than protection against pathogenic fungi.

4.2. Fungal endophytes parasitic on trees

Fungal pathogens with a latent phase are common factors that affect tree health. For example the pathogenic fungus, *Guignardia citricarpa* was recovered as an endophyte from leaves of *Citrus limon* in Argentina (Durán *et al*. 2005). This fungus has, however, been shown to be a pathogen where environmental conditions are unfavourable to the tree. Likewise, *Cytospora chrysosperma*, a well-known pathogen of poplar (Chapela 1989), was frequently isolated from both healthy and dying tissues of *Populus tremula* from northern Spain (Santamaria and Diez 2005). In another similar study, *Colletotrichum gloeosporioides,* which is pathogenic on orchids in the northeast Argentina (Cabrera *et al*. 2003), was isolated as endophyte from *Taxus mairei* in Taiwan (Wang *et al*. 2008). Some fungi can thus exist as endophytes on one host and potential mutualists on another (Espinosa-Garcia and Langenheim 1990). A significant issue here is that such studies are plagued by taxonomic

issues. For example, what is called *C. gloeosporioides* refers to a complex of species which can be confusing and other examples are treated later. More knowledge is clearly needed on the role of factors such as host genotype, environmental stress and evolutionary changes in order to thoroughly understand the ecological relevance of an endophyte in endophyte-tree associations.

4.3. Infection and transmission of tree endophytes

Fungal endophytes of woody plants are typically non-systemic infections as compared to the systemic infections of grasses. Factors such as tree size, the complex morphology of the tree and time taken for trees to reach maturity probably all limit the potential for systemic growth of endophytes (Saikkonen *et al*. 2004). Tree endophytes are mainly transmitted horizontally (Arnold *et al*. 2003) and tend to show high diversity as compared to single species dominance of endophytes in grasses (Carroll 1991; Petrini 1991). This is attributed to multiple infections caused by different fungal endophytes on a particular part of a host plant (McCutcheon *et al*. 1993). Fungal endophytes on trees also tend to show high levels of specificity to the host genotype they infect or to a particular host tissue of plant part in which they occur (Carroll 1988; Saikkonen 2007). Thus, Ahlmolm *et al*. (2002*a*) proposed that successful infections of host-genotypes by certain endophyte-genotypes rely on co-evolution that started from the time the hosts' fitness was affected by the endophyte. It is clear that more studies on the infection and transmission of these fungal endophytes are needed before we can fully understand the relationship between trees and their endophytes.

5.0. Botryosphaeriaceae as endophytes of trees

Many species belonging to the Botryosphaeriaceae have been reported as endophytes on various tree hosts (Slippers and Wingfield 2007). However, not much is known regarding their ecological role on these tree hosts. Their existence as latent pathogens has been documented worldwide (although mostly in plantation or fruit trees), with no information on the role they might play as mutualists. There is especially little information about their role in natural environments.

5.1. Botryosphaeriaceae as latent pathogens on *Eucalyptus*

Eucalyptus spp. are amongst the most important commercially planted tree crops in many countries around the world. Various fungal pathogens are known to cause diseases on this tree. Among them are species of Botryosphaeriaceae. Species of Botryosphaeriaceae have also been reported as latent pathogens on *Eucalyptus* worldwide (Smith *et al*. 1996*a*; Slippers *et al*. 2004*c*, Mohali *et al*. 2006).

In the native environment of these trees, species of Botryosphaeriaceae (then reported as *B. dothidea*) were isolated as endophytes from leaves, xylem and bark of *E. nitens* in Australia (Fisher *et al*. 1993). Furthermore, *N. eucalypticola* and *N. eucalyptorum* were isolated from native *Eucalyptus* trees in eastern Australia (Slippers *et al*. 2004*c*). More recently, *N. australe* was found to be the most common endophyte species in the leaves of *E. globulus* in plantations in Western Australia and from native *Eucalyptus* spp. in Western Australia (Burgess *et al*. 2005). In addition, *N. parvum*, *D. eucalypti* and *N. macroclavatum* were also isolated as endophytes in the latter study. More recently *Lasiodiplodia rubropurpurea* was isolated from cankers of *Eucalyptus grandis* in Queensland (Burgess *et al*. 2006*a*).

The presence of species of Botryosphaeriaceae as endophytes and pathogens has also been reported from countries where eucalypts are not native. In Venezuela, *Neofusicoccum andinum*, *Pseudofusicoccum stromaticum* and *B. mamane* were isolated from asymptomatic branches and stems of a *Eucalyptus urophylla* x *Eucalyptus grandis* hybrid, as well as from *Acacia* spp. (Mohali *et al*. 2006). In Ethiopia, Congo and Chile, *N. parvum* was found to be the major cause of *Botryosphaeria* stem canker in *Eucalyptus* plantations (Roux *et al*. 2000; Ahumada 2003; Gezahgne *et al*. 2004). In South Africa, members of the genus *Botryosphaeria* were observed on *Eucalyptus* foliage in the 1980's (Crous *et al*. 1989). *Botryosphaeria dothidea*, which was later identified as either *N. parvum* or *N. eucalyptorum*, was regarded to be amongst the most important canker and dieback pathogens of this tree species in the country (Smith *et al*. 1996*a*; Slippers *et al*. 2004*c*; Crous *et al*. 2006).

Eucalyptus spp. are affected by some species of the Botryosphaeriaceae that are also endophytes and cause diseases on various other hosts. Thus, *Lasiodiplodia theobromae* (= *B. rhodina*), a well known latent pathogen, has been reported from more than 500 hosts (Punithalingam 1976). For example, *L. theobromae* was found to be the cause of stem end

rot on mango, and to cause wood discoloration on *Pinus*, *Acacia* and *Eucalyptus* spp. in Venezuela (Johnson *et al*. 1992; Jacobs 2002; Mohali 2005; Burgess *et al*. 2006*a*). Diseases such as reduced germination of seeds, stem canker and dieback in various African countries have been reported to be associated with this fungus on *Pinus* and *Eucalyptus* spp. (Cilliers *et al*. 1993; Roux *et al*. 2001). *Neofusicoccum parvum* (= *B. parva*) is another common fungus known to cause diseases on variety of hosts (Slippers *et al*. 2004*c*; Crous *et al*. 2006). It is known to cause canker and dieback and stem end rots on hosts such as *Eucalyptus*, Avocado (*Persea americana*) and Mango (*Mangifera indica*) in countries like South Africa, Australia, Chile and New Zealand (Jacobs 2002; Hartill and Everett 2002; Ahumada 2003; Slippers *et al*. 2004*c*; Burgess *et al*. 2005). Given the wide host range of some Botryosphaeriaceae, movement of such fungi with *Eucalyptus* germplasm or seeds to new areas could be detrimental to the native plant communities in those areas.

5.2. Taxonomic history of Botryosphaeriaceae endophytes on trees

The Botryosphaeriaceae have been plagued by considerable taxonomic confusion in the past. This confusion has had a significant negative effect on studies of the endophytic nature of the members of Botryosphaeriaceae and thus deserves discussion here. The taxonomic confusion regarding these fungi arose from the fact that their taxonomy initially relied on teleomorph morphology and the host association. This approach proved to be unreliable because the species have overlapping morphological characteristics especially in the case of their teleomorph structures. Furthermore, many species also colonise more than one host and naming new species based on the hosts on which they occur was particularly misleading. In more recent studies, identification of species of the Botryosphaeriaceae has been based predominantly on anamorph morphology which is more frequently observed in nature. Studies combining both DNA sequence data and morphological characteristics has, however, made the greatest contribution to reliable species identification (Jacobs and Rehner 1998; Denman *et al*. 2000; Slippers *et al*. 2004*a*; Crous *et al*. 2006).

The most recent and extensive attempt to resolve the taxonomy of the Botryosphaeriaceae based on DNA sequence data and morphology was by Crous *et al*. (2006), which separated multiple lineages within the Botryosphaeriaceae into distinct genera. These genera include *Diplodia* / *Lasiodiplodia*, *Botryosphaeria*, *Macrophomina*,

Neoscytalidium, *Dothidotthia*, *Botryosphaeria*, *Neofusicoccum*, *Pseudofusicoccum*, "*Botryosphaeria*" *quercuum*, *Saccharata*, *Guignardia*, *Camarosporium* and *Stenocarpella*. *Botryosphaeria dothidea* provides an excellent example of how modern tools have affected studies of Botryosphaeriaceae as endophytes. This fungus was previously thought to be common as an endophyte infecting *Eucalyptus* in South Africa (Smith *et al*. 1996*b*). It was later shown that this identification rather represents *Neofusicoccum parvum*, *N. eucalyptorum* and *N. eucalypticola* (Slippers *et al*. 2004*c*). These species all occur as endophytes and are also associated with canker and dieback of *Eucalyptus* in South Africa (Smith *et al*. 2001; Slippers *et al*. 2004*c*). A number of previously unknown species were also discovered in other countries by combining morphological and molecular data. In Venezuela, for example *N. andinum* and *Pseudofusicoccum stromaticum* were discovered and described from asymptomatic branches and stems of *Eucalyptus* and *Acacia* spp. (Mohali *et al*. 2006). Likewise, in Western Australia, *N. macroclavatum* was described from *Eucalyptus globulus* as an endophyte (Burgess *et al*. 2005).

Many species of the Botryosphaeriaceae known as endophytes of trees other than *Eucalyptus* have also undergone taxonomic changes in recent years. The fungus previously treated as *Diplodia pinea* (= *Sphaeropsis sapinea*) on pine, has been shown to represent a species complex including two forms of *D. pinea* and *D. scrobiculata* (de Wet *et al*. 2003). Furthermore, the Botryosphaeriaceae that had previously been identified as *Dothiorella* or *Natrassia* from mango (Johnson *et al*. 1992) were re-examined and found to represent *N. parvum*, *N. mangiferum*, *F. aesculi* (teleomorph *B. dothidea*) and an undescribed *Neofusicoccum* sp. (Slippers *et al*. 2005). Clearly, wide variety of hosts are affected by members of the Botryosphaeriaceae and a thorough taxonomic resolution of these species will serve as a basis for understanding their biology as endophytes.

Studies based on ITS sequence data, together with morphological characteristics, paved the way towards more consistent identification and more reliable interpretation of the biology of the Botryosphaeriaceae as endophytes (Jacobs and Rehner 1998; Smith *et al*. 2001; Pavlic *et al*. 2004). It is important, however, to note that in the case of closely related species, a single gene region might not be sufficiently variable to distinguish these cryptic species. For this reason, multiple gene sequence data are needed to clearly distinguish between these cryptic species (de Wet *et al*. 2003; Slippers *et al*. 2004*a*, *b*, *c*; Burgess *et al*.

2005; Phillips *et al*. 2005, Alves *et al*. 2008). Future studies will need to focus on the correct identification of unrecognized species and cryptic species using tools of appropriate resolution.

Earlier work on the Botryosphaeriaceae as endophytes is confusing as the species names used do not reflect the current nomenclature of these fungi. For example, it is almost impossible to use reports that were made prior to the application of DNA sequence data for the taxonomy of the Botryosphaeriaceae, to understand the specificity, infection and distribution of these fungi as endophytes. The early literature should, therefore, be interpreted with caution or at least with an understanding that the names are probably not accurate.

5.3. Mode of infection, transmission and dispersal

Botryosphaeriaceae infect their hosts through both natural openings and wounds, although wounds were previously thought to be the primary mode of entry into the host for many of these fungi (von Arx and Müller 1954; Schreiber 1964; Punithalingam and Holliday 1973; McGlohon 1982; Michailides 1991; Smith 1995, 2001). Smith (1995) showed *E. grandis* leaf infection by *Neofusicoccum* spp. (identified as *B. dothidea*), where germ tubes from conidia entered through the stomatal openings. The same situation was also observed on leaves, rachises and shoots of pistachio (Michailides 1991). This enables a fungus to be established inside a leaf tissue with no apparent disease symptoms, therefore causing a latent infection.

Species of Botryosphaeriaceae reproduce sexually and asexually in their life cycle through the production of ascospores and conidia, respectively (Sutton 1981). Conidia are more frequently encountered in nature than ascospores (Michailides 1991; personal observations). The conidia are thus expected to be the primary source of inoculum and are transmitted horizontally from one plant to another (Smith 2001). These conidia are mostly wind dispersed or are dispersed through rain splash (Weaver 1974; Michailides and Owaga 1986; Cresswell and Milholland 1988; Pusey 1989). This is consistent with the findings of Stanosz *et al*. (2005) who reported 88 % higher frequency detection of *D. pinea* in seedlings that were near a source of *D. pinea* conidia than those that were distant from it in nurseries.

Germination and infection is strongly influenced by environmental conditions (Swart *et al*. 1987; Ntahimpera *et al*. 2002; Ahimera *et al* 2003). For example, Michailides and Owaga (1986) reported that infections on pistachio caused by *B. dothidea* developed rapidly in the late spring and summer when maximum temperature ranged from 21–40 ºC. Everett and Pak (2002) on the other hand reported that in latent pathogens such as *N. parvum*, which causes fruit rot on avocado, spore germination could occur at low temperatures. Low temperatures were shown to influence rapid proliferation of '*Botryosphaeria' ribis* that invades stems of *Melaleuca quinquenervia* (Rayachhetry *et al*. 1996). There are other factors that also contribute to ascospore and conidial germination of the Botryosphaeriaceae. Relative humidity has thus been shown to influence ascospore and conidia germination of *Diplodia seriata* (= *Botryosphaeria obtusa*) and most spores achieve maximum germination at 95 % RH or higher. If free water and relative humidity decrease, germination is also decreased (Arauz and Sutton 1989). Closer attention needs to be paid to the role of temperature and moisture in influencing the mode of infection, transmission and dispersal of these fungi.

Physical response to Botryosphaeriaceae infection has been documented on hosts such as *Melaleuca quinquenervia* (Rayachhetry *et al*. 1996). Callus cells are formed around the invaded tissue and these limit pathogen invasion. However, there are mechanisms that exist by which the pathogens can overcome host resistance. In the case of *D. pinea*, this is aided by enzymatic activity that degrades the cell walls in the initial phase of pathogenicity (Chou 1978) and this work was done before the fungus was known to exist as an endophyte. However, this topic has been poorly studied and more intensive investigations on the plants' response to Botryosphaeriaceae infections is needed if we are to better understand their mode of infection and how the plants' resistance is overcome by these fungi.

Some species of Botryosphaeriaceae are known to be seed-borne. For example, *D. rosulata* has been isolated from healthy seeds of *Podocarpus falcatus* and *Prunus africana*, while *L. theobromae* and *D. pinea* has been isolated from coniferous seeds (Cilliers *et al*. 1993, 1995; Smith *et al*. 1996*a*; Gure *et al*. 2005). It has been suggested that the seed-borne nature of these fungi has led to the global movement of the Botryosphaeriaceae around the world (Smith *et al*. 2000; Burgess *et al*. 2001, 2004). There is no conclusive evidence that endophytic infections result in systemic infection of the entire plant, but most evidence

points to the plant being continuously re-infected with these endophytes from the environment (Slippers and Wingfield 2007). There is clearly a need for additional studies to address these questions and thus to better understand the infection biology and distribution of species of Botryosphaeriaceae as endophytes.

5.4. Stress as an inducer of disease expression

Environmental conditions are important for disease expression of Botryosphaeriaceae living as latent pathogens. Visual disease symptoms are typically observed as soon as conditions become unfavorable for the host. This probably results in a lowering of the hosts natural defenses, which in turn results in their becoming susceptible to fungal colonization and disease development (Schoeneweiss 1981; Pusey 1989; Old *et al*. 1990; Smith *et al.* 1994; Brown-Rytlewski and McManus 2000). It has also been shown that a decrease in water potential increases colonization of plants by Botryosphaeriaceae (Schoeneweiss 1981). In addition, it has been shown that freezing and defoliation stress are important factors that favour disease development (Schoeneweiss 1981). Death of *E. radiata* trees in species selection trials has been attributed to increased susceptibility resulting from drought or low rainfall (Shearer *et al*. 1987). Likewise, 92 % of dead *Pinus* seedlings with collar rot symptoms, which develop due to water stress, were infected with *D. pinea* (Stanosz *et al*. 2001). The seedlings displayed symptoms of collar rot, which normally develops due to internal water deficit. Similarly ring necrosis that develops under dry conditions has been shown to promote susceptibility to species of Botryosphaeriaceae causing post harvest disease of avocado fruits in New Zealand (Hartill 1991). The damage that these fungi can cause to their hosts under stressful conditions can be quite severe, and is expected to increase with increasing stress on trees due to climate change (Desprez-Loustau *et al*. 2006).

5.5. Disease symptoms

Species of Botryosphaeriaceae have been associated with diseases of many woody plants and economically important agricultural crops (Sutton 1980; Ciesla *et al*. 1996; Old 2000; Old and Davidson 2000). Disease symptoms are often associated with environmental stress resulting from abiotic and biotic factors (Schoeneweiss 1981; Swart and Wingfield 1991; Smith *et al*. 1994; Paoletti *et. al*. 2001). Symptoms associated with the

Botryosphaeriaceae infections are also very variable. They can for example include twig and branch dieback of walnut trees induced by *N. ribis* (Rumbos 1987), root diseases of *Pinus taeda* and *P. eliottii* caused by *B. dothidea* in Hawaii (Hodges 1983), gummosis of peach trees caused by three species of Botryosphaeriaceae (*B. dothidea*, *D. seriata* and *L. theobromae*) (Britton and Hendrix 1989), and bleeding necrosis of sweet gum caused by *N. ribis* in Illinois and India (Neely 1968).

On *Eucalyptus,* disease symptoms associated with the Botryosphaeriaceae have been reported in many countries of the world. Members of this family of fungi are known to cause diseases such as seed capsule abortion and twig dieback of *E. camaldulensis* in South Florida, USA (Webb 1983), twig, branch and upper trunk cankers of *E. marginata* in Western Australia (Davison and Tay 1983), death of *E. radiata* in species selection trials in Australia (Shear *et al*. 1987), basal cankers and coppice failure of *E. grandis* in Florida (Barnard *et al*. 1987). In South Africa, *B. dothidea* (later shown to represent *N. parvum* and *N. eucalyptorum*) has been shown to cause cankers and dieback on this host throughout South Africa (Smith *et al*. 1996*a*; Smith *et al*. 2001; Slippers *et al*. 2004*c*).

6.0. Endophytes and quarantine

Many endophytic fungi are seed-borne or can be present on asymptomatic host material. Their movement around the world is of great concern and they must be considered when developing quarantine strategies (Wingfield *et al*. 2001; Burgess and Wingfield 2002*a*, 2002*b*). As latent pathogens occur in the absence of symptoms for part of their life cycle, they can easily be overlooked. This must clearly facilitate their introduction into new areas. For example, Slippers *et al*. (2004*c*) found *N. eucalyptorum* and *N. eucalypticola* to be the most common species on Eucalypts in eastern Australia. They are probably native to that region. Their occurrence on *Eucalyptus* spp. in South Africa and Chile was possibly due to the introduction of these exotic hosts into these two countries (Smith *et al*. 2001; Ahumada 2003).

Species of Botryosphaeriaceae have the ability to jump hosts and to co-infect native as well as closely related introduced tree species. For example, *N. parvum* has been reported to occur on *Eucalyptus* spp. in many countries around the world, but it is also common on native trees in South Africa (Ahumada 2003; Nakabonge 2002; Gezahgne *et al*. 2004;

Slippers 2003, 2004*c*). Pavlic *et al*. (2007) identified eight species of Botryosphaeriaceae on the native species *Syzygium cordatum* in South Africa and some of these fungi have also been reported to occur on *Eucalyptus* (Slippers *et al*. 2004*a*, *b*).

Neofusicoccum australe was found to commonly occur on different hosts native to Australia, including *Eucalyptus*, but has been isolated from native *Acacia* spp., fruit trees (apple, pear, plum and almond), *Syzygium cordatum* and grapevine in South Africa (Slippers *et al*. 2004*b*; van Niekerk *et al*. 2004; Pavlic *et al*. 2007; Slippers *et al*. 2007). No barriers were detected in the movement of *N. australe* between commercial *E. globulus* plantations and native forests in Western Australia (Burgess *et al*. 2006*b*). Since it is known that introduction of exotics can introduce pathogens into new areas, stringent quarantine measures are needed to control detrimental effect that these pathogens could have on tree hosts, both native and introduced.

7.0. Conclusions

Fungal endophytes can assume many different ecological roles. They can be beneficial (mutualism), exist without being harmful (commensalism) or can be harmful to the hosts at some point in their lifecycle (parasitism). Much of the work done on grasses focused on endophytes as mutualists. More research is clearly is needed to better understand other ecological roles that these fungi might have. Endophyte infected grasses in the natural, undisturbed stands have also been less well studied, and such studies could substantially expand the understanding of the biology of endophytes.

Many reports show that endophytes of trees can become parasitic. These species include members of the family Botryosphaeriaceae that are well known latent pathogens of many woody hosts. However, most studies focused on planted and managed trees or tree crops and there is little information available on the ecological role these fungi play as endophytes in natural ecosystems. The possibility of these species existing as mutualists needs to be further investigated, more so now that evidence of other mutualistic endophytes on woody hosts is slowly emerging.

Factors such as environmental stress clearly contribute to the change from an endophytic lifestyle to a parasitic one. Many agricultural crops and hosts of commercial importance are planted on marginal sites, and increasingly so due to limited space and

climate change. These plants will be under constant attack from latent opportunistic pathogens such as the Botryosphaeriaceae. Future studies should focus on understanding the interaction of these pathogens with their hosts under varying environmental conditions. The mode of reproduction, infection and distribution of these fungi under such conditions can contribute substantially in assessing risk and developing control measures. Resistant cultivars could also be selected based on a better understanding of the tree-fungal-environment interaction.

The existence of fungi such as the Botryosphaeriaceae as latent pathogens, pose a threat to indigenous flora, plantation forestry and agricultural crops, especially because these fungal pathogens often go undetected by quarantine procedures. Rapid identification tools should thus be designed for early detection of these fungal pathogens in order to help address this problem. This process should be based on a fuller understanding of the true diversity of species belonging to the Botryosphaeriaceae. Databases on fungal pathogens should also be frequently up-dated, to keep track of newly emerging latent pathogens with the aim of trying to minimize their spread.

8.0. References

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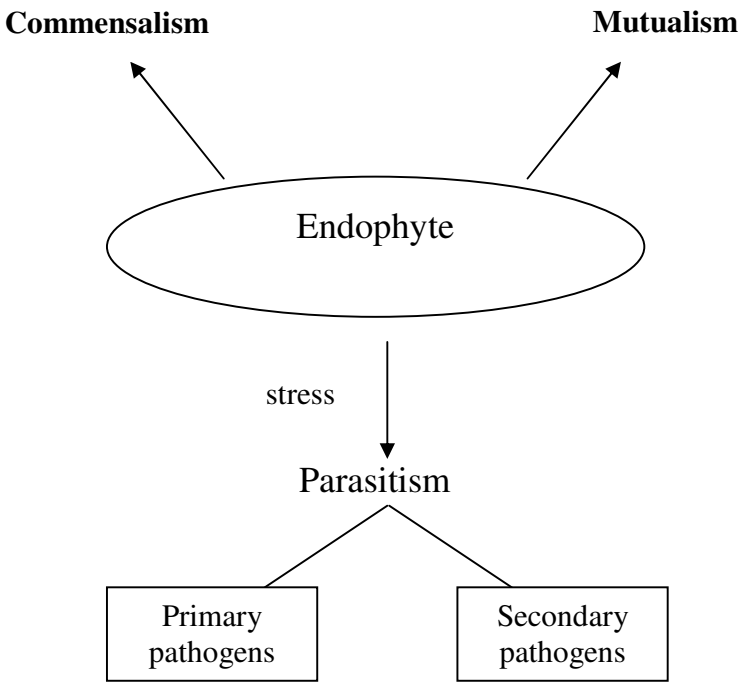

Fig. 1. Different ecological roles that an endophyte can adopt.

Chapter 2

Botryosphaeriaceae, including two new species, identified from a *Eucalyptus* arboretum in Pretoria, South Africa

Abstract

Disease symptoms that are typical for the Botryosphaeriaceae were observed in a small *Eucalyptus* arboretum in Pretoria, which is maintained to provide feed for Koala bears at the Pretoria Zoo. The aim of this study was to identify and characterize species of Botryosphaeriaceae from the 20 *Eucalyptus* species maintained in this arboretum and to determine their pathogenicity. Isolations were made from diseased and asymptomatic twigs and leaves. All isolates resembling species of Botryosphaeriaceae were induced to sporulate in culture and were subsequently characterized based on anamorph morphology. DNA sequence data for the ITS rDNA, translation elongation factor 1-α (EF- 1 α) and β-tubulin region of selected isolates were compared with known species to establish their identity. A previously developed PCR-RFLP technique was used to separate isolates of *Neofusicoccum parvum* and *N. ribis*. Isolates that did not sporulate in culture were also distinguished using a PCR-RFLP fingerprinting technique. Results revealed five species of the Botryosphaeriaceae including *B. dothidea, N. parvum*, *N. eucalypti* comb. nov. and two new species described here as *Neofusicoccum ursorum* sp. nov. and *N. crypto-australe* sp. nov. While there was no indication of host specificity, all five species were pathogenic on *Eucalyptus*.

Introduction

The Botryosphaeriaceae are among the most common fungi that cause diseases of various commercially grown trees. These fungi have been reported on branches and leaves of gymnosperms and angiosperms, culms of monocotyledons, herbaceous stalks or leaves of dicotyledons, worldwide (von Arx 1987). The various Botryosphaeriaceae are associated with disease symptoms such as cankers and dieback, leaf lesions and tip blight, fruit rots and even death of the trees (Shearer *et al*. 1987; Crous *et al*. 1989; Smith *et al*. 1994). Species of Botryosphaeriaceae can also exist in plant tissue as endophytes or latent pathogens. These fungi typically do not cause any visible symptoms until the onset of stressful environmental conditions such as drought, hail and frost damage, hot and cold winds and damage caused by hail and frost (Pusey 1989; Old *et al*. 1990).

The taxonomy of the Botryosphaeriaceae has been confused in the past. Identification was commonly achieved based on morphological characteristics or the host on which species were found (e.g. von Arx and Müller 1954; Shoemaker 1964; Pennycook and Samuels 1985). The many overlapping morphological characteristics among different species and the fact that some morphological features change with age lead to a substantially misleading taxonomy for these fungi. Recent studies, combining molecular and morphological characters, have led to an extensive revision of the taxonomy of the Botryosphaeriaceae (Denman *et al*. 2000; Slippers *et al*. 2004*a*; Crous *et al*. 2006). For example, *B*. *dothidea* (Moug.: Fr.) Ces. & De Notaris, previously considered one of the most important canker and dieback causing fungi on *Eucalyptus* in South Africa, was shown to represent *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers and A.J.L. Phillips and *N*. *eucalyptorum* (Crous, H. Smith. & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (Slippers *et al*. 2004*b*).

Eucalyptus have been planted as exotics in many parts of the world, including South Africa. It has been previously suggested that the introduction of these trees can also introduce pathogens into new areas via planting stock or seed (Wingfield *et al*. 2001). Evidence of the presence of pathogenic fungi such as species of Botryosphaeriaceae on seeds has been documented on *Eucalyptus* and other hosts (Webb 1983; Cilliers *et al*. 1993; Lupo *et al*. 2001; Gure *et al*. 2005). Due to their seed-borne nature and existence in

asymptomatic plant material, species of Botryosphaeriaceae can be expected to be easily moved unnoticed into new areas together with *Eucalyptus* (Slippers and Wingfield 2007).

Botryosphaeriaceae have a wide host range with the ability to move between native and introduced hosts (Slippers *et al*. 2005; Slippers and Wingfield 2007). For example, no restrictions of the gene flow of canker pathogen *N. australe* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips were detected between non-native *Eucalyptus* globulus plantations and native eucalypt forests in Western Australia (Burgess *et al*. 2006). As another example, all species of Botryosphaeriaceae found on the native *Syzygium cordatum* in South Africa, were found to be more pathogenic on *Eucalyptus*, with a number of these species overlapping in occurrence between the two hosts (Pavlic *et al*. 2007). Consequently, *Eucalyptus* can be expected to acquire new species of Botryosphaeriaceae from the surrounding trees in a new area, and to donate species to native plant communities.

An arboretum of 20 different *Eucalyptus* spp. has been established in Pretoria, South Africa. This resource was established to provide feed for Koala bears housed at the Pretoria Zoo. As part of an overall disease evaluation, a survey was conducted to identify and characterize species of Botryosphaeriaceae on these trees and to compare them to species found on naturally regenerated *Eucalyptus* trees surrounding the arboretum.

Materials and methods

Isolates

Eighty four isolates were obtained from 20 *Eucalyptus* spp. growing in an arboretum in Pretoria, South Africa, as well as from surrounding wild *Eucalyptus* trees (Table 1). The arboretum consisted of 12 blocks (20 rows in a block), with each row having 11 trees of each *Eucalyptus* sp. Three trees (tree 1, 5 and 10 of each row) of each species were sampled from three of the blocks (block 1, 6 and 7). Twenty five *Eucalyptus* trees surrounding the arboretum were sampled. Isolations were made using the protocol described by Pavlic *et al*. (2004). All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphology and cultural characteristics

Isolates were induced to sporulate on sterilized pine needles placed on 2 % water agar (WA) (Agar; Biolab, South Africa) and incubated at 25 °C under near-UV light. Pycnidia formed on pine needles after two to three weeks of incubation. A mass of conidia oozing from the pycnidia were mounted in 85 % lactic acid on microscopic slides and examined using a light microscope. Images were captured using an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd., Münhen, Germany). Conidia (20–50) and 50 conidiogenous cells were measured for each isolate. Colony morphology and colour were noted for cultures grown on 2 % malt extract agar (MEA) (Biolab, South Africa) at 25 °C and culture colours were defined by comparison to the colour charts of Rayner (1970). Growth studies were conducted for selected isolates representing each species of Botryosphaeriaceae at temperatures ranging from 10–35 °C in the dark. Each culture was grown in duplicate and subsequently growth was measured perpendicularly. Culture growth at different temperatures was measured daily until 90 mm plates had been covered by mycelium.

DNA extraction and PCR amplification

Single conidial cultures were grown on 2 % MEA at 25 °C for 7 days. The mycelium was scraped directly from the medium and transferred to Eppendorf tubes (1.5 mL) and 300 µL of an extraction buffer (200 mM Tris-HCl pH 8.5, 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added. A modified phenol:chloroform method for DNA extraction was followed (Raeder and Broda 1985, Slippers *et al*. 2004*a*). The resulting DNA pellets were re-suspended in 30 µL sterile SABAX water. RNAse (1mg/ µL) was added to DNA suspensions and left overnight at the room temperature for RNA degradation. DNA electrophoresis was performed on a 1.5 % agarose gel, stained with ethidium bromide. Bands were visualised under ultra-violet light. DNA concentration was estimated against a λ standard size marker.

The ITS region was amplified using primers ITS 1 and ITS 4 (White *et al*. 1990) and part of the β-tubulin gene region was amplified using the primers Bt2a and Bt2b (Glass and Donaldson 1995). A portion of the EF-1α gene region, was amplified with primer set EF-AF (5´ CAT CGA GAA GTT CGA GAA 3´) and EF-BR (5´ CRA TGG

TGA TAC CRC GCT C 3´) (Sakalidis 2004). The reaction mixture contained 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California), 10 × PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM dNTPs and 10 mM of each primer. The reaction mixture was made up to the final volume of 25 µL with sterile water. The following PCR program was used: denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing temperature at 55 °C for 45s, elongation at 72 °C for 1½ min and a final elongation step at 72 °C for 5 min. The PCR amplicons were viewed on a 1 % agarose gel, stained with ethidium bromide under UV-light. To estimate the band sizes, a 100 bp marker was used.

PCR-RFLP analysis

Two groups of isolates could be separated based on conidial morphology. Variations observed in the largest group indicated that there can be more than one species. A PCR-RFLP technique developed by Slippers (2003) was used to distinguish the species identified in this study based on morphology. ITS rDNA amplicon digestion with the restriction enzymes (RE) *Hha*I and *Ksp*I could separate three species from isolates identified using morphology. Isolates identified to belong to the *Neofusicoccum parvum* / *N. ribis* complex based on this ITS rDNA PCR-RFLP technique, were further identified by PCR-RFLP analysis of the amplicon obtained using primers *Bot 15* and *Bot 16* (Slippers *et al*. 2004*b*) and RE *Hha*I. The PCR-RFLP reaction mixture for all the above reactions consisted of 20 µL PCR product, 0.3 µL RE *Hha*I or *Ksp*I and 2.5 µL of the matching enzyme buffer (Fermentas, South Africa). The reaction mixture was incubated at 37 °C overnight. Digested fragments were separated on a 3 % agarose gel ran at a low voltage (60V) for 1 hour.

DNA sequencing and analysis

Representative isolates from each group (twenty six in total) including those that could not be identified based on morphology and PCR-RFLP technique, were sequenced using the primers that were used for the PCR amplification. The ITS sequences were compared to those of known Botryosphaeriaceae obtained from GenBank, with a particular focus on those previously isolates from *Eucalyptus* (Table 1). Two sequences of

Lasiodiplodia theobromae (Pat. Griffon and Maubl.) were used as outgroup. Five isolates that grouped separately from known species were further sequenced using EF-1α and β-tubulin primers in order to confirm their identity. Sequencing of the purified products was carried out by using ABI PRISM 3100^{TM} automated DNA sequencer (Perkin-Elmer). Nucleotide sequences were analysed and edited using SEQUENCE NAVIGATOR version 1.0.1. (Perkin- Elmer Applied Bio-Systems, Foster City, CA) software. Online software, MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/ server/), was used for original alignments (Katoh *et al*. 2002), after which manual adjustments were made. Phylogenetic analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999) with gaps treated as the fifth character and all characters assumed to be unordered and of equal weight. The heuristic search function was used to obtain the maximum parsimony tree and random stepwise addition and tree bisection and reconstruction (TBR) were chosen as the branch swapping algorithm. A thousand (1000) bootstrap replicates were used as a measure of branch support (Felsenstein 1985).

Pathogenicity

Thirteen isolates representing five species of the Botryosphaeriaceae that were identified in this study were used in pathogenicity tests in the greenhouse (Table 1). Two or three of the fastest growing isolates (on MEA) per species were selected and grown on MEA at 25 ^{o}C under continuous near-fluorescent light for seven days before inoculations.

Two-year-old trees of an *Eucalyptus grandis* clone (ZG-14) were maintained in the green house for approximately three weeks prior to inoculations for acclimatization. The greenhouse was exposed to natural day and night light cycles at a constant temperature of about 25 ^{o}C. Ten trees were inoculated with each of the 13 isolates selected. As a control, 30 trees were inoculated with sterile MEA plugs. Wounds were made on the stems of trees using 8 mm diameter cork borer approximately 250 mm above the soil level. Mycelial plugs were prepared from seven-day-old cultures, with the same size cork borer. Mycelial plugs were placed into wounds with the mycelium facing the exposed cambium and sealed with laboratory film (Parafilm “M”, Pechiney Plastic Packaging, Chicago, U.S.A) to prevent drying out and contamination. Lesion lengths were measured six weeks after inoculation. Re-isolation of the fungi from resulting lesions was done by cutting small

pieces of the wood from the edge of lesions and plating it on 2 % MEA at 25 ^{o}C. The entire trial was repeated once to verify the pathogenicity of all isolates under the same conditions.

Lesion lengths that developed six weeks after inoculation were used as a measure of the pathogenicity. SAS® version 8.2 (2001) was used for statistical analysis of the data produced. The data were analyzed separately for each of the two trials and because there were no significant difference between them, the data were combined. P-values generated after the statistical analysis of the combined dataset were used as a measure of determining the significant differences amongst lesions produced.

Results

Morphology and cultural characteristics

Forty four of the 84 isolates produced anamorph structures on WA amended with pine needles. No teleomorph structures were observed. Two groups were obtained based on length to width (L/W) ratio of conidial morphology (Table 2). Isolates representing the first group with the L/W ratio of 2.8–4.0 could not be distinguished to species level based on conidial and cultural morphology, therefore PCR-RFLP technique and sequencing was used. In that group, few isolates were distinguished from others based on yellow pigmentation in culture. The second group could be easily distinguished from other species based on a higher L/W ratio. All isolates produced conidia typical for the *Neofusicoccum* species, and two groups were identified to species level. The morphology of the other three groups could not be linked to species with confidence and are described in detail later under the taxonomy section, following molecular identification.

PCR-RFLP analysis

Three profiles (restriction length polymorphism fingerprints) were observed after digesting ITS rDNA PCR product with RE *Hha*I (Fig. 4) and were matched with those specified by Slippers (2003). Based on this comparison, forty isolates represented *Neofusicoccum parvum / N. ribis* (profile A), 28 isolates represented either *D. eucalypti* or one of the two undescribed *Neofusicoccum* species identified using sequencing (profile B) and 10 isolates represented *Botryosphaeria dothidea* (profile C). The ITS rDNA

amplicons of all isolates were then digested with (RE) *Ksp*I and three profiles were observed (Fig. 5). *Neofusicoccum* sp. 1 (clade VII) (profile B) could be separated from the rest following this digestion with (RE) *Ksp*I. All the remaining isolates had the same profile (profile A) and their identity were confirmed with DNA sequence data (*D. eucalypti* and *Neofusicoccum* sp. 2).

All forty isolates that were identified as belonging to the *N. parvum* / *N. ribis* complex produced the same profile following *Hha*I digestion of the *Bot 15* and *Bot 16* primers amplicons (Fig. 6). This profile matched that of *N. parvum* as described by Slippers (2003). With the use of these PCR-RFLP profiles and DNA sequence data, the number of isolates per species obtained from both *Eucalyptus* in the arboretum, as well as in the surrounding *Eucalyptus* spp. could be confirmed for all 84 isolates (Table 4).

DNA sequencing and analysis

Sequences of approximately 550 bp (ITS rDNA), 300 bp (EF-1α) and 390 bp (β-tubulin) were obtained. Aligned ITS dataset consists of 60 in-group sequences of which 26 represent isolates obtained in this study and 34 sequences of known species of Botryosphaeriaceae that occur on *Eucalyptus*, obtained from GenBank (Table 1). The primary analysis produced 112 most parsimonious (MP) trees of 154 steps (consistency index (CI) = 0.747, retention index (RI) = 0.952) and one of them is presented (Fig. 1).

Twelve terminal clades were observed for all isolates considered in the phylogenetic analyses of ITS sequence data (Fig.1). Most isolates obtained in this study grouped with other species of *Neofusicoccum* and some isolates grouped with *Botryosphaeria*. The *Neofusicoccum* clade consisted of *N. ribis* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade I), *N. parvum* (clade II), *N. macroclavatum* (T. Burgess, Barber & G.E Hardy) T. Burgess, Barber and G.E Hardy (clade III), *D. eucalypti* (G. Winter) B. Sutton (clade IV), *N. vitifusiforme* (van Niekerk & Crous) Crous, Slippers and A.J.L Phillips (clade V), an unknown species (*Neofusicoccum* sp. 1) closely related to *Dichomera eucalypti* (clade VI), an unknown species (*Neofusicoccum* sp. 2) closely related to *N. australe* (clade VII), *N. australe* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade VIII), *N. luteum* (Pennycook & Samuels) Crous, Slippers and A.J.L. Phillips (clade IX), *N. eucalypticola*

(Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade X), *N. eucalyptorum* (Crous, H. Smith. ter & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade XI). The other major clade represents *B. dothidea* (clade XII). All isolates obtained in this study grouped into five different clades, namely clades II (*N. parvum*), IV (*D. eucalypti*), VI (*Neofusicoccum* sp. 1) and VII (*Neofusicoccum* sp. 2), XII (*B. dothidea*) (Fig. 1).

A subset of isolates were analysed using EF-1α data to strengthen support for the branches observed using ITS sequences and to test congruence between the trees. EF-1α aligned sequences contained 42 taxa and 360 characters of which 123 characters were parsimony informative. The data for the EF-1α gene region produced 100 MP trees of 390 steps (consistency index (CI) = 0.831, retention index (RI) = 0.957). A partition homogeneity test produced a P-value = 0.1, showing that the ITS rDNA and EF-1α sequence data are combinable (Cunningham 1997). The ITS rDNA and EF-1α data sets were, therefore, combined and the aligned dataset consists of 40 in-group sequences, of which fourteen sequences represent isolates from this study and the rest were obtained from GenBank (Table 1). Of the 882 characters in the combined dataset, 200 characters were parsimony informative. Combined data for the ITS rDNA and EF-1α gene regions produced 88 MP trees of 369 steps (consistency index (CI) = 0.805, retention index (RI) = 0.953), of which one is presented (Fig. 2). Twelve terminal clades were observed (Fig. 2) and they were consistent with those observed in the tree based on ITS rDNA sequence data.

In the ITS rDNA sequence dataset, Clade VII included an isolate previously isolated from *Wollemia nobilis* and three from *Syzygium cordatum*. This clade was very closely related to clade VIII (*N. australe*) and was, therefore, also characterized using sequence data from third gene region namely β-tubulin. The three datasets contained 525 (ITS rDNA), 274 (EF-1α) and 388 (β-tubulin) characters, of which 3, 7 and 3 characters in each datasets respectively, were indicated in the analysis. All the variable characters in all three gene regions were parsimony informative. One MP tree was obtained for ITS rDNA, (Tree length = 3, consistency index (CI) = 1.00, retention index (RI) = 1.00); three for EF-1α (Tree length = 8, CI = 0.875, RI = 0.971) and one for β-tubulin (Tree length = 3, CI = 1.00, RI = 1.00) (Fig. 3). Fixed, unique polymorphism were identified in all three gene

regions, which produced congruent trees from the individual loci that separated clade VII (*Neofusicoccum* sp. 2) and clade VIII (*N. australe*), supporting the view that they are distinct species (Fig. 3; Table 3).

Taxonomy

Two previously undescribed species of the Botryosphaeriaceae were identified in this study. They reside in *Neofusicoccum* and were distinguished from known *Neofusicoccum* species based on anamorph morphology, DNA sequence data and PCR-RFLP analysis. These species are described below. Furthermore, *Dichomera eucalypti* was shown in this and previous studies to belong to *Neofusicoccum*, and a new combination is therefore proposed in this genus.

Neofusicoccum ursorum. *Maleme*, *Pavlic*, *Slippers sp. nov. Mycobank MB 512478* (Fig. 7)

Etymology: Name refers to the Koala 'bears', that feed on *Eucalyptus* spp. that were sampled in this study

Teleomorph: Unknown

Conidiomata: pycnidia solitaria subimmersa papillata, pilis hyphalibus tecta, nigra, usque ad 645 µm diametro. *Conidia* hyalina laevia granulis tenuibus, non septata, fusiformia vel ellipsoidea, (20.8–)22–26(–28.4) × (5.6–)6.5–8(–8.2) µm. *Cellulae conidiogenae* hyalinae holoblasticae cylindricae vel subcylindricae (8.8–)10–14(–15.5) × (2.0–)2.5–3(–3.3) µm.

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 645 µm diam. *Conidia:* hyaline, smooth with contents having fine granular appearence, aseptate, fusiform to ellipsoid (20.8–)22–26(–28.4) × (5.6–)6.5–8(–8.2) µm (ave. of 50 conidia 24 × 7.1 µm, l/w 3.4). *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (8.8–)10–14(–15.5) × (2.0–)2.5–3(–3.3) µm (ave. of 50 conidiogenous cells 12.1 × 2.8 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium changing after 3-4 days to pale olivaceous grey from the middle of the colony (both sides), margins regular. Optimum temperature for growth 30 ^{o}C, colonies grown on MEA covering a 90 mm diam plate after 7 days of incubation in the dark.

Habitat: Endophytic in healthy leaves of *Eucalyptus* spp.

Known distribution: Pretoria, South Africa

Material examined: South Africa, Pretoria, Gauteng province, from branches and leaves of living *Eucalyptus* trees, May 2005, H.M. Maleme (Holotype PREM 59815) (Culture CMW 24480); Gauteng province, *Eucalyptus* trees, H.M. Maleme (Paratype PREM 59816) (Culture CMW 23790).

Neofusicoccum crypto-australe. *Maleme, Pavlic, Slippers sp. nov. Mycobank MB 512477* (Fig. 8)

Etymology: Referring to cryptic species closely related to *N. australe*

Teleomorph: Unknown

Conidiomata: pycnidia solitaria subimmersa papillata, pilis hyphalibus tecta, nigra usque ad 575 µm diametro. *Conidia* hyalina laevia contentis granularibus, non septata, fusiformia apicibus rotundatis (21.0–)18–20.5(–26.4) × (4.9–)5–6(–6.2) µm, cum maturitate brunnescentia et semel vel bis septata. Cellulae conidiogenae hyalinae holoblasticae cylindricae vel subcylindricae (11–)11.5–12.5(–12.9) × (2.0–)2.2–2.7(–2.9) µm.

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 575 µm diam. *Conidia:* hyaline, smooth with granular contents, aseptate, fusiform, apices rounded (18–)20.5–21.0(–26.4) × (4.9–)5–6(–6.2) µm (ave. of 50 conidia 19 × 5.5 µm, l/w 3.5), becoming brown and one or two-septate with maturation. *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (11–)11.5–12.5(–12.9) × (2.0–)2.2–2.7(–2.9) µm (ave. of 50 conidiogenous cells 11.9 × 2.4 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium, changing to straw yellow after 3 days of incubation. After 4-7 days the colour changed to pale olivaceous grey from the middle of the colony from the top and reverse, margins regular. Optimum temperature for growth at 25 ^{o}C, covering a 90 mm diam MEA plate after 3 days of incubation in the dark.

Habitat: Endophytic in healthy leaves of *Eucalyptus* spp.

Known distribution: Pretoria, South Africa

Material examined: South Africa, Pretoria, Gauteng province from branches and leaves of living *Eucalyptus* trees, May 2005, H.M. Maleme (Holotype PREM 59817) (Culture

CMW 23785); Gauteng province, *Eucalyptus* trees, H.M. Maleme (Paratype PREM 59818) (Culture CMW 20738); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60063) (Culture CMW 23787); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60064) (Culture CMW 23784); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60065) (Culture CMW 23786).

Neofusicoccum eucalypti. *(Winter) Maleme*, *Pavlic*, *Slippers comb. nov. Mycobank MB 512501* (Fig. 9)

Basionym: Camarosporium eucalypti G. Winter (1886).

≡*D. eucalypti* B. Sutton, *Mycol. Pap.* **138**: 182 (1975).

≡*Camarosporellum eucalypti* (G. Winter) Tassi, *Bull*, *Lab*, *ort. Bot.* Siena **5**: 62 (1902).

=*Coryneum viminale* Cooke and Massee, Grevillea **20**: 36 (1891).

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 625 µm diam. *Conidia:* hyaline, smooth with granular contents, aseptate, fusiform, apices rounded (16.8–)17.7–19.7(–21.6) × (4.1–)4.9–5.9(–6.3) µm (ave. of 50 conidia 18.7 × 5.4 µm, l/w 3.5). *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (9.8–)11–15(–15.5) × (3.0–)3.5–4(–3.3) µm (ave. of 50 conidiogenous cells 13.1 × 3.8 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium changing after 3-4 days to pale olivaceous grey from the middle of the colony (both sides), margins regular. Optimum temperature for growth 30 ^{o}C, colonies grown on MEA covering a 90 mm diam plate after 7 days of incubation in the dark.

Notes: *Dichomera eucalypti* was formally described by Sutton (1975), based on *Camarosporium eucalypti* (G. Winter) and including *Coryneum viminale* Cke and Massee as synomym. This description was based on similarity of the conidial morphology. These species are known to form irregular, globose to pyriform conidia that are brown and muriform with transverse, longitudinal and oblique septa. Barber *et al.* (2005) confirmed these observations by examining type material. Barber *et al.* (2005) also designated an

epitype specimen and ex-epitype isolate for this species. Sequence data of this ex-epitype isolate is linked here to isolates obtained from *Eucalyptus* in our study.

'*Dichomera*' *eucalypti* groups within the *Neofusicoccum* clade in this and other recently published studies (Barber *et al*. 2005; Burgess *et al*. 2005; Crous *et al*. 2006). *Neofusicoccum* is represented by species with *Fusicoccum*-like conidia sometimes having *Dichomera*-like synanamorphs (Crous *et al*. 2006). *Fusicoccum*-like conidia were observed in this study as opposed to muriform, globose conidia observed in the previous studies. Based on these morphological observations and the phylogenetic grouping of our isolates with isolates from Barber *et al*. (2005) (Fig. 1, clade IV), which were morphologically linked to the epitype, we transfer this taxon to *Neofusicoccum*.

Pathogenicity

All isolates inoculated in this study on the *Eucalyptus* clone ZG-14 produced lesions after six weeks that were significantly different to those of the controls (R-square = 0.58, Coefficient variable = 39.7, Root MES = 16.2), confirming their pathogenicity on this host (Fig. 10). The isolates could be recovered by re-isolations from the lesions. Some lesions were observed on trees used as controls, but no Botryosphaeriaceae were re-isolated from these lesions.

Isolates could be separated into two virulence classes (high and low) based on the mean lesion lengths produced. Virulence variation was observed amongst isolates within species, such that individual isolates of a species could group into either the higher or lower virulence category. The more virulent group of isolates is typified by one isolate of *N. eucalypti* (CMW 24571) and two isolates of *N. crypto-australe* (CMW 23785 and CMW 23786). The lesions produced by these isolates were significantly longer than those of all other isolates used for inoculations. The less virulent group represented by isolates from each species with the variation providing a continuous range (Fig. 10). On average, *B. dothidea* isolates were the least virulent and *N. crypto-australe* and *N. parvum* the most virulent (Fig. 10).

Discussion

Five species of the Botryosphaeriaceae were identified in this study, including *Botryosphaeria dothidea, Neofusicoccum parvum*, *N. eucalypti*, and the newly described species, *N. ursorum* and *N. crypto-australe.* These species were found in a small plot on 20 *Eucalyptus* spp. planted in a Pretoria arboretum and a few surrounding *Eucalyptus* trees. No host specificity was evident amongst these Botryosphaeriaceae and *Eucalyptus* spp. from which they were collected. Furthermore, three of these species of Botryosphaeriaceae are recorded on *Eucalyptus* in South Africa for the first time, which is interesting given the numerous previous studies considering these fungi on this host in the country.

Neofusicoccum parvum was the most commonly isolated species from *Eucalyptus* in this study. Subsequent to its first description from kiwifruit in New Zealand in 1985 (Pennycook and Samuels 1985), this species has been described from a wide variety of hosts in various parts of the world (Phillips *et al*. 2002; Gure *et al*. 2005; Pavlic *et al*. 2007). On *Eucalyptus,* it is commonly reported as the cause of canker and dieback, in countries including Uganda, Chile, Ethiopia, South Africa, Australia and Venezuela (Nakabonge 2002; Ahumada 2003; Gezahgne *et al*. 2004; Slippers *et al*. 2004*b*; Barber *et al*. 2005; Mohali *et al*. 2006). Its common occurrence on *Eucalyptus* in this study is thus not surprising. *Neofusicoccum parvum* is also known from native Myrtaceae in South Africa including *Heteropyxis natalensis* and *Syzygium cordatum* (Smith *et al*. 2001; Slippers *et al*. 2004*b*; Pavlic *et al*. 2007). Individual *N. parvum* isolates were pathogenic, but not the most virulent in this study. On average they were at least as virulent as any other species.

Neofusicoccum crypto-australe is described here as a cryptic sister species to *N. australe*. This species has previously been isolated from *Wollemia nobilis*, a native conifer in eastern Australia (Slippers *et al*. 2005) and on native *Syzygium cordatum* trees in South Africa (Pavlic *et al*. 2007). In those studies, it was not provided with a formal description due to the small number of isolates available. The occurrence of *N. crypto-australe* on two different native hosts in Australia and South Africa and now on non-native *Eucalyptus* in South Africa, makes it difficult to suggest a possible origin for the fungus. In this study, five isolates were obtained from *Eucalyptus* spp. in the arboretum and the surrounding

wild *Eucalyptus* trees. Its existence on *Eucalyptus* spp. could be explained by the movement of species of Botryosphaeriaceae from different continents through plant material, possibly from Australia where *Eucalyptus* is native. Alternatively, it could have jumped hosts from native *Syzygium cordatum* to introduced *Eucalyptus*, or *visa versa* in South Africa since both hosts were shown to share similar pathogens in the work done by Pavlic *et al*. (2007). Its wide host and geographic range, as well as the high level of virulence revealed in this study, makes it a potential threat to both native and non-native hosts in South Africa and Australia.

Neofusicoccum ursorum was represented by two isolates in this study. Both isolates were obtained from *Eucalyptus* trees surrounding the arboretum. *Neofusicoccum ursorum* has, to the best of our knowledge also never been isolated from any other area or host. It is thus possible that this species is native to the region and could be more common on other native trees surrounding the arboretum. A situation similar to this has previously been observed (Pavlic *et al*. 2007; Slippers and Wingfield 2007). *Neofusicoccum ursorum* was pathogenic, but only mildly virulent when compared to other species studied here.

Morphology alone is typically insufficient when identifying closely related fungal species and this approach tends to underestimate the true diversity of fungi on a host or in an area. This is also true for the identification of cryptic species of the Botryosphaeriaceae such as *N. crypto-australe* and *N. ursorum* discovered in this study. Molecular tools coupled with morphology have, however, been very successfully applied in recent years to overcome this problem in the Botryosphaeriaceae (Slippers and Wingfield 2007). *Neofusicoccum ursorum* is for example virtually indistinguishable from *N. parvum* and *N. eucalypti* in this study based on morphology, but clearly distinct from them based on phylogenetic analysis of ITS rDNA sequence data.

Some cryptic species may be very difficult to distinguish with confidence when considering only one gene region and, therefore, the application of multiple gene regions has been promoted for identification of the Botryosphaeriaceae (De Wet *et al*. 2003; Slippers *et al*. 2004*a*, *b*, *c*; Alves *et al*. 2008). In this study, *N. crypto-australe*, the sister species to *N. australe*, could have gone unidentified if only one gene region had been analysed. An analysis of concordance between three gene regions, ITS rDNA, EF-1α and β-tubulin, were needed to confidently separate these two species. Such concordance

between gene regions can only emerge in the absence of recombination between the groups.

'*Dichomera*' *eucalypti* (= *Camarasporellum eucalypti*) was described from *Eucalyptus* spp. in Australia (Sutton 1975) as producing globose, subglobose, obvoid, obpyriform, muriform or somewhat fusiform with septate conidia. This was confirmed by Barber *et al*. (2005) who designated an epitype specimen (and ex-type culture) for '*D. eucalypti*'. The isolates obtained in the present study were identical to these ex-type cultures in ITS rDNA and Ef-1α sequence data, but did not show the morphological characteristics described above. Instead, they produced *Fusicoccum*-like hyaline, aseptate, fusiform to ellipsoid conidia. This observation, together with the consistent grouping with other species of *Neofusicoccum*, validates its description here as *N. eucalypti*. Some other *Neofusicoccum* species (e.g. *N. parvum*, *N. australe*, see Barber *et al*. (2005) are also known to produce both *Fusicoccum*-like as well as *Dichomera*-like conidia, but is interesting that some isolates produce one of these very distinct conidial forms and not the other. The genetic basis of this unusual characteristic deserves further study.

Neofusicoccum eucalypti (= '*D. eucalypti*') is well known from woody tissues, foliage and bark samples of *Eucalyptus* spp. in Australia (Sutton 1975; Barber *et al*. 2005; Burgess *et al*. 2005). In this study, two isolates of this species were identified as endophytes from asymptomatic leaves in the *Eucalyptus* arboretum and one from the surrounding *Eucalyptus* spp. This is the first report of this fungus on *Eucalyptus* in South Africa. Its occurrence on non-native *Eucalyptus* in South Africa might have been anticipated due to its common association with *Eucalyptus* in Australia and the fact that the trees sampled in this study were generated from seed introduced from that country. Although the isolates of *N. eucalypti* varied significantly in virulence, it is noteworthy that one of the isolates was the second most virulent. The presence of *N. eucalypti* in South Africa, albeit at low levels currently, poses a potential threat to this host and its presence should be monitored.

Five isolates of *Botryosphaeria dothidea* were obtained in this study. This pathogen has been documented on many hosts worldwide, including *Eucalyptus* (Farr *et al*. 1989; Fisher *et al*. 1993; Smith *et al*. 1996). Recent studies have, however, indicated that this pathogen is not common on *Eucalyptus* and other related hosts (Slippers *et al*.

2004*b*; Pavlic *et al*. 2007). *Botryosphaeria dothidea* was found to be only mildly virulent in this study, which is consistent with recent studies that have considered its pathogenicity on species of Myrtaceae in South Africa, Venezuela and Colombia (Rodas 2003; Mohali 2005; Pavlic *et al*. 2007). Therefore, the previous assumption that *B*. *dothidea* is an important canker and dieback pathogen on *Eucalyptus*, appears to be incorrect.

Fifteen species of Botryosphaeriaceae have been identified in the past five years on *Eucalyptus*, applying both morphological characters and DNA sequence data (Slippers *et al.* 2007). Some are thought to be host specific and/or with a local distribution, while others have a broad host range and are more widely distributed. For example, *Lasiodiplodia theobromae* has been reported to occur on more that 500 host species (Punithalingam 1976). On *Eucalyptus* it has been documented in countries such as South Africa, Venezuela, Congo and Uganda (Roux *et al*. 2000, 2001; Burgess *et al*. 2003; Mohali *et al*. 2007). Species of the Botryosphaeriaceae, such as *B*. *mamane*, *N*. *stromaticum* and *N*. *andinum*, have, however, only been identified on *Eucalyptus* in Venezuela (Mohali *et*. *al*. 2006). The current study adds to this global picture of a combination of a few common generalists and some rare, apparently specific, species of the Botryosphaeriaceae that infect *Eucalyptus* at any given site. Future studies should also focus on native hosts surrounding plots, such as the one studied here, to better understand host and geographic distribution of certain species. Furthermore, it would be valuable to study the distribution of these Botryosphaeriaceae at a finer spatial scale on the trees in this plot, in order to better understand their biology and interaction.

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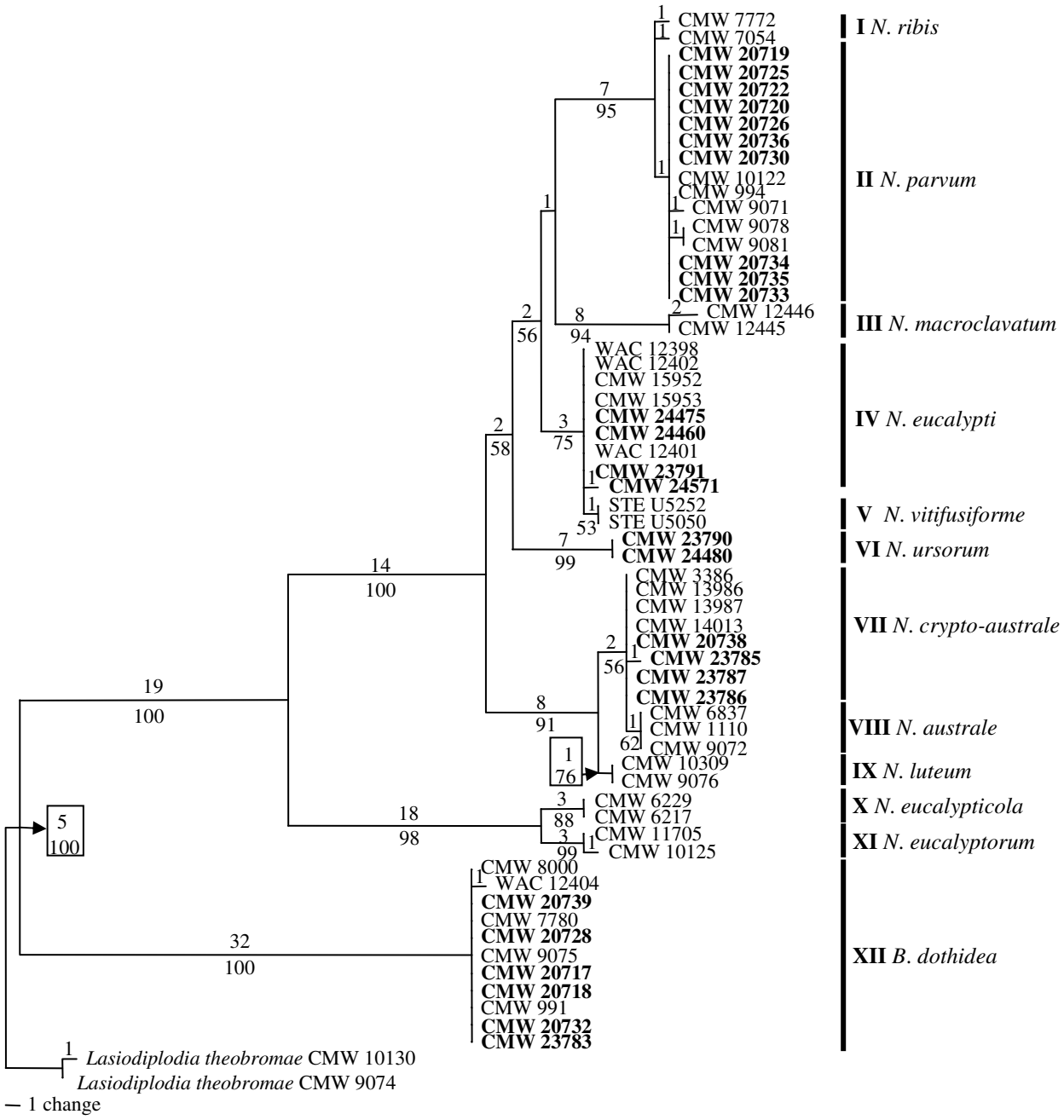
Fig. 1. One of the 112 most parsimonious trees obtained from sequence data of the ITS rDNA locus (tree length = 154 steps, CI = 0.747, RI = 0.952). Bootstrap values based on 1000 bootstrap replicates are shown below the branches and number of steps above the branches. Isolates sequenced in this study are in bold. The tree is rooted to *Lasiodiplodia theobromae*.


CMW 7772
CMW 7054
CMW 20719
CMW 20725
CMW 20722
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CMW 994
CMW 9071
CMW 9078
CMW 9081
CMW 20734
CMW 20735
CMW 20733
CMW 12446
CMW 12445
WAC 12398
WAC 12402
CMW 15952
CMW 15953
CMW 24475
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STE U5050
CMW 23790
CMW 24480
CMW 3386
CMW 13986
CMW 13987
CMW 14013
CMW 20738
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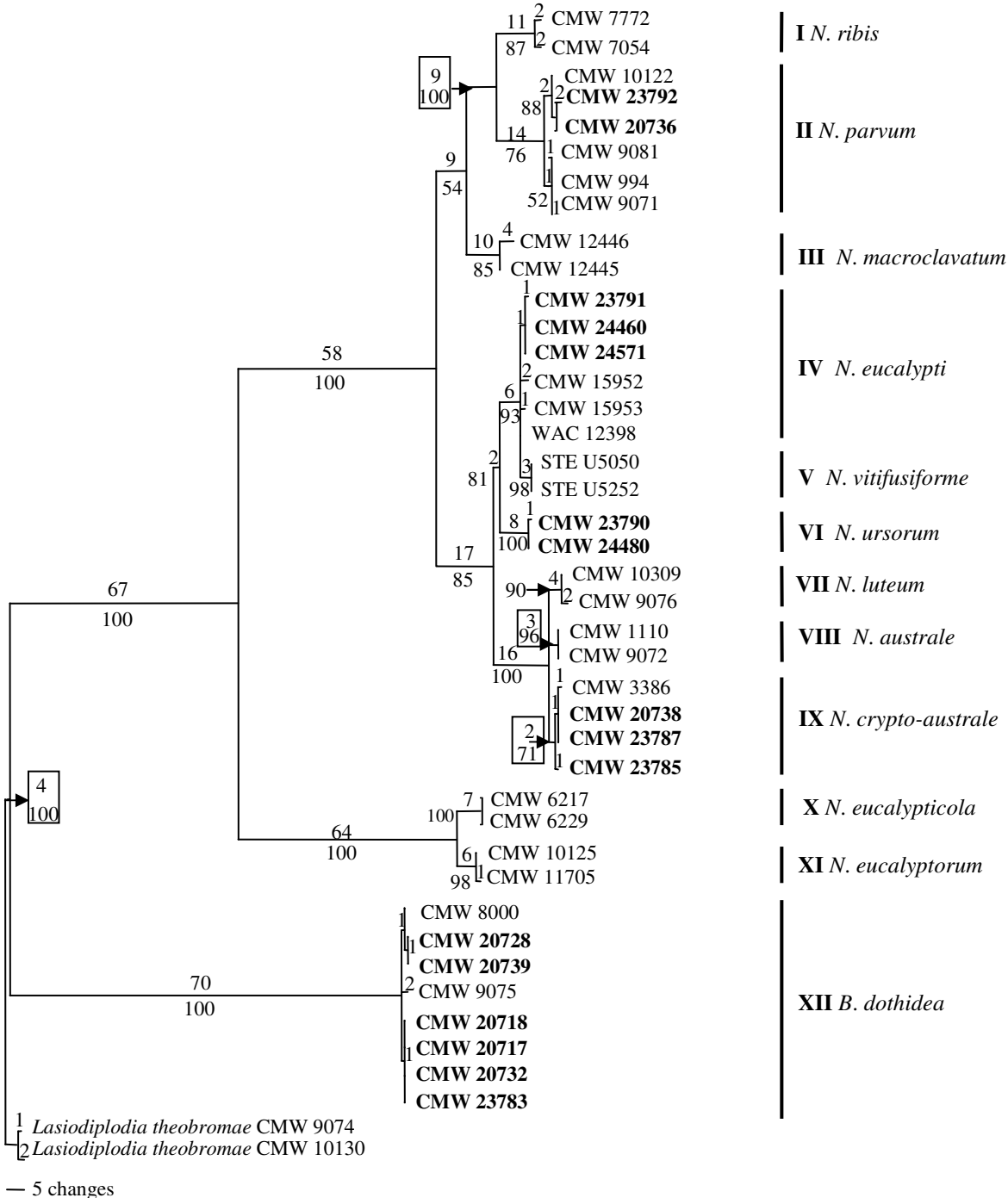
CMW 23787
CMW 23786
CMW 6837
CMW 1110
CMW 9072
CMW 10309
CMW 9076
CMW 6229
CMW 6217
CMW 11705
CMW 10125
CMW 8000
WAC 12404
CMW 7780
CMW 20739
CMW 20728
CMW 9075
CMW 20717
CMW 20718
CMW 991
CMW 20732
CMW 23783
Lasiodiplodia theobromae CMW 10130
Lasiodiplodia theobromae CMW 9074
1 change
I N. ribis
II N. parvum
III N. macroclavatum
IV N. eucalypti
V N. vitifusiforme
VI N. ursorum
VII N. crypto-australe
VIII N. australe
IX N. luteum
X N. eucalypticola
XI N. eucalyptorum
XII B. dothidea

Fig. 2. One of the 88 most parsimonious trees obtained from the combined sequence datasets of the ITS rDNA and EF-1α loci (tree length = 369 steps, CI = 0.805, RI = 0.953). Bootstrap values based on 1000 bootstrap replicates are shown below the branches and number of steps above the branches. Isolates sequenced in this study are in bold. The tree is rooted to *Lasiodiplodia theobromae*.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CMW 7054
CMW 10122
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CMW 24460
CMW 24571
CMW 15952
CMW 15953
WAC 12398
STE U5050
STE U5252
CMW 23790
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CMW 9076
CMW 1110
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CMW 3386
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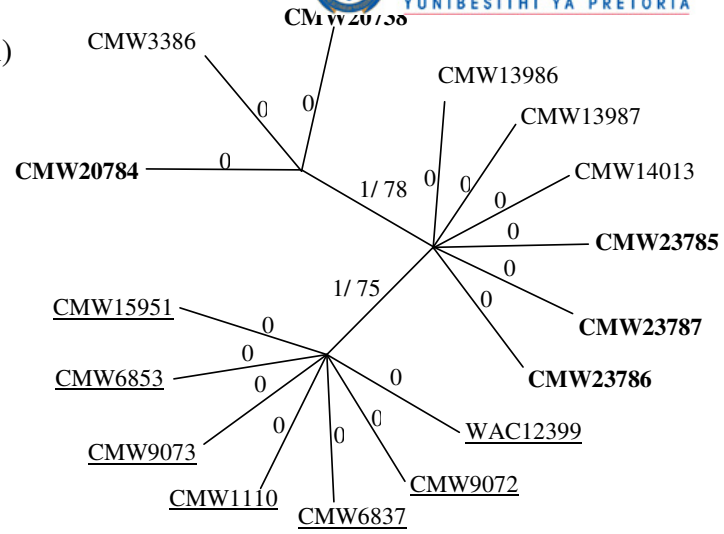
CMW 9075
CMW 20718
CMW 20717
CMW 20732
CMW 23783
Lasiodiplodia theobromae CMW 9074
Lasiodiplodia theobromae CMW 10130
— 5 changes
I N. ribis
II N. parvum
III N. macroclavatum
IV N. eucalypti
V N. vitifusiforme
VI N. ursorum
VII N. luteum
VIII N. australe
IX N. crypto-australe
X N. eucalypticola
XI N. eucalyptorum
XII B. dothidea

Fig. 3. Unrooted cladograms obtained from DNA sequence comparison of three gene regions, (*A*) ITS rDNA, (*B*) EF-1α, and (*C*) β-tubulin. The trees represent isolates belonging to *N. australe* (underlined) and *N. crypto-australe* which group congruently in analyses of the three loci. Isolates sequenced in this study are in bold. The first numbers represent branch lengths and the second numbers bootstrap values (1000 replicates).

(A)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CMW20784
CMW14013
1/ 78
CMW23785
1/ 75
CMW23787
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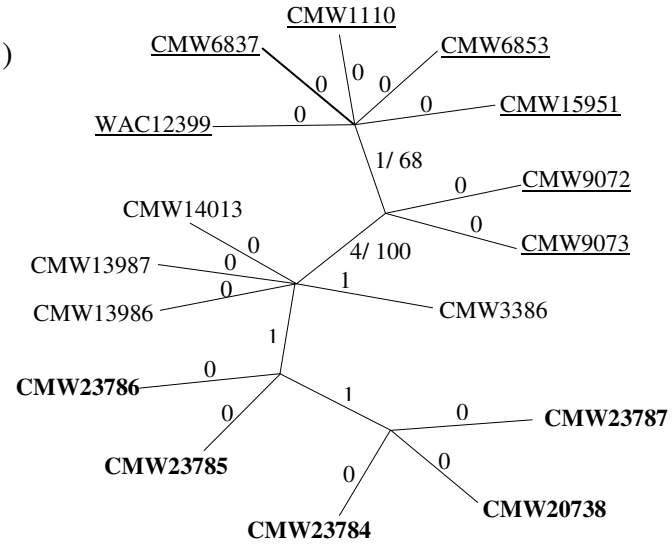
CMW23786
CMW6853
WAC12399
CMW9073
CMW9072
CMW1110
CMW6837

(B)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1/ 68
CMW9072
CMW14013
CMW9073
4/ 100
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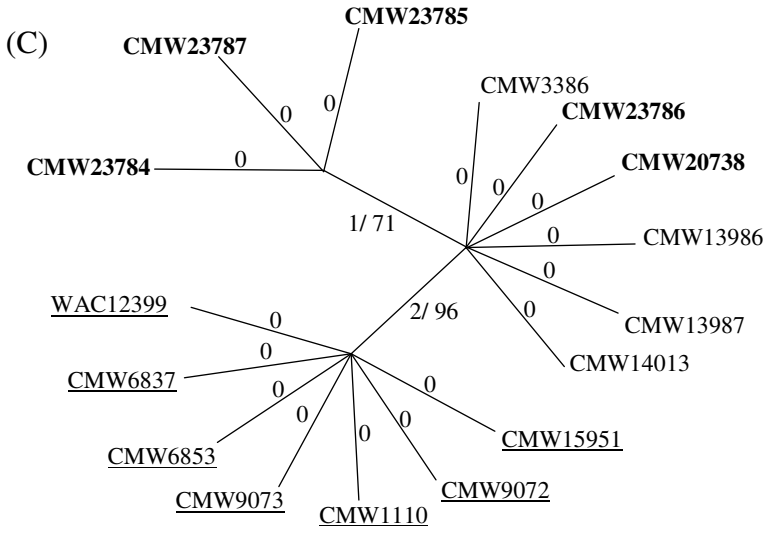
CMW13986
CMW3386
CMW23786
CMW23787
CMW23785
CMW20738
CMW23784

(C)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CMW3386
CMW23786
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1/ 71
CMW13986
CMW13987
WAC12399
2/ 96
CMW14013
CMW6837
CMW15951
CMW6853
CMW9072
CMW9073
CMW1110

Fig. 4. Restriction fragment length polymorphism maps of five species of Botryosphaeriaceae from *Eucalyptus* after digestion of the ITS rDNA PCR products with the (RE) *Hha*I. *N. parvum* / *N. ribis* (*A*), *N. eucalypti*; *N. ursorum*; *N. crypto-australe* (*B*) and *B. dothidea* (*C*). The total length of each fragment is given in brackets and the fragment lengths are given below each line.

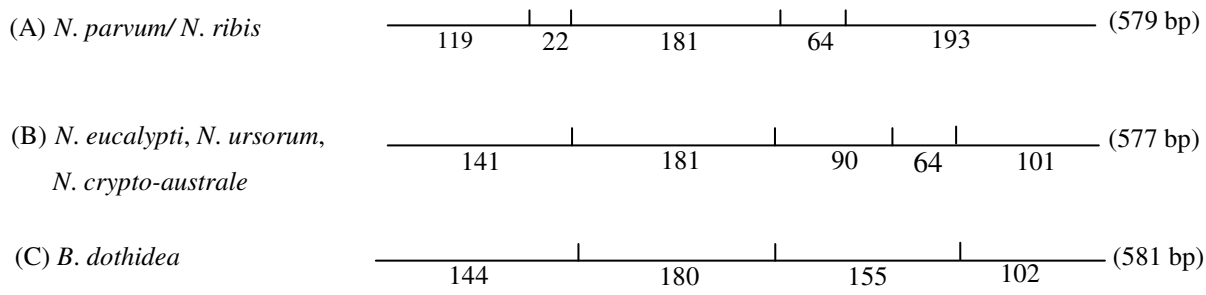
(A) N. parvum/ N. ribis
119
22
181
64
193
(579 bp)
(B) N. eucalypti, N. ursorum,
N. crypto-australe
141
181
90
64
101
(577 bp)
(C) B. dothidea
144
180
155
102
(581 bp)

Fig. 5. Restriction fragment length polymorphism maps of five species of Botryosphaeriaceae obtained from digestion of the ITS rDNA PCR products with (RE) *Ksp*I. *N. parvum*, *N. ursorum*, *N. eucalypti* (*A*), *B. dothidea* (*B*), *N. crypto-australe* (*C*). The total length of each fragment is given in brackets and the fragment lengths are given below each line.

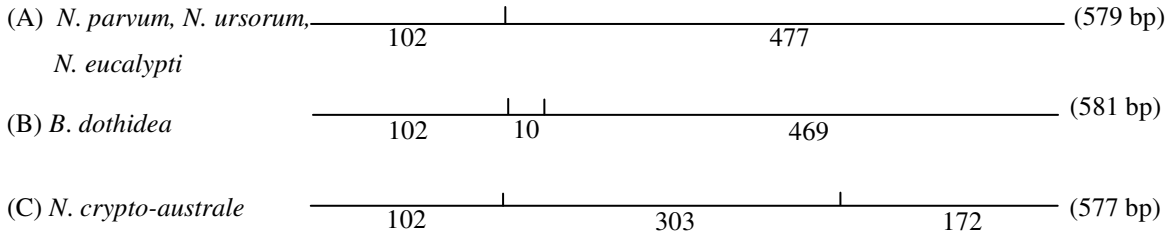
(A) N. parvum, N. ursorum, N. eucalypti
102
477
(579 bp)
(B) B. dothidea
102
10
469
(581 bp)
(C) N. crypto-australe
102
303
172
(577 bp)

Fig. 6. Restriction fragment length polymorphism maps of *N. ribis* (*A*) and *N. parvum* (*B*). PCR amplicons were amplified with primers *Bot 15* and *Bot 16* and digested with (RE) *Hha*I. The total length of each fragment is given in brackets and the fragment lengths are given below each line.

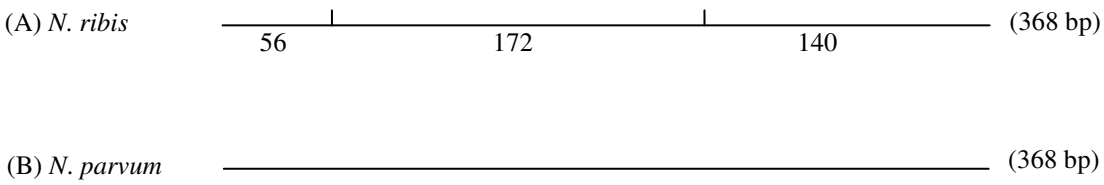
(A) N. ribis
56
172
140
(368 bp)
(B) N. parvum
(368 bp)

Fig. 7. Light micrographs of *Neofusicoccum ursorum*: (*A*) Pycnidia, (*B*,*C*) Conidiogenous cells, (*D*) Mature conidia, (*E*,*F*) Germinating conidia. Scale bar (*A*) = 500 µm, Scale bars (*B*-*F*) = 10 µm.

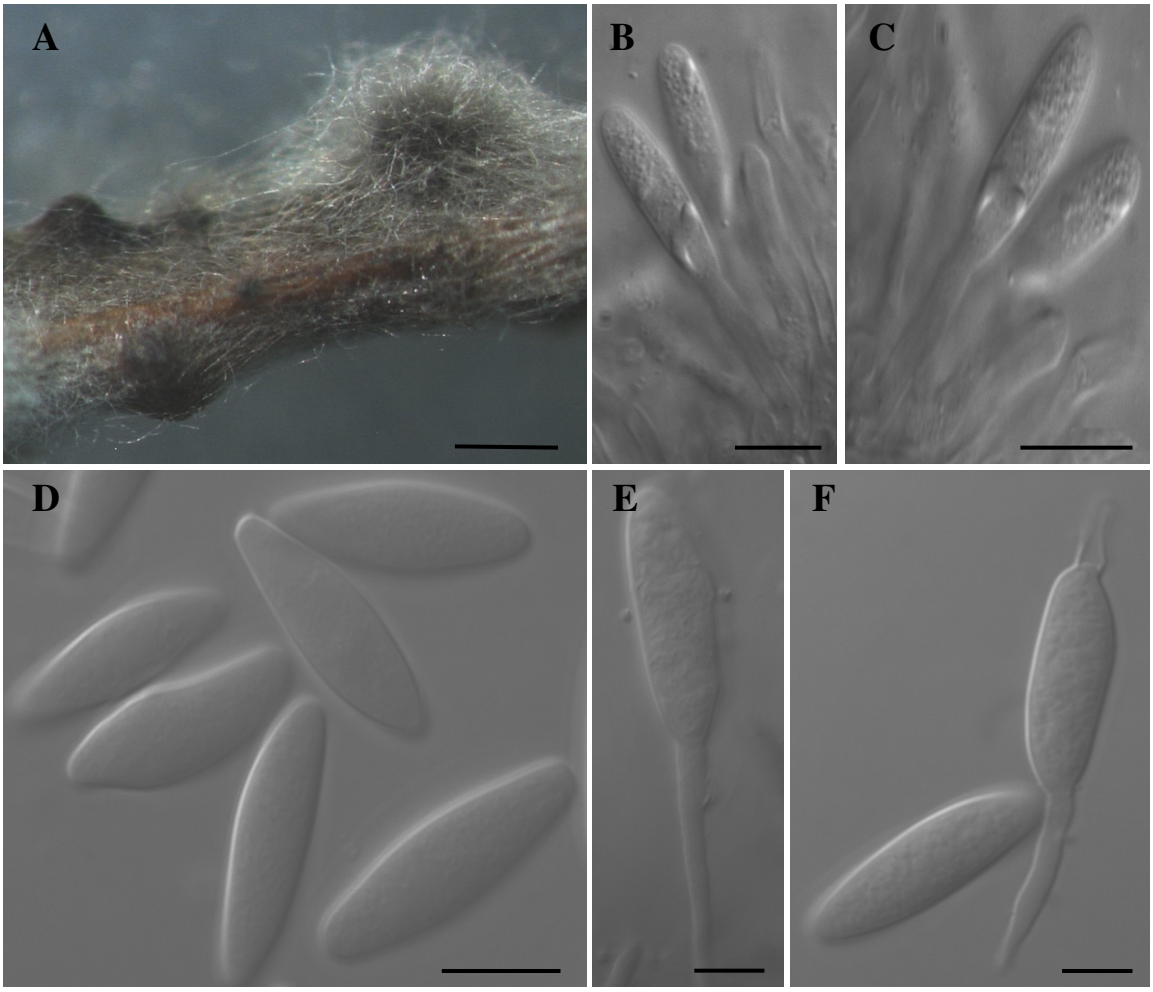
A
B
C
D
E
F

Fig. 8. Light micrographs of *Neofusicoccum crypto-australe*: (*A*) Pycnidium, (*B*) Conidiogenous cells, (*C*) 2-septate conidium, (*D*) One and two-septate germinating conidia, (*E*) aseptate conidia, (*F*) One and two-septate dark conidia. Scale bars (*A*) = 500 µm, Scale bar (*B-F*) = 10 µm.

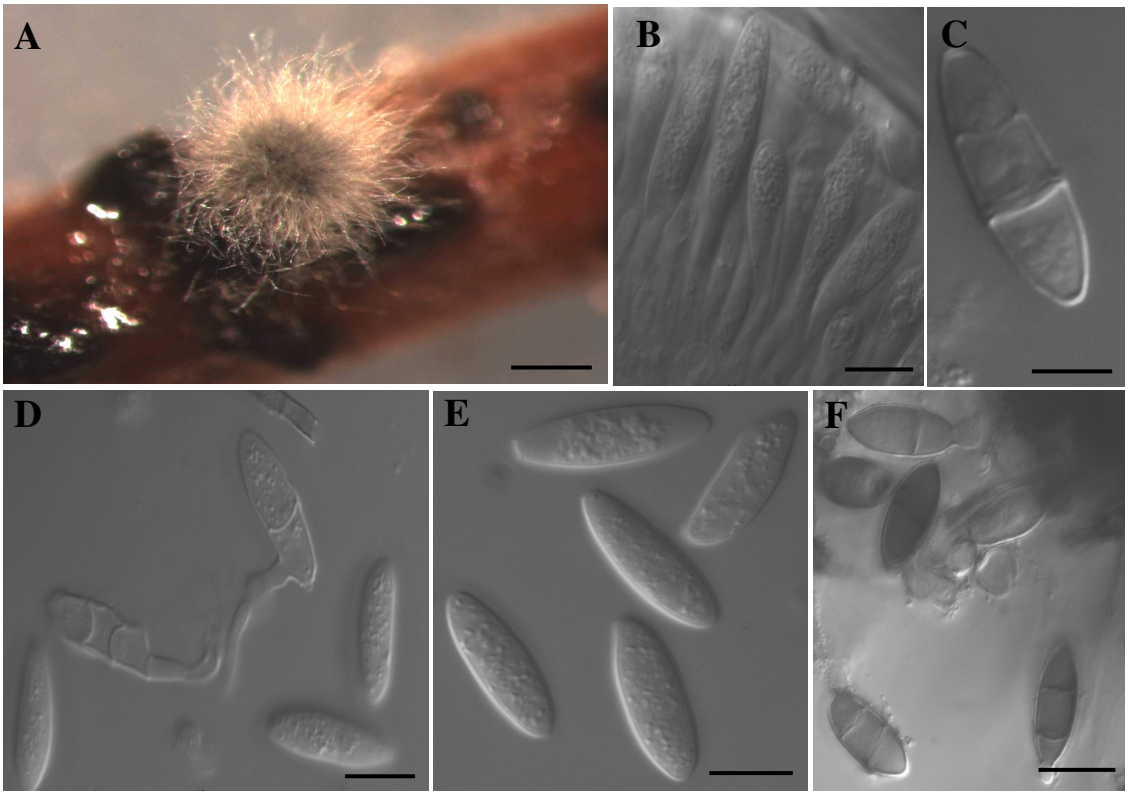
A
B
C
D
E
F

Fig. 9. Light micrograph of *N. eucalypti* conidia. Scale bar = 10 µm.

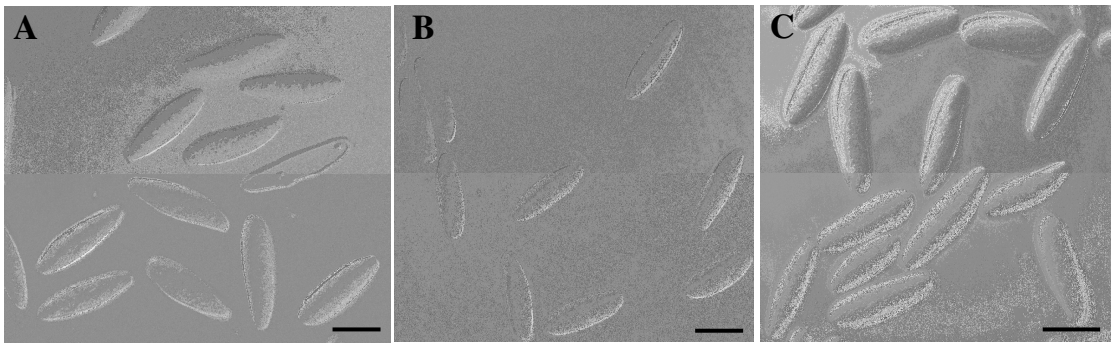
A
B
C

Fig. 10. Mean lesion lengths (mm) for each isolate of five species of Botryosphaeriaceae after inoculation on a *Eucalyptus grandis* clone (ZG-14), including *Botryosphaeria dothidea*, *Neofusicoccum eucalypti*, *Neofusicoccum crypto-australe*, *Neofusicoccum parvum*, *Neofusicoccum ursorum*. Control inoculations were done with MEA agar. Bars indicate the 95 % confidence limit for each isolate.

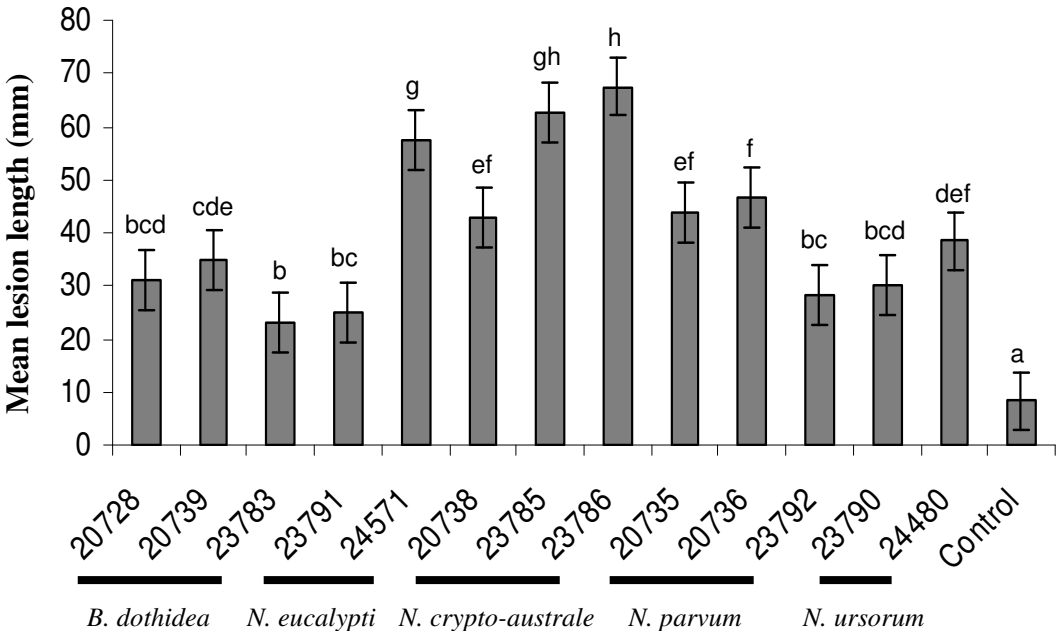
Mean lesion length (mm)
80
70
60
50
40
30
20
10
0
bcd
cde
b
bc
g
ef
gh
h
ef
f
bc
bcd
def
a
20728
20739
23783
23791
24571
20738
23785
23786
20735
20736
23792
23790
24480
Control
B. dothidea
N. eucalypti
N. crypto-australe
N. parvum
N. ursorum

Table 1. Isolates representing species of the Botryosphaeriaceae considered in a phylogenetic study and pathogenicity test

Culture no.[A,B]	Other no.[A]	Identity	Host	Location	Isolator	Genbank		
						ITS	EF	Bt
CMW7772		*Neofusicoccum ribis*	*Ribes* sp.	New York	B. Slippers & G. Hudler	AY236935	AY236877	AY236906
CMW7054	CBS 121.26	*N. ribis*	*R. rubrum*	New York	N. E. Stevens	AF241177	AY236879	AY236908
CMW10122		*Neofusicoccum parvum*	*Eucalyptus grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283681	AF283681	AF283681
CMW994	ATCC58189	*N. parvum*	*Malus Sylvestris*	New Zealand	G. J. Samuels	AF243395	AY236883	AY236912
CMW9071		*N. parvum*	*Ribes* sp.	Australia	M. J. Wingfield	AY236938	AY236880	AY236909
CMW9081	ICMP8003	*N. parvum*	*Populus nigra*	New Zealand	G. J. Samuels	AY236943	AY236888	AY236917
CMW9078	ICMP7925	*N. parvum*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236940	AY236885	AY236914
CMW23792[C]		*N. parvum*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			
CMW20736[C]		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme			
CMW20727		*N. parvum*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20719		*N. parvum*	*E. ovata*	Pretoria, S. Africa	H. M. Maleme			
CMW20724		*N. parvum*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20722		*N. parvum*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20720		*N. parvum*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20726		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme			
CMW20735[C]		*N. parvum*	*E. nicholii*	Pretoria, S. Africa	H. M. Maleme			
CMW20725		*N. parvum*	*E. scorparia*	Pretoria, S. Africa	H. M. Maleme			
CMW20730		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20733		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20734		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW3386		*Neofusicoccum crypto-australe*	*Wollemia nobilis*	Queensland, Australia	M. Ivory	AY615165	AY615157	AY615179
CMW13986		*N. crypto-australe*	*Syzygium cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615166		
CMW13987		*N. crypto-australe*	*S. cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615167		
CMW14013		*N. crypto-australe*	*S. cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615168		
CMW20738[C]		*N. crypto-australe*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW23787		*N. crypto-australe*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			

Table 1. Continued

CMW23784		*N. crypto-australe*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW23785[C]		*N. crypto-australe*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW23786[C]		*N. crypto-australe*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW9072		*N. australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY339260	AY339268	AY339252
CMW1110		*N. australe*	*Widdringtonia nodiflora*	Cape Province, S. Africa	W. J. Swart	AY615166	AY615158	AY615150
CMW9073		*N. australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY339261	AY615269	AY339253
CMW6837		*N. australe*	*Acacia* sp.	Australia	M. J. Wingfield	AY339262	AY339270	AY339254
CMW6853		*N. australe*	*Sequiadendron giganteum*	Australia	B. Slippers, T. Burgess/K.-	AY339263	AY339271	AY339255
CMW15951		*N. australe*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	DQ093201	DQ093225	DQ093212
WAC12399		*N. australe*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	AY744374	DQ093222	DQ093209
CMW10309	CAP 002	*Neofusicoccum luteum*	*Vitis vinifera*	Portugal	A. J. L. Phillips	AY339258	AY339266	AY339250
CMW9076	ICMP 7818	*N. luteum*	*Malus domestica*	New Zealand	S. R. Pennycook	AY236946	AY236893	AY615126
CMW6217	CBS 115766	*Neofusicoccum eucalypticola*	*E. rossi*	Australia	B. Slippers	AY615143	AY615135	AY615127
CMW6229		*N. eucalypticola*	*E. rossi*	Australia	B. Slippers	AY615142	AY615134	AY615126
CMW10125	CBS 115791	*Neofusicoccum eucalyptorum*	*E. grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283686	AY236891	AY236920
CMW11705		*N. eucalyptorum*	*E. nitens*	South Africa	B. Slippers	AY339248	AY339264	AY339256
CMW15948	WAC 12445	*Neofusicoccum macroclavatum*	*E. globulus*	Western Australia	T. Burgess	DQ093197	DQ093218	DQ093207
CMW15949	WAC 12446	*N. macroclavatum*	*E. globulus*	Western Australia	T. Burgess	DQ093198	DQ093219	DQ093208
CMW23790[C]		*Neofusicoccum ursorum*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW24480[C]		*N. ursorum*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW15952		*Neofusicoccum eucalypti*	*E. diversicolor*	Australia	T. Burgess	DQ093194	DQ093215	DQ093204
CMW15953		*N. eucalypti*	*E. diversicolor*	Australia	T. Burgess	DQ093195	DQ093216	DQ093205
CMW24571		*N. eucalypti*	*E. paniculata*	Pretoria, S. Africa	H. M. Maleme			
CMW23791[C]		*N. eucalypti*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
WAC12401		*N. eucalypti*	*E. pauciflora*	Victoria, Australia	P. J. Keane	AY744371		
WAC12402		*N. eucalypti*	*E. camaldulensis*	Victoria, Australia	G. Whyte	AY744372		
WAC12398		*N. eucalypti*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	AY744373	DQ093214	DQ093203
CMW24460		*N. eucalypti*	*E. pilularis*	Pretoria, S. Africa	H. M. Maleme			
CBS110887	STE-U 5252	*Neofusicoccum vitifusiforme*	*Vitis vinifera*	South Africa	J. M. van Niekerk	AY343383	AY343343	
CBS110880	STE-U 5050	*N. vitifusiforme*	*V. vinifera*	South Africa	J. M. van Niekerk	AY343382	AY343344	
CMW8000		*Botryosphaeria dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898	AY236927

Table 1. Continued

CMW9075	ICMP 8019	*B. dothidea*	*P. nigra*	New Zealand	G. J. Samuels	AY236950	AY236899	AY236928
CMW991		*B. dothidea*	*P. nigra*	New Zealand	B. Slippers	AY236947	AY236896	AY236925
CMW20739[C]		*B. dothidea*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20728		*B. dothidea*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20718		*B. dothidea*	*E. teresticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20717		*B. dothidea*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW20732		*B. dothidea*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW23783		*B. dothidea*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			
CMW10130		*L. theobromae*	*Vitex doniana*	Uganda	J. Roux	AY236951	AY236900	AY236929
CMW9074		*L. theobromae*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901	AY236930

[A]Culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ATCC = American Type Culture Collection, Fairfax, VA, USA; BRIP = Plant Pathology Herbarium, Department of Primary industries, Queensland, Australia, CAP = Culture collection of A. J. L. Phillips, Lisbon, Portugal; CBS = Centraalbureau voor isolates Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plant, Auckland, New Zealand.
[B]Isolates sequenced in this study are given in bold.
[C]Isolates used in the pathogenicity tests.

Table 2. Conidial measurements of five species of the Botryosphaeriaceae identified in this study

Identity	CMW no.[A]	Conidial measurement (µm)[B]		
		Length	Width	L/W
Neofusicoccum parvum	20719	(16.4) 18.7 (22.3)	(5.3) 6.8 (7.9)	2.8
	20725	(16.6) 18.5 (22.6)	(5.6) 6.1 (6.8)	3.1
	20724	(13.0) 16.0 (17.6)	(4.9) 5.3 (5.8)	3
	20722	(16.5) 18.7 (22.2)	(4.7) 4.8 (5.7)	3.9
	20720	(18.8) 18.5 (23.2)	(6.0) 5.1 (5.2)	3.7
	20726	(15.5) 17.7 (21.7)	(6.3) 5.8 (5.3)	3.3
	20736	(17.1) 18.6 (21.7)	(4.0) 4.8 (5.7)	3.9
	20730	(15.5) 18.7 (21.2)	(4.1) 5.8 (5.4)	3.3
	20734	(14.3) 16.5 (17.8)	(4.0) 5.4 (5.5)	3.3
	20735	(17.8) 18.8 (20.2)	(6.0) 5.4 (4.7)	3.5
	20733	(18.2) 18.9 (19.8)	(4.9) 5.4 (6.2)	3.5
	20723	(15.7) 16.9 (18.5)	(4.9) 5.1 (5.8)	3.3
	23792	(15.3) 17.1 (18.8)	(4.1) 5.4 (6.4)	3.2
	20727	(17.4) 18.6 (21.3)	(5.3) 6.4 (7.4)	3
Neofusicoccum eucalypti	23791	(20.1) 21.8 (23.6)	(5.1) 6.1 (7.4)	3.6
	24571	(14.9) 18.6 (18.7)	(4.8) 4.7 (5.8)	3.6
Neofusicoccum crypto-australe	20738	(17.8) 21.0 (22.2)	(5.2) 6.0 (6.0)	3.5
	23787	(16.2) 18.8 (20.7)	(4.8) 5.6 (6.4)	3.4
	23786	(19.2) 19.8 (22.3)	(4.9) 5.5 (6.5)	3.6
	23785	(18.2) 19.7 (21.6)	(4.9) 5.5 (6.2)	3.6
Neofusicoccum ursorum	23790	(20.8) 23.9 (28.4)	(5.6) 7.1 (8.2)	3.4
	24480	(21.0) 24.0 (26.4)	(5.7) 6.9 (8.4)	3.5
Botryosphaeria dothidea	20728	(21.6) 25.6 (28.4)	(5.7) 6.0 (6.7)	4.3
	20739	(22.6) 25.8 (26.6)	(3.8) 5.7 (5.8)	4.5
	20718	(22.8) 26.1 (30.1)	(4.6) 5.3 (5.9)	4.9
	20717	(20.4) 25.8 (30.6)	(4.4) 5.7 (7.4)	4.5
	23783	(22.2) 23.8 (29.6)	(5.1) 5.7 (6.3)	4.2

[A]CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[B]Measurements in brackets are actual ranges. Values outside brackets are averages of 30 conidia.

Table 3. Fixed polymorphic nucleotides from the sequence data of ITS rDNA, EF-1α and β-tubulin between isolates of *N. australe* and *N. crypto-australe*

Culture no.[A, B]	ITS	EF- 1α			β- tubulin		Identity
	463	55	240	248	44	58	
CMW9072	T	T	T	C	C	T	
CMW6837	-	-	-	-	-	-	
CMW1110	-	-	-	-	-	-	
CMW9073	-	-	-	-	-	-	*Neofusicoccum australe*
CMW6853	-	-	-	-	-	-	
CMW15951	-	-	-	-	-	-	
WAC12399	-	-	-	-	-	-	
CMW13986	C	C	C	T	T	C	
CMW14013	C	C	C	T	T	C	
CMW13987	C	C	C	T	T	C	
CMW 3386	C	C	C	T	T	C	
CMW 23787	**C**	**C**	**C**	**T**	**T**	**C**	*N. crypto-australe*
CMW 23786	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 23785	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 20738	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 23784	**C**	**C**	**C**	**T**	**T**	**C**	

[A] Culture Collections: CMW= Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; [B]WAC= Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia; KJ = Jacobs and Rehner (1998).
Isolates sequenced in this study are in bold.

Table 4. Distribution of five Botryosphaeriaceae isolated from 20 different *Eucalyptus* spp. in the Pretoria arboretum and from surrounding *Eucalyptus* trees

Species of Botryosphaeriaceae	*Eucalyptus* spp. surrounding the arboretum	*E. camaldulensis*	*E. nicholii*	*E. microcorys*	*E. punctata*	*E. terreticorris*	*E. dorrrigoensis*	*E. propinqua*	*E. citriodora*	*E. saligna*	*E.obligua*	*E. coniocalyx*	*E. ovata*	*E. sideroxlon*	*E. pilularis*	*E. maculata*	*E. botryoides*	*E. paniculata*	*E. scorparia*	*E. uiminalis*	*E. robusta*	Total
N. parvum	13	-	5	3	-	9	6	2	2	5	-	-	4	3	-	1	4	5	2	-	2	66
N. eucalypti	1	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	1	-	-	1	5
N. ursorum	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
N. crypto-australe	1	-	-	-	1	1	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	5
B. dothidea	4	2	-	2	-	2	2	-	2	-	-	-	-	-	-	2	-	-	-	-	-	16

Chapter 3

Development of species-specific primers for five species of the Botryosphaeriaceae

Abstract

Certain species of the Botryosphaeriaceae have been reported to cause destructive canker and dieback diseases of trees such as *Eucalyptus* in South Africa and other parts of the world. These fungi can also live latently inside healthy *Eucalyptus* tissue. Species of Botryosphaeriaceae are morphologically difficult to distinguish making it necessary to develop DNA based identification techniques. Such techniques are also potentially useful in determining the presence of these fungi directly from plant material. The aim of this study was to design species-specific primers for rapid and reliable *in-vivo* identification of *Neofusicoccum parvum*, *N. eucalypti* prov. nom., *N. ursorum* prov. nom., *N. crypto-australe* prov. nom. and *B. dothidea*, which were reported from *Eucalyptus* in Pretoria in a recent study. Five species-specific primers were designed by exploiting differences in the translation elongation factor 1α (EF-1α) gene region. The general primer (EF-Br) was used in all cases as the complimentary primer in the amplification. Primer specificity was tested on DNA templates extracted from isolates of all the five species, in known samples and a blind test. The sensitivity was determined by serial dilutions with the purified DNA in sterilized distilled water and plant DNA solution from *Eucalyptus* leaves. All primers detected as little as 10 pg of fungal DNA when serially diluted with water and with plant DNA. In a preliminary *in vivo* experiment *N. parvum* was identified directly from the plant material. The sensitivity of these primers will allow reliable and rapid detection of the five species *in vivo*. The strategy used to develop these primers could be extended to detect other endophytes and latent pathogens.

Introduction

Species of the Botryosphaeriaceae are among the most destructive pathogens of forest trees worldwide, including *Eucalyptus*. Most *Eucalyptus* spp. originate from Australia (Poynton 1979) and many species are grown commercially in many countries around the world. Introduction of *Eucalyptus* spp. in the form of seed and cuttings around the world is thought to have resulted in the concomitant introduction of their fungal pathogens (Burgess *et al*. 2002*a*; 2002*b*; Slippers *et al*. 2004). Among these pathogens are species of Botryosphaeriaceae. These fungi are endophytes and / or latent pathogens on woody hosts, and they often remain undetected until the host suffers from some sort of environmental stress (Schoeneweiss 1981; Old *et al*. 1990; Smith *et al*. 1996; Slippers and Wingfield 2007).

The endophytic occurrence of the Botryosphaeriaceae on leaves have been reported on different hosts around the world (e.g. Johnston 1998; Swart *et al*. 2000; Denman *et al*. 2003; Pavlic *et al*. 2007). In South Africa, Botryosphaeriaceae were first reported on *Eucalyptus* foliage in the 1980's (Crous *et al*. 1989). Smith (1995) demonstrated penetration of *Eucalyptus* leafs through the stomatal openings by germinating conidia of *Botryosphaeria dothidea*, and also found Botryosphaeriaceae on leaves of *E. smithii*, *E. camaldulensis*, *E. grandis* and *E. nitens*. *Botryosphaeria dothidea* was also thought to be the cause of canker and dieback of *Eucalyptus* in South Africa (Smith *et al*. 1996). In the recent study it was shown that *N. parvum* was the most common pathogen causing canker and dieback of *Eucalyptus* in South Africa, while *B. dothidea* was rarely isolated (Slippers *et al*. 2004).

Identification of species of Botryosphaeriaceae is often based on anamorph morphology, because the sexual structures (teleomorph) are rarely encountered in nature and are not easily induced in culture. During the last decade DNA based tools have provided more robust methods to identify the members of the Botryosphaeriaceae (e.g. Denman *et al*. 2000; Crous *et al*. 2006; Slippers and Wingfield 2007). These studies have resulted in a much clearer view of the diversity of Botryosphaeriaceae that infect the particular hosts in certain areas. For example, a recent study revealed the presence of five species (*Neofusicoccum* and *Fusicoccum*) in a small *Eucalyptus* arboretum in Pretoria (Chapter 2).

The standard method of detecting species of Botryosphaeriaceae from asymptomatic tissue has been by primary isolation from leaves and twigs on agar media. This method can be time consuming and slow growing species may be undetected or under represented (Zhang *et al*. 2006). The aim of this study is to design species-specific primers for rapid detection of five species of Botryosphaeriaceae isolated from *Eucalyptus* in Pretoria, South Africa (see Chapter 2). For this purpose, variations among the translation elongation factor (EF-1α) gene region sequences of the five species were targeted. The second aim was to test the ability of these primers to detect these species *in-vivo* in *Eucalyptus* leaves.

Materials and Methods

Isolates and DNA extraction

Twenty-two isolates representing six species of the Botryosphaeriaceae were used in this study (Table 1). These isolates were obtained from a recent study of Botryosphaeriaceae on *Eucalyptus* spp. in Pretoria, South Africa (Chapter 2) and from culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

. DNA template was obtained from fungal cultures by using the modified version of phenol:chloroform DNA extraction method described in Pavlic *et al*. (2007). The concentration of the extracted DNA was determined by using the nanodrop (ND-1000 v3.3.0; Inqaba biotech, South Africa) and accompanied software.

DNA was also extracted from *Eucalyptus* leaves. Visually healthy leaves were randomly sampled from *Eucalyptus* trees. All leaves were surface sterilized by placing them sequentially for 1 min in 96 % ethanol, undiluted bleach (3.5–5 % available chlorine) and 70 % ethanol, and rinsed in sterile distilled water. The leaves were freeze dried with liquid nitrogen and crushed with a mortar and pestle. The DNA was extracted by using a CTAB extraction method (Doyle and Doyle 1987).

PCR with general primers

The translation elongation factor 1α (EF-1α) gene region of all isolates was amplified with the general primers EF-AF (5' CAT CGA GAA GTT CGA GAA GG 3')

and EF-BR (5' CRA TGG TGA TAC CRC GCT C 3') (Sakalidis 2004). A PCR reaction mixture contained 1 µL of template DNA (60-100 ng/µL), 10mM of each primer, 0.2 mM of dNTPs, PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda California), and the final reaction mixture volume was 25 µL. PCR conditions were as follows: denaturation at 95 ^{o}C for 2 min, followed by 35 cycles of denaturation (95 ^{o}C for 20s), annealing (55 ^{o}C for 45s) and elongation (72 ^{o}C for 1½ min), followed by a final elongation step at 72 ^{o}C for 7 min. PCR amplicons were viewed on a 1.5 % agarose gel stained with ethidium bromide under UV-light.

DNA sequencing and primer design

After purifying PCR products using the High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) amplicons were sequenced in both directions using the same primers that were used in PCR reactions. The ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Warrington, U.K.) was used for sequencing reactions as specified by the manufacturer and reactions were run on an ABI PRISM 3100^{TM} automated DNA sequencer (Perkin-Elmer, Warrington, U.K.). The nucleotide sequences were aligned with MAFFT software version 5.667 (http://timpani.genome.ad.jp/~mafft/ server/) (Katoh *et al*. 2002).

The variations between the EF-1α gene region sequence data of five species of Botryosphaeriaceae were used for designing forward primers. The species-specific primers were designed by using the software Primer 3 (v. 0.4.0) (http://frodo.wi.mit.edu/) (Rozen and Skaletsky 2000). Online software Netprimer (www.PremierBiosoft.com) was used to further check for potential primer dimer and hairpin formation, and presence of repeated sequences on all selected putative primer sequences. All putative primer sequences were compared using BLAST (http://www.ncbi.nlm.nih.gov) against known species of Botryosphaeriaceae from GenBank in order to confirm their compatibility to relevant species. The primer EF-BR was used as complement to all the designed species-specific primers.

Specificity and sensitivity tests

All five primers were optimized by adjusting temperature to the relevant annealing temperatures as well as the amount of $MgCl_2$ added for each species and tested for specificity on isolates representing five species of Botryosphaeriaceae of known identity (Table 1). DNA extracted from representatives of five species of the Botryosphaeriaceae was diluted to a starting concentration of ~100 ng/µL with sterile distilled water. DNA concentration was verified by using nanodrop (ND-1000 v3.3.0) and accompanied software. The DNA was further diluted to 50, 25, 12.5, 4, 0.01, 0.001, 0.0001 ng/µL and was used in the PCR amplification with the species-specific primers designed in this study to determine the detection limits of the technique using these primers. PCR conditions were as described above, except that annealing temperatures were adjusted (Table 2). Both sterilized distilled water and DNA extracted from the youngest *Eucalyptus* leaves (expected to be endophyte free) were used as dilutents. DNA extracted from the youngest *Eucalyptus* leaves was checked for the presence of species of Botryosphaeriaceae by amplifying with EF-1α primers EF-AF and EF-BR and the designed species-specific primers (Table 2).

DNA from fifteen isolates representing six species of Botryosphaeriaceae of known identity were used for performing a blind test. Prior to this analysis all isolates were renamed and a DNA sample (1 µL) with a concentration between 60-100 ng/µL was used for each in PCR reactions. PCR amplification of DNA template with all five (BdeF, NueF, NceF, NeeF, NpeF) species-specific primer pairs was performed separately. DNA template of *N. ribis*, a close relative of *N. parvum*, was also included in the blind test to check the specificity of the *N. parvum* primer (NpeF). The PCR conditions for this test were the same as those described for PCR with the general EF-1α primers.

Detection of species of Botryosphaeriaceae from naturally infected Eucalyptus *leaves*

Sections of about 2 x 2 cm in size were cut from visually healthy leaves that were randomly sampled from *Eucalyptus* trees in a Pretoria arboretum. All leaves were cut into 8 segments and labelled following surface sterilization by sequential emersion for 1 min in 96 % ethanol, undiluted bleach (3.5–5 % available chlorine) and 70 % ethanol, and rinsed in sterile distilled water. Samples were then freeze dried with liquid nitrogen and crushed

with a mortar and pestle. DNA extraction was done from each segment using the CTAB extraction method for *Eucalyptus* leaves (Doyle and Doyle 1987). DNA amplification from each section was performed by using all the species-specific primers pairs separately; using PCR conditions described above and appropriate annealing temperatures (Table 2). The DNA from known species of Botryosphaeriaceae was used as positive controls.

Results

PCR with general primers

Amplification of DNA templates with the EF-1α general primers showed the expected amplicons of about 330 bp in size for all isolates used (Fig. 1). Comparison of the EF-1α DNA sequences of five species of Botryosphaeriaceae revealed conserved sequences when compared to the ITS sequence data.

DNA sequencing and primer design

Analysis of the DNA sequences of the EF-1α gene region revealed conserved regions within species which differed between the five species of Botryosphaeriaceae analysed. Forward primers were designed based on the differences between the species (Table 2 and 3). The BLAST results from the analysis of the putative primer designed for *Botryosphaeria dothidea* (BdeF) and *Neofusicoccum parvum* (NpeF), showed that the sequence corresponded to these species. However, the *N. eucalypti* prov. nom. primer (NeeF) is identical to a corresponding locus in *N. vitifusiforme*. The latter species does not occur on *Eucalyptus* and this primer should still be useful for detecting *N. eucalypti*. *N. ursorum* (NueF) and *N. crypto-australe* (NceF) primer sequences did not result in any identical matches within any of the known Botryosphaeriaceae from the GenBank. This is expected as these two species have been recently characterized (Chapter 2) and their sequences have been submitted. However, *N. crypto-australe* (NceF) primer sequence was expected to amplify its close relatives *N. australe* and *N. luteum*.

Specificity and sensitivity test

The species-specific primers only amplified DNA fragments from the species for which they were designed for (Fig. 2). An amplicon of about 330 bp was observed for

each species under different annealing temperatures (Table 2). The sensitivity test performed with serial dilutions of the genomic DNA of all species of Botryosphaeriaceae revealed that 10 pg of the fungal genomic DNA could be detected when diluted with both sterile distilled water and a plant DNA solution (Fig. 3). No species of Botryosphaeriaceae were detected from the DNA isolated from surface sterilized *Eucalyptus* leaves that were used as a dilutent in performing serial dilutions. The identity of all isolates that were randomly selected and used for the blind test were correctly determined using the species-specific primers (Fig. 4).

Detection of species of Botryosphaeriaceae from naturally infected Eucalyptus *leaves*

Neofusicoccum parvum was the only species that was detected from six of the eight leaf segments tested using the species-specific primers NpeF and EF-BR (Fig. 5). The PCR amplicons of the positive control was similar in size (330 bp) to that of the tested samples. No other species were detected when using the rest of the designed primers (Table 2).

Discussion

In this study, five species-specific primers were developed for the identification of *Neofusicoccum parvum*, *N. ursorum*, *N. eucalypti*, *N. crypto-australe* and *Botryosphaeria dothidea* based on the interspecies sequence variations of the translation elongation factor 1α gene fragment. Three species tested in this study have been previously reported as endophytes and latent pathogens on *Eucalyptus* in different parts of the world (Smith *et al*. 1996; Slippers *et al*. 2004; Burgess *et al*. 2006). *Neofusicoccum ursorum* and *N. crypto-australe* have only been previously reported from *Eucalyptus* in Pretoria (Chapter 2). The development of these primers provides a promising method for the detection of these species from healthy leaves.

The EF-1α gene region was targeted in this study for designing species-specific primers. Previously, ITS rDNA sequences have been used to design species-specific primers for identification of the Botryosphaeriaceae as well as other fungal species. For example, species-specific primers were designed from ITS rDNA sequence data for identification of *Fusicoccum* spp. from pistachio in California (Ma and Michailides 2002),

Diplodia pinea from *Pinus* in Lexington, Wisconson and South Africa (Flowers *et al*. 2003) and soil microfungi in Oregon USA (Martin and Rygiewicz 2005). The ITS rDNA sequence is, however, not always ideal for this purpose as differences amongst species of the Botryosphaeriaceae are often small. Furthermore, the most variable region of the ITS rDNA is also highly repetitive in many Botryosphaeriaceae, making primer design difficult. Therefore, exploiting variations in gene regions other than ITS might in some cases be necessary for designing primers for the identification of multiple species belonging to this group. EF-1α is useful for this purpose as it has been characterized in a fairly large number of species of Botryosphaeriaceae. It is also less repetitive and contains many indels that enhance specificity of primers as seen in (Table 3).

In this study, the primers designed could detect as little as 10 pg/µL of fungal DNA when used in a dilution series with both sterile distilled water and plant DNA as dilitents. These results are consistent with findings of Smith and Stanosz (2006) where the same detection limit was found following amplification with species-specific primers for detecting the latent Botryosphaeriaceous pathogen, *Diplodia pinea* in *Pinus*. Amplification using all the primers, even in the presence of high concentration of plant DNA, shows that these primers have the potential to be used for *in vivo* detection for the Botryosphaeriaceae.

In the preliminary tests on DNA isolated from sections of naturally infected *Eucalyptus* leaves from Pretoria arboretum, only *N. parvum* was detected using the species-specific primer NpeF and general primer EF-BR. This finding was expected since *N. parvum* was found to be the most abundant species on *Eucalyptus* in the arboretum (Chapter 2). The small sample size and rare occurrence of other *Neofusicoccum* species in the *Eucalyptus* arboretum could explain the failure to detect them from the leaves sampled in this study.

Neofusicoccum ribis was also included amongst the isolates selected for the blind test. All species were correctly identified using the species-specific primers with the exception of *N. ribis* where an amplicon was produced in the reaction containing the NpeF primer designed for identification of *N. parvum*. This was not unexpected as these two species are closely related to each other (Slippers 2003; Slippers *et al*. 2004). *Neofusicoccum australe* and *N. luteum* also share the DNA sequence which was used for

designing the species-specific primer for *N. crypto-australe* (NceF), and DNA from the former species would be expected to result in an amplification product using this primer. However, *N. australe* and *N. luteum* are not known from *Eucalyptus* in South Africa. The primer (NeeF) specific for *N. eucalypti* would also possibly amplify a locus in *N. vitifusiforme* as it has significant similarity to the sequence of this gene region in that species. The latter species, however, has not been identified from *Eucalyptus*. In cases such as this, the use of molecular tools like PCR-RFLP fingerprinting, as well as phylogenetic analysis can be used to further verify the identity of pathogens where necessary (Slippers *et al*. 2004; Alves *et al*. 2005; Pavlic *et al*. 2007).

With the species-specific primers developed in this study identification of endophytic Botryosphaeriaceae directly from the plant material can be achieved relatively easily. The species-specific primers apparently allows for the detection of small amounts of fungal cells. This technique can thus provide an easy way for future studies in trying to understand the relative abundance and distribution of species of the Botryosphaeriaceae that colonize *Eucalyptus* leaves.

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Fig. 1. An agarose gel (1.5 %) showing amplification of a fragment of the elongation factor 1-α gene region in species of Botryosphaeriaceae, using primers EF-AF and EF-BR. PCR products of each species are indicated in the gel. Lane 15 is negative control (PCR without DNA template); M = 100 bp marker.

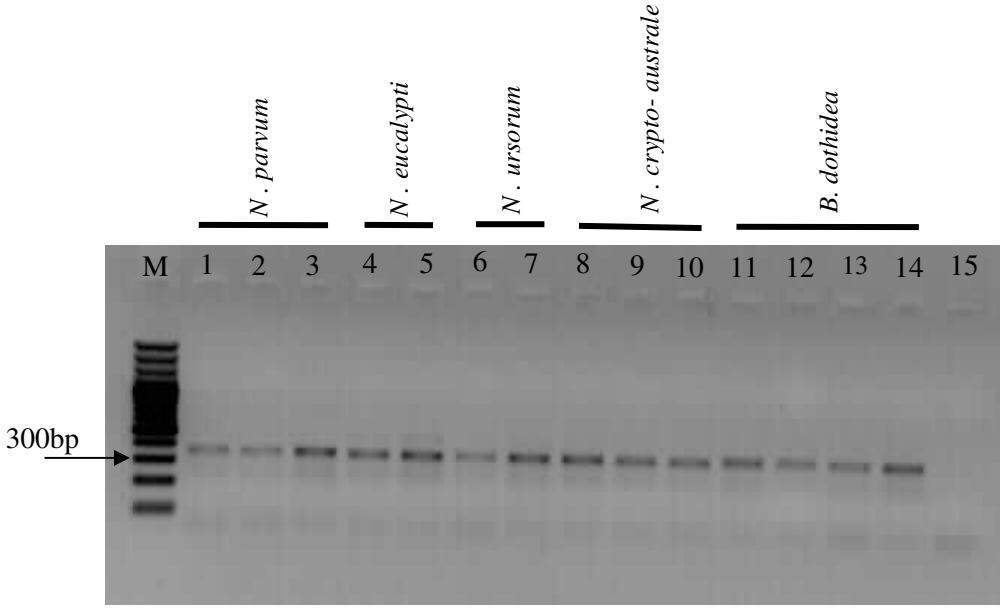
N . parvum
N . eucalypti
N . ursorum
N . crypto- australe
B. dothidea
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
300bp

Fig. 2. An agarose gel (1.5 %) showing the specificity of the different primers. For each set of lanes, two lanes contain PCR product from DNA of the intended species, while the next four contain PCR product where DNA from the other four species were included. Thus, the gels contain *N. parvum* (*A*, lane 1 and 2), *B. dothidea* (*A*, lane 7 and 8), *N. crypto-australe* (*B*, lane 13 and 14), *N. ursorum* (*B*, lane 19 and 20), *N. eucalypti* (*C*, lane 25 and 26). M = 100 bp marker.

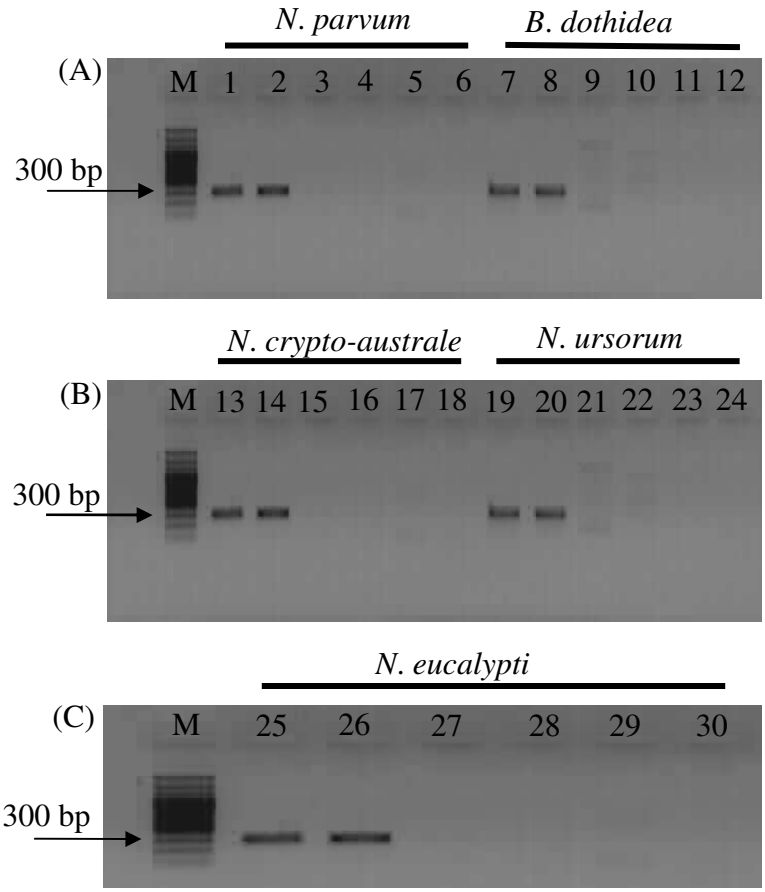
N. parvum
B. dothidea
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12
300 bp
N. crypto-australe
N. ursorum
(B)
M 13 14 15 16 17 18 19 20 21 22 23 24
300 bp
N. eucalypti
(C)
M 25 26 27 28 29 30
300 bp

Fig. 3. An agarose gel (1.5 %) showing the smallest concentration of DNA for which an amplicon could be detected. Lanes 1-8 (*A*) shows the amplicons produced using EF-1α primers (EF-AF and EF-BR) and lanes 1-8 (*B*) shows using the forward BdeF primer and EF-Br reverse primer, as an example. Lanes 1-8 (*A* and *B*) represents different concentrations of the DNA template (50, 25, 12.5, 4, 0.01, 0.001, 0.0001 and 0.00001 ng/µL). M (*A* and *B*) = 100 bp marker.

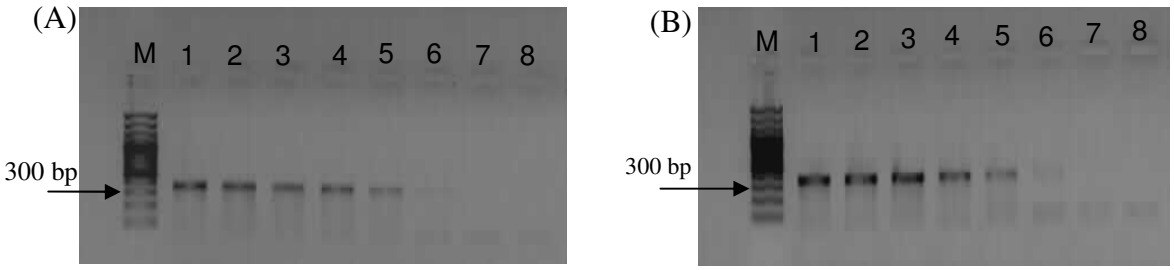
(A)
M 1 2 3 4 5 6 7 8
300 bp
(B)
M 1 2 3 4 5 6 7 8
300 bp

Fig. 4. An agarose gel (1.5 %) showing the specificity of primers for five species of Botryosphaeriaceae in the blind test. Isolates of the five species of Botryosphaeriaceae of known identity were randomly selected and relabeled. Amplification was done with each of the five species specific primers namely NpeF (*N. parvum*), NueF (*N. ursorum*), NeeF (*N. eucalypti*), NceF (*N. crypto-australe*) and BdeF (*B. dothidea*). PCR products amplified for each species are indicated in gels (*A-D*); M = 100 bp marker.

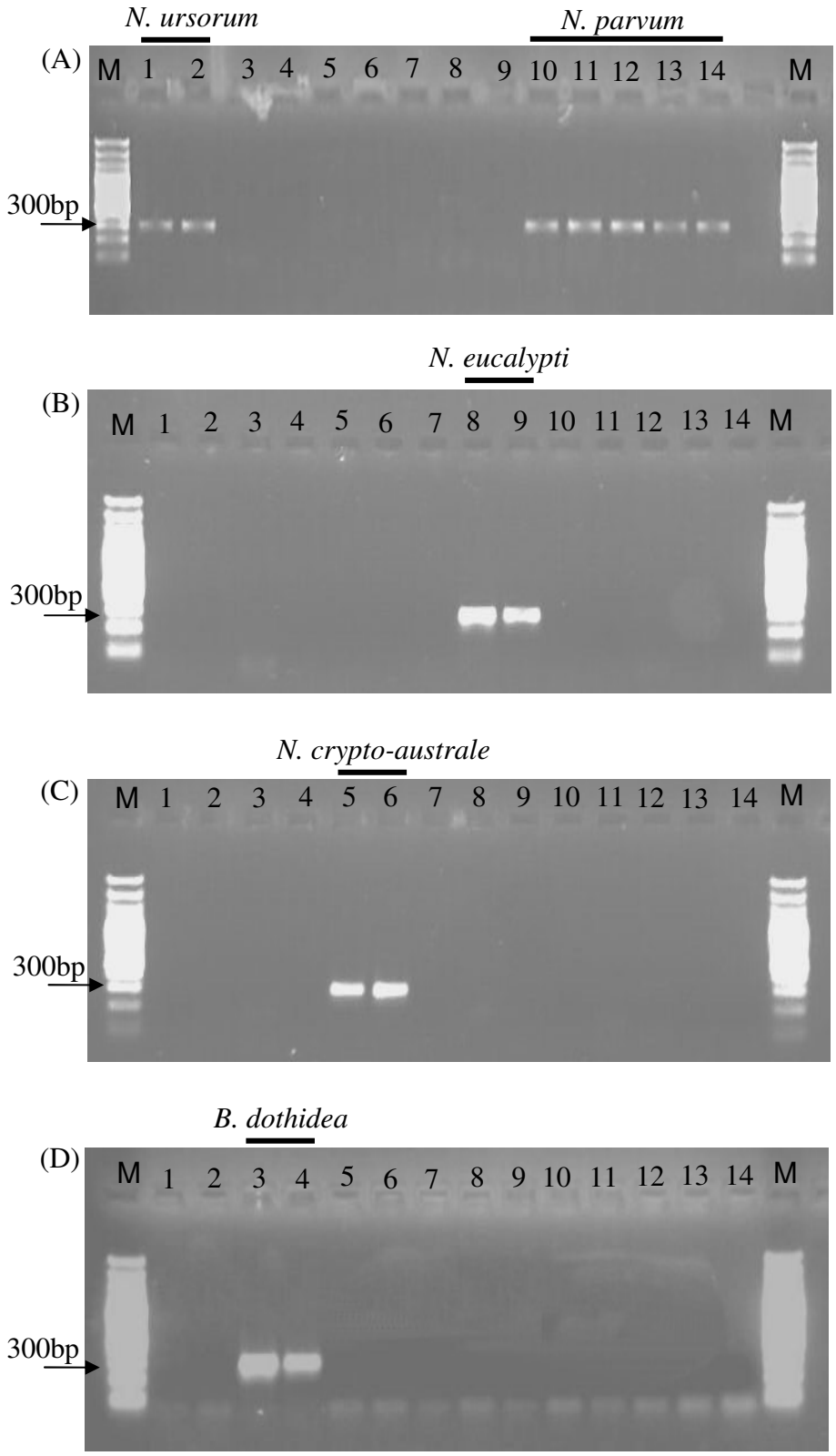
N. ursorum
N. parvum
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
N. eucalypti
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
N. crypto-australe
(C)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
B. dothidea
(D)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp

Fig. 5. An agarose gel (1.5 %) showing detection of *N. parvum* directly from *Eucalyptus* leaf segments. Lanes 1-8 show the amplicons produced using NpeF species specific primer together with EF-1α reverse primer (EF-BR). Lane 9 is negative control (PCR product without DNA template). Lane 10 shows positive control of purified genomic DNA of *N. parvum*. M = 100 bp marker.

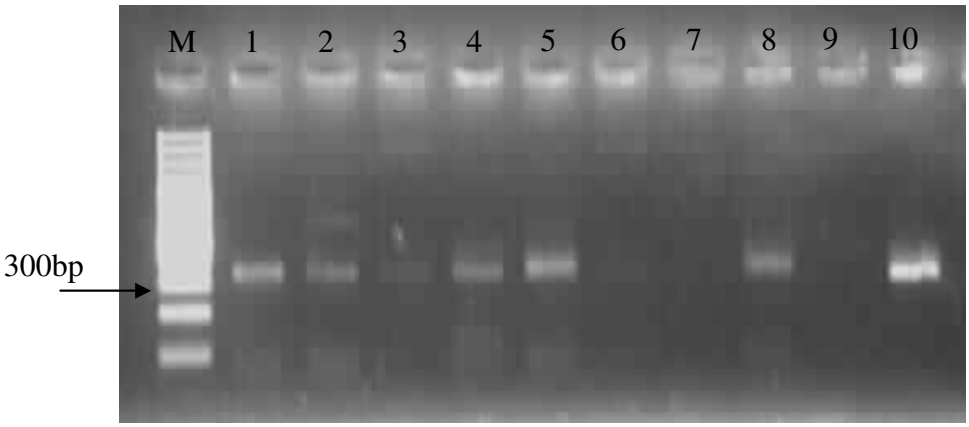
M
1
2
3
4
5
6
7
8
9
10
300bp

Table 1. Isolates representing species of Botryosphaeriaceae used in the specificity tests and primer design

Culture no.[A,B]	Other no.	Identity	Host	Location	Collector	Genbank EF
CMW7772 [A]		*Neofusicoccum ribis*	*Ribes* sp.	New York	B. Slippers & G. Hudler	AY236877
CMW7054 [A]	CBS 121.26	*N. ribis*	*R. rubrum*	New York	N. E. Stevens	AY236879
CMW14035[A, B]		*N. ribis*	*Syzygium cordatum*	Kwambonambi, S. Africa	D. Pavlic	AF283681
CMW14140 [A, B]		*N. ribis*	*S. cordatum*	Tzaneen, S. Africa	D. Pavlic	AY236885
CMW9078 [A]	ICMP 7925	*Neofusicoccum parvum*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236885
CMW10122 [A]		*N. parvum*	*Eucalyptus grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283681
CMW14021 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14022 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14019 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14018 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW20736 [A, B]		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme	
CMW23792 [A, B]		*N. parvum*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme	
CMW24571 [A, B]		*N. eucalypti*	*E. paniculata*	Pretoria, S. Africa	H. M. Maleme	
CMW23791 [A, B]		*N. eucalypti*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW23790 [A, B]		*Neofusicoccum ursorum*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW24480 [A, B]		*N. ursorum*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW9076 [A,]	ICMP 7818	*Neofusicoccum luteum*	*Malus domestica*	New Zealand	S. R. Pennycook	AY236893
CMW10309 [A,]	CAP 002	*N. luteum*	*Vitis vinifera*	Portugal	A. J. L. Phillips	AY339266
CMW3386 [A]		*Neofusicoccum crypto-australe*	*Wollemia nobilis*	Queensland, Australia	M. Ivory	AY615157
CMW20738 [A, B]		*N. crypto-australe*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme	
CMW23787 [A, B]		*N. crypto-australe*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW9073 [A]		*Neofusicoccum australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY615269
CMW1110 [A]		*N. australe*	*Widdringtonia nodiflora*	Cape Province, S. Africa	W. J. Swart	AY615158
CMW8000 [A]		*Botryosphaeria dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236898
CMW9075 [A]	ICMP 8019	*B. dothidea*	*P. nigra*	New Zealand	G. J. Samuels	AY236899
CMW20728 [A, B]		*B. dothidea*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme	
CMW20739 [A, B]		*B. dothidea*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme	

[A]Culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; CAP = Culture collection of A. J. L. Phillips, Lisbon, Portugal; CBS =Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[A]Sequences of isolates used for designing primers.

[B]Isolates used in the blind test.

Table 2. Species-specific primers designed for five species of Botryosphaeriaceae and their PCR annealing temperatures

Identity	Primer	Primer sequences	Annealing tempetature (^{o}C)
Neofusicoccum parvum	NpeF	5' CTG GGT GCC AGG TGC 3'	60
N. eucalypti	NeeF	5' GTT TTT CCG CTG CAC GCA 3'	55
N. ursorum	NueF	5' GCA CGC TGG GTG GTG GGT 3'	60
N. crypto-australe	NceF	5' GCG ATG GTC CTG GGA TC 3'	60
Botryosphaeria dothidea	BdeF	5' GTT CCT GCG CCG AAT T 3'	54

Table 3. Multiple aligned sequences of representative isolates of Botryosphaeriaceae used for designing species specific primers. Primer sequences designed are in bold. Only the portion where primers where designed is shown. Each couloured block represent primer sequences for different species of Botryosphaeriaceae. (*Neofusicoccum ribis*), (*N. parvum*), (*N. eucalypti*), (*N. vitifusiforme*), (*N. ursorum*), (*N. luteum*), (*N. australe*), (*N. crypto-australe*) and (*Botryosphaeria dothidea*)

```
CMW_7772     cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acg c-- gct ggg tgc tgg gtg ctg ggt gct ggg tgc tgg gtt ccc gca ctc aat ttg
CMW_7054     cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg tgg gtg ctg ggt gct ggg tgc tgg gtt ccc gca ctc aat ttg
CMW_9078     cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg cag gtg ctg ggt --- --- --- --- --t ccc gca ctc aat ttg
CMW_10122    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg cag gtg ctg ggt --- --- --- --- --t ccc gca ctc aat ttg
CMW_23792    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tgc cag gtg ctg ggt --- --- --- --- --t tcc gca ctc aat ttg
CMW_20736    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tgc cag gtg ctg ggt --- --- --- --- --t tcc gca ctc aat ttg

CMW_15952    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_15953    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc agc cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_23791    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_24460    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_24571    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
STE_U5252    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
STE_U5050    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_23790    cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acc cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_24480    cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gca ctc aat ttg

CMW_10309    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_9076     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_9073     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gct gca ctc aat ttg
CMW_1110     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gct gca ctc aat ttg
CMW_3386     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_20738    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_23787    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_8000     cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_9075     cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_23787    cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_20739    cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
```