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DIVERSITY AND HOST RANGE OF THE CRYPHONECTRIACEAE IN SOUTHERN AFRICA

MARCELE VERMEULEN

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Diversity and host range of the Cryphonectriaceae in southern Africa

by

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DECLARATION

I, Marcele Vermeulen declare that the thesis/dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

September 2011

This thesis is dedicated to my grandmother

Rachel Maria Elisabeth Alexander

Thank you for always believing in me

Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us.' We ask ourselves, who am I to be brilliant, gorgeous, talented, fabulous? Actually, who are you not to be? You are a child of God. Your playing small does not serve the world. There's nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we're liberated from our own fear, our presence automatically liberates others.

Marianne Williamson: A Return to Love

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PREFACE

Members of the Cryphonectriaceae include important tree pathogens, some of which have had a considerable impact on commercial forestry in the tropics and Southern Hemisphere. Species of *Chrysoporthe*, especially, have shaped the face of commercial plantation forestry using non-native species of *Eucalyptus* in South Africa, South America and Asia. Species residing in four genera of the Cryphonectriaceae are known to be present in Africa, including *Aurifilum*, *Celoporthe, Chrysoporthe* and *Holocryphia*. All of these genera include tree pathogens of native as well as non-native tree species. The aim of this thesis was to conduct expanded surveys in southern Africa and to characterize the Cryphonectriaceae found.

The literature review presented in Chapter One of this thesis treats the Cryphonectriaceae that have been reported from southern Africa. This overview summarizes the taxonomic history of *Aurifilum*, *Celoporthe*, *Chrysoporthe* and *Holocryphia* species. The pathology, possible origins, host range, distribution, symptoms, impact and possible control of these fungi are also discussed.

Recent studies have greatly expanded our knowledge on the distribution of the Cryphonectriaceae in Africa. However, several regions in Africa remain unexplored despite the fact that they could harbour important or potentially important tree pathogens. Chapter Two describes the results of expanded surveys for the Cryphonectriaceae on the Myrtales in southern Africa. A particular aim is to improve the base of knowledge regarding their species diversity and distribution on the continent.

Chrysoporthe austroafricana is a serious fungal pathogen of plantation-grown *Eucalyptus* species in southern and eastern Africa. This pathogen is also known to infect native *Syzygium*

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cordatum, *S. guineense* and non-native *Tibouchina granulosa.* The exact origin and routes of movement of the fungus among hosts and countries in southern Africa are unknown. Furthermore, apart from South Africa, nothing is known regarding the population diversity of the fungus on different hosts and in different countries in the region. Chapter Three aims to determine the population structure and diversity of populations of *Chr. austroafricana* from Malawi, Mozambique, Namibia and Zambia based on vegetative compatibility groups (VCG's).

Chapter Four represents a taxonomic study of isolates of *Celoporthe* from South Africa, Zambia and Namibia. The aim of the study was to determine whether these represent more than one species. DNA sequence data for multiple gene regions, as well as extensive comparisons based on morphology are used. Furthermore, I considered whether isolates from different hosts and countries differ in pathogenicity on a *Eucalyptus* clone and *S. cordatum* seedlings.

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Chapter 1

Literature review

The Cryphonectriaceae in Africa with particular reference to species in southern Africa

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1 INTRODUCTION

The Cryphonectriaceae is a recently established family in the Diaporthales and was previously known as the *Cryphonectria-Endothia* complex (Castlebury *et al*. 2002, Gryzenhout *et al.* 2006c). Members of the Cryphonectriaceae include important tree pathogens. Perhaps the most important species in this group is *Cryphonectria parasitica* (Murrill) M.E. Barr, the causal agent of chestnut blight, which virtually wiped out the American chestnut [*Castanea dentata* (Marsh.) Borkh.] in North America and seriously impacted European chestnut [*Castanea sativa* Mill] in Europe (Heiniger and Rigling 1994). The American chestnut tree can now mostly be found only as small shrubs in contrast to the once dominant canopy species of the past (Anagnostakis 1987).

The Cryphonectriaceae have had a considerable impact on commercial forestry based on non–native tree species in the tropics and Southern Hemisphere. In this regard, the trees most seriously affected are commercially planted species of *Eucalyptus* in Africa, South America and Asia, infected by *Chrysoporthe* species (Hodges *et al*. 1979, Sharma *et al.* 1985, Florence *et al.* 1986, Conradie *et al.* 1990, Wingfield 2003, Gryzenhout *et al*. 2004). One member of this group, *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf., seriously threatened clonal eucalypt plantations in South Africa when it caused large scale death of one of the first *Eucalyptus grandis* Hill. ex Maid. clones, known for its excellent timber qualities (Wingfield 2003).

In recent years, the taxonomy of the Cryphonectriaceae and the genera included in this family have undergone dramatic change. The family now includes thirteen genera, ie. *Amphilogia*, *Aurapex*, *Aurifilum*, *Celoporthe, Chrysoporthe*, *Cryphonectria, Cryptometrion, Endothia,*

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Foliocryphia, Holocryphia, Microthia, Rostraureum, *Ursicollum* and the anamorph genus *Endothiella* (Cheewangkoon *et al*. 2009, Gryzenhout *et al*. 2009, 2010a, Begoude *et al*. 2010). The Cryphonectriaceae can be differentiated from other families in the Diaporthales by DNA sequence data. Morphological characteristics such as distinct orange stromatic tissue at some stage of the life cycle, the purple discoloration of the stromatic tissue in 3% potassium hydroxide (KOH) and a yellow reaction in lactic acid ($C_3H_6O_3$) also distinguish the Cryphonectriaceae from other Diaporthalean fungi (Castlebury *et al*. 2002, Gryzenhout *et al*. 2009).

Species residing in four genera of the Cryphonectriaceae are known to be present in Africa. These include *Aurifilum* (Begoude *et al*. 2010), *Celoporthe* (Nakabonge *et al.* 2006a), *Chrysoporthe* (Gryzenhout *et al*. 2004) and *Holocryphia* (Gryzenhout *et al*. 2006a). Species in these genera include introduced pathogens that infect *Eucalyptus* species in plantations, as well as fungi thought to be native and that cross-infect non-native trees such as *Eucalyptus* species. *Chrysoporthe austroafricana*, for example, most likely originated on native African Myrtales and adapted the capacity to infect non-native *Eucalyptus* species (Heath *et al.* 2006). In contrast, *Chrysoporthe cubensis* (Bruner) Gryzenh. & M.J. Wingf. and *Chr. deuterocubensis* Gryzenh. & M.J. Wingf. appear to have been introduced to the African continent (Nakabonge *et al.* 2006b, 2007, Van der Merwe *et al*. 2010). The aim of this review is to provide an overview of the taxonomy, pathology, possible origins, host range and distribution of those Cryphonectriaceae that have been reported from Africa.

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2 *CHRYSOPORTHE*

2.1 TAXONOMY AND HISTORY OF *CHRYSOPORTHE* SPECIES

In 1917, a stem canker disease affecting *Eucalyptus* species was reported from Cuba, and the causal agent was described as *Diaporthe cubensis* Bruner (Bruner 1917). Hodges (1980) renamed *D. cubensis* as *Cryphonectria cubensis* (Bruner) Hodges, based on the fact that the *Eucalyptus* pathogen had one-septate ascospores, prosenchymatous stromata and orange stromatal tissue surrounding the perithecia. This description is more similar to the characteristics of *Cryphonectria* than those of *Diaporthe*. *Diaporthe* in contrast, is characterised by perithecia formed within a pseudostromata circumscribed by a black zone (Hodges 1980). Another fungus, *Endothia eugeniae* J. Reid & C. Booth causing clove die-back in Indonesia, Malaysia and Zanzibar (Nutman and Roberts 1952), was later shown to be conspecific with *C. cubensis* based on morphological comparisons, cultural studies, protein profiles, isoenzyme analyses and inoculation studies (Hodges *et al.* 1986, Micales *et al.* 1987). The conspecificity of these two fungi was later confirmed using DNA sequence comparisons (Myburg *et al.* 1999, 2003).

Recent research has led to the discovery that *C. cubensis* should not reside in *Cryphonectria* and that it represents more than one species. The genus *Chrysoporthe* was thus described for *C. cubensis* (Gryzenhout *et al.* 2004) and currently includes *C. cubensis*, now known as *Chr. cubensis*, and the new species *Chr. austroafricana*, *Chr. deuterocubensis, Chr. doradensis* Gryzenh. & M.J. Wingf., *Chr. hodgesiana* (Gryzenh. & M.J. Wingf.) Gryzenh. & M.J. Wingf., *Chr. inopina* Gryzenh. & M.J. Wingf., *Chr. syzygiicola* Chungu, Gryzenh. & Jol.

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Roux and *Chr. zambiensis* Chungu, Gryzenh. & Jol. Roux (Gryzenhout *et al.* 2004, 2005b, 2006d, Chungu *et al*. 2010, Van der Merwe *et al.* 2011) (TABLE 1, FIG. 1).

Chrysoporthe hodgesiana was originally described as *Chrysoporthella hodgesiana* Gryzenh. & M.J. Wingf. to accommodate the asexual structures of *Chrysoporthe* (Gryzenhout *et al.* 2004) (TABLE 1, FIG. 1). The use of the anamorphic genus *Chrysoporthella* in this small but well known genus, *Chrysoporthe,* led to confusion. It was, therefore, suggested that the more well known holomorph is to be used (Chungu *et al*. 2010). Therefore, although only the asexual state of both *Chr. syzygiicola* and *Chr. zambiensis* is known these species were also described in the more well-known holomorph genus *Chrysoporthe* (Chungu *et al.* 2010).

Chrysoporthe species can be distinguished from species in *Cryphonectria* and other genera in the Cryphonectriaceae based on distinct morphological features. These include superficial, black, pyriform conidiomata, ascostromata with orange prosenchymatous stromatic tissue, reduced stromatic development, perithecial necks covered with dark brown tissue extending beyond the stromatal surface, single septate ascospores and oblong conidia (Gryzenhout *et al.* 2004, 2009). The genera that are morphologically most similar to *Chrysoporthe* are *Aurapex* and *Celoporthe.* All three of these genera have black conidiomata and the teleomorph of both *Celoporthe* and *Chrysoporthe* do not have extensive and obvious orange stromatic tissue (Nakabonge *et al.* 2006a, Gryzenhout *et al.* 2006b, 2009). *Aurapex* can be distinguished from *Chrysoporthe* based on its orange necks and brick red spore drops. *Chrysoporthe* can be differentiated from *Celoporthe* based on its conidiomata that are pulvinate to conical with short necks (Nakabonge *et al*. 2006a), whereas those of *Chrysoporthe* are pulvinate to pyriform with distinct necks (Gryzenhout *et al.* 2004, 2009). All other genera in the

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Cryphonectriaceae have distinct orange stromata in both the anamorph and teleomorph states, and orange perithecial necks (Gryzenhout *et al.* 2009).

Chrysoporthe cubensis can be differentiated from other *Chrysoporthe* species based on ascospore, ascus and conidial morphology as well as optimum temperature for growth in culture (TABLE 1). For example, *Chr. austroafricana* has ascospores that are relatively long with tapered apices as opposed to the shorter asci with rounded apices in *Chr. cubensis* (Gryzenhout *et al.* 2004, 2009). *Chrysoporthe inopina* can be distinguished from *Chr. cubensis* based on its longer asci and from other *Chrysoporthe* species based on its wider ascospores (Gryzenhout *et al.* 2006d, 2009).

Chrysoporthe doradensis has conidia of variable shape, and cream-colored conidial spore masses, compared to more uniform, oblong to oval conidia that are exuded in yellow masses in other *Chrysoporthe* species (Gryzenhout *et al*. 2005b, 2009) (TABLE 1). *Chrysoporthe hodgesiana* and *Chr. inopina* can also be differentiated from other *Chrysoporthe* species based on an optimum growth in culture at 25°C as opposed to 30°C for other *Chrysoporthe* species (Gryzenhout *et al*. 2004, 2006d, 2009). Lastly, *Chr*. *syzygiicola* and *Chr*. *zambiensis* can be differentiated from other *Chrysoporthe* species based on their conidiomata. The conidiomata of *Chr. syzygiicola* are darker than those of other species and are rostrate in shape, while those of *Chr*. *zambiensis* have globose to ovoid bases with tapering necks and a rust brown color (Chungu *et al.* 2010).

Based on phylogenetic inference using sequences for the Internal Transcribed Spacer (ITS) regions of the ribosomal operon, two regions within the β-tubulin genes and one region of the histone *H3* gene, *Chr. cubensis* could be divided into two sub-clades (Myburg *et al.* 1999,

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2002b, Gryzenhout *et al.* 2004) (FIG. 1). One of the sub-clades in this phylogeny includes isolates from South and Central America, and central Africa, and the other sub-clade accommodates isolates from Southeast Asia, Australia, eastern Africa and Hawaii. Isolates in the two sub-clades give rise to the same disease symptoms and they share hosts in the different regions in which they occur (Boerboom and Maas 1970, Hodges *et al.* 1976, 1979, Sharma *et al.* 1985, Florence *et al.* 1986, Gryzenhout *et al.* 2006d). Although there are clear phylogenetic differences for these two sub-clades, they were not at first described as distinct taxa as no obvious morphological or biological differences could be found to distinguish between them (Myburg *et al.* 2002b, Gryzenhout *et al.* 2004). Based on phylogenetic and population genetic analyses the Asian sub-clade was recently described by Van der Merwe *et al.* (2010) as *Chr. deuterocubensis*. *Chrysoporthe cubensis* and *Chr. deuterocubensis* can be distinguished from each other based on fixed polymorphisms in five gene regions, namely Actin, β-tubulin, Translation Elongation Factor 1-α, Histone *H3* and ITS (Van der Merwe *et al*. 2010). These two species are known from distinct geographical regions with no overlap in their distribution (Van der Merwe *et al*. 2011).

2.2 DISTRIBUTION AND HOST RANGE

Chrysoporthe species generally occur in the tropics and sub-tropics, preferring hot and humid conditions for infection and growth (Boerboom and Maas 1970, Sharma *et al.* 1985, Conradie *et al.* 1990, Wingfield 2003). They typically occur on trees at latitudes between the 30˚ North and South (Sharma *et al.* 1985). Five *Chrysoporthe* species (TABLE 2) are known in Africa, namely *Chr. austroafricana*, *Chr. cubensis, Chr. deuterocubensis, Chr. syzygiicola* and *Chr. zambiensis* (Gryzenhout *et al.* 2009, Chungu *et al.* 2010, Van der Merwe *et al*. 2010). Only one species of *Chrysoporthe* is known from Asia and Australasia, namely *Chr.*

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deuterocubensis (Van der Merwe *et al.*, 2010) and four species from the Americas, namely *Chr. cubensis*, *Chr. doradensis, Chr. hodgesiana* and *Chr. inopina* (Gryzenhout *et al*. 2009). Because of the overlapping taxonomies of *Chrysoporthe* great care should be taken in interpreting published data on their host range and distribution. In the following section the distribution and host range of *Chrysoporthe* species are reported based on the most recent information and based on species identities which have been confirmed with DNA sequence data.

Chrysoporthe cubensis has been reported from countries in Africa (Gibson 1981, Hodges *et al.* 1986, Roux *et al.* 2003) and the Americas (Boerboom and Maas 1970, Hodges *et al.* 1976, 1979) (FIG. 2). *Chrysoporthe cubensis* was first reported in Africa when it was detected in the Democratic Republic of Congo in 1960 on *Eucalyptus saligna* Sm. but was at that time wrongly identified as *Endothia havanensis* Bruner (Hodges *et al.* 1986, Micales *et al.* 1987). *Chrysoporthe cubensis* was, however, formally reported from Africa for the first time in Cameroon in 1981 on a *Eucalyptus* sp. (Gibson 1981) and is now known from several countries in western Africa, namely Cameroon (Gibson 1981), Democratic Republic of Congo (Hodges *et al.* 1986, Micales *et al.* 1987), Ghana (Roux and Apetorgbor 2009) and Republic of Congo (Roux *et al.* 2003).

Chrysoporthe deuterocubensis is known from countries in eastern Africa (Nakabonge *et al.* 2006b) and from Asia (Sharma *et al.* 1985, Florence *et al.* 1986, Van Heerden *et al.* 1997), Australia (Davison and Coates 1991, Pegg *et al*. 2010) and Hawaii (Hodges *et al.* 1979) (FIG. 2). In Africa, *Chr. deuterocubensis*, was previously reported as *C. cubensis*, *Chr. cubensis* and *E. eugeniae* and is known from several countries in eastern Africa namely Kenya,

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Malawi, Mozambique and Tanzania, including the island of Zanzibar (Nakabonge *et al.* 2006b).

Chrysoporthe cubensis has been reported from many hosts in the past and due to the recent description of the new species *Chr. deuterocubensis* for the Asian sub-clade of *Chr. cubensis*, it is worthwhile to delimit the host ranges again. *Chrysoporthe cubensis* has been reported from a number of *Eucalyptus* species (Hodges *et al.* 1979, Hodges 1980) and *Syzygium aromaticum* (L.) Merr & Perry. (Hodges *et al.* 1986) in the Myrtaceae, and *Clidemia sericea* D. Don., *Miconia rubiginosa* (Bonpl.) DC., *M. theaezans* (Bonpl.) Cogn. (Rodas *et al.* 2005) and *Rhynchanthera mexicana* DC. (Gryzenhout *et al.* 2006d) in the Melastomaceae. Recently it was shown also to occur on *Lagerstroemia indica L.* in the Lythraceae (Gryzenhout *et al.* 2006d). *Chrysoporthe deuterocubensis* has been reported from *Eucalyptus* species (Sharma *et al.* 1985) and *S. aromaticum* (Nutman and Roberts 1952, Hodges *et al.* 1986) in the Myrtaceae and *Melastoma malabathricum* L. (Gryzenhout *et al.* 2006d), *Tibouchina urvilleana* Cogn. & Longn. (Gryzenhout *et al.* 2006d) and *Tibouchina heteromalla* Cogn. (Pegg *et al.* 2010) in the Melastomaceae. On the African continent *Chr. cubensis* is known only from *Eucalyptus* species and *Chr. deuterocubensis* is known from *Eucalyptus* species and *S. aromaticum* (Nutman and Roberts 1952, Gibson 1981).

Chrysoporthe austroafricana is known only from Africa (TABLE 2) and was first reported from South Africa as *C. cubensis* (Wingfield *et al.* 1989). It has since been reported from Malawi, Mozambique, Zambia (Nakabonge *et al.* 2006b) and Namibia (Vermeulen 2006) (FIG. 2). *Chrysoporthe austroafricana* has been reported from *Eucalyptus* species (Wingfield *et al.* 1989, Nakabonge *et al.* 2006b), *Syzygium cordatum* Hachst. and *Syzygium guineense* (CD.) in the Myrtaceae (Heath *et al.* 2006, Vermeulen 2006) and *Tibouchina granulosa*

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Cogn.: Britton (Myburg *et al.* 2002a) in the Melastomataceae. *Chrysoporthe syzygiicola* and *Chr. zambiensis* has to date only been reported from two locations in Zambia. *Chrysoporthe syzygiicola* was isolated from *S. guineense* in a native forest in Samfya and *Chr. zambiensis* from *E. grandis* trees in Kapweshi (Chungu *et al.* 2010).

Chrysoporthe inopina infects *T. lepidota* variety Alstonville (Melastomataceae) while *Chr. doradensis* infects *E. deglupta* Blume and *E. grandis* (Myrtaceae), and both have been shown to be pathogenic to *T. urvilleana* in artificial inoculation trials (Gryzenhout *et al.* 2005b, Gryzenhout *et al.* 2006d). *Chrysoporthe hodgesiana* has been isolated from cankers on *T. lepidota* Baill, *T. semidecandra* Cogn. and *T. urvilleana*. (Wingfield *et al*. 2001, Gryzenhout *et al.* 2004), as well as from *M. theaezans* (Rodas *et al*. 2005). It was also pathogenic to *E. grandis* in pathogenicity trials but has not been reported from naturally infected trees in the field (Wingfield *et al*. 2001).

Chrysoporthe syzygiicola and *Chr. zambiensis* are only known from Zambia, and the remaining species in this genus; *Chr. hodgesiana* and *Chr. inopina* are known from only two countries in South America. *Chrysoporthe hodgesiana* and *Chr. inopina* have been recorded only from Colombia (Gryzenhout *et al*. 2004, 2006d), while *Chr. doradensis* is known from neighboring Ecuador (Gryzenhout *et al*. 2005b) (FIG. 2). The restricted geographical range of these fungi may, however, reflect a lack of surveys on native vegetation and related tree species.

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2.3 ORIGIN OF *CHRYSOPORTHE* SPECIES

There are several hypotheses regarding the origin of *Chr. austroafricana, Chr. cubensis* and *Chr. deuterocubensis,* the most widely studied and devastating species in the genus. These hypotheses regarding geographic origin are based on the hosts on which the *Chrysoporthe* species occur in various countries. Population biology studies based on Vegetative Compatibility Groups (VCG) (TABLE 3) and polymorphic molecular markers in the form of microsatellite/simple sequence repeats (TABLE 4) (Van Heerden *et al.* 1997, Van Zyl *et al.* 1998, Van der Merwe 2000, Van Heerden and Wingfield 2001, Van der Merwe *et al.* 2003, Heath 2004, Nakabonge 2006, Nakabonge *et al.* 2007) also added more data towards elucidating the origin of these species.

2.3.1 *Chrysoporthe cubensis* and *Chr. deuterocubensis*

For a long time *Chr. cubensis* was thought of as a pathogen occurring worldwide in tropical to subtropical areas. Several hypotheses were suggested to explain the origin of *Chr. cubensis.* One of these was that the pathogen is native to the Indonesian Molucca Islands, where it occurs as a mild pathogen on native *S. aromaticum* (Hodges *et al.* 1986). The world-wide spread of *Chr. cubensis* could then most likely be linked to the establishment of clove plantations in Africa, the Caribbean and South America for the spice trade, as well as to clove trees being planted in numerous botanical gardens throughout the tropics (Hodges *et al.* 1986, Myburg *et al.* 1999, Wingfield *et al.* 2001, Wingfield 2003). The hypothesis that *Chr. cubensis* originated from Southeast Asia was supported by the occurrence of *Chr. cubensis* on native *M. malabathricum* in Southeast Asia and its ability to infect *S. aromaticum* that is native to the Molucca Islands

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(Gryzenhout *et al.* 2006d). Furthermore, isolates collected from Indonesia have a high phenotypic diversity based on VCGs (Van Heerden *et al.* 1997).

Wingfield *et al*. (2001) hypothesized that *Chr. cubensis* might have had its origin on native Melastomataceae from Central and South America (Wingfield *et al.* 2001, Wingfield 2003). This view was based on the fact that the fungus occurs on several native Melastomataceae in Colombia and Mexico (Rodas *et al.* 2005, Gryzenhout *et al.* 2006d). The hypothesis was further supported by its high phenotypic diversity based on VCGs in isolates from South American countries such as Venezuela and Brazil (Van Heerden *et al.* 1997, Van Zyl *et al*. 1998).

The updated taxonomy of *Chr. cubensis* and the description of the Asian sub-clade as the separate species *Chr. deuterocubensis* indicate that these two species have separate continental origins (Gryzenhout *et al*. 2006d). *Chrysoporthe cubensis* most likely has an origin in Central to South America, while the origin of *Chr. deuterocubensis* is in Southeast Asia. This is supported by the fact that both the Asian and South American species demonstrated high degrees of diversity based on VCG's (Van Heerden *et al.* 1997).

Recent surveys by Roux *et al*. (2003, 2005) and Nakabonge *et al.* (2006b) have significantly expanded our knowledge regarding the distribution of *Chr. cubensis* and *Chr. deuterocubensis* in Africa. These authors found that isolates from the western part of Africa group in *Chr. cubensis* (Roux *et al.* 2003, Roux and Apetorgbor 2009). This is different from the situation in eastern Africa where isolates group in *Chr. deuterocubensis* (Nakabonge *et al.* 2006b).

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A very low population diversity was observed amongst *Chr. deuterocubensis* isolates from *Eucalyptus* in Kenya, Malawi and Mozambique (Nakabonge *et al.* 2007). This low diversity could indicate that the isolates of *Chr. deuterocubensis* do not have an African origin (Nakabonge *et al*. 2007) and that the fungus has recently been introduced into the region. This hypothesis is further supported by the fact that *Chr. deuterocubensis* has not been observed on any native trees in Africa, making this continent a highly unlikely centre of origin for it (Nakabonge *et al.* 2006b). It is further believed that the presence of *Chr. cubensis* and *Chr. deuterocubensis* in Africa was due to introductions from both South America and Southeast Asia into Africa on separate occasions (Nakabonge *et al.* 2006b, Van der Merwe *et al*. 2010).

2.3.2 *Chrysoporthe austroafricana*

An early view concerning the origin of *Chr. austroafricana* was that it had been introduced into South Africa, either from Latin America or Asia (Van Heerden and Wingfield 2001). This was substantiated by a low phenotypic diversity based on VCG's of this fungus on *Eucalyptus* species in South Africa. This hypothesis was, however, based on the misconception that *Chr. austroafricana* and *Chr. cubensis* represented a single species.

The discovery of *Chr. austroafricana* on a native host in South Africa led Heath *et al.* (2006) to hypothesize that *Chr. austroafricana* is native to South Africa and that it could have originated from native Myrtaceae in the country. This was also based on pathogenicity trials showing that native *S. cordatum* was more tolerant to inoculation by *Chr. austroafricana* than the non-native *Eucalyptus* clones tested (Heath *et al.*

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2006). Furthermore, VCG data showed a high phenotypic diversity within the population collected from native *S. cordatum* (Heath 2004) and *S. guineense* (Vermeulen 2006) (TABLE 3). The fact that *Chr. austroafricana* has also been found more widely on *S. cordatum* in other southern African countries further supports this view (Nakabonge *et al.* 2006b).

Recent studies on the diversity of *Chr. austroafricana* isolates collected from native and introduced hosts in South Africa based on microsatellite data supports the view that *Chr. austroafricana* is native to Africa (Heath 2004). A high diversity based on microsatellite data was observed for populations from non-native *Eucalyptus* and *Tibouchina* as opposed to a low diversity observed for isolates from native *Syzygium* (Heath 2004). This suggests that the centre of diversity for *Chr. austroafricana* could be a native host other than *S. cordatum* (Heath 2004), since the high diversity observed on *Eucalyptus* and *Tibouchina* supports other data that suggest that it is an African fungus (Heath 2004).

2.3.3 Other species of *Chrysoporthe*

Reports of *Chr. doradensis, Chr. hodgesiana, Chr. inopina*, *Chr. syzygiicola* and *Chr. zambiensis* are very limited, making strong inferences regarding their origins difficult. *Chrysoporthe inopina* has been reported only from native *T. lepidota* at a single locality in Colombia (Gryzenhout *et al.* 2006d). *Chrysoporthe hodgesiana* is also known only from Colombia were it infects native *Melastomataceae* (Gryzenhout *et al.* 2004). Likewise, *Chr. doradensis* is known only from *Eucalyptus* species in Ecuador (Gryzenhout *et al.* 2005b). It is suspected that *Chr. doradensis* occurs on native

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melastomes in Ecuador, which would indicate that it is native to the area. The fact other *Chrysoporthe* species such as *Chr. cubensis*, *Chr. hodgesiana* and *Chr. inopina* occur on native melastomes in neighbouring countries suggest that *Chr. doradensis* also could occur on these hosts. Furthermore, to date *Chr. doradensis* is known only from non-native *Eucalyptus* species in Ecuador, and is not known from other countries, including Australia where *Eucalyptus* species are native (Gryzenhout *et al.* 2005b). *Chrysoporthe syzygiicola* and *Chr. zambiensis* are only known from Zambia on native *S. guineense* and non-native *E. grandis* respectively.

More extensive surveys on *Eucalyptus* species, and especially native Myrtales, should be undertaken in an attempt to expand the host and geographical ranges of these fungi before any hypotheses on their origin is promoted with confidence. In this regard, it is also important that samples collected are identified based on both DNA sequence data as well as morphology, in order to negate problems linked with misidentifications of cryptic species. However, currently it is hypothesised that the three species from South America are native to South America, due to their absence from other continents and the presence of both *Chr. hodgesiana* and *Chr. inopina* on native hosts in Colombia (Gryzenhout *et al.* 2004, 2006d). Similarly, *Chr. syzygiicola* and *Chr. zambiensis* could be native to Zambia due to their absence in other countries and the presence of *Chr. syzygiicola* on native *S. guineense* (Chungu *et al.* 2010).

2.4 SYMPTOMS AND IMPACT

Chrysoporthe species are all canker pathogens that characteristically kill the cambium of trees and form cankers on stems and branches (Gryzenhout *et al.* 2009). Of the seven

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Chrysoporthe species, five are known to cause disease on *Eucalyptus* species. These are *Chr. austroafricana* (Wingfield *et al.* 1989), *Chr. cubensis* (Hodges *et al.* 1979), *Chr. deuterocubensis* (Van der Merwe *et al.* 2010), *Chr. doradensis* (Gryzenhout *et al*. 2006d) and *Chr. zambiensis* (Chungu *et al.* 2010). *Chrysoporthe hodgesiana* (Gryzenhout *et al.* 2004, Wingfield *et al.* 2001) and *Chr. syzygiicola* (Chungu *et al.* 2010) has, however, also been shown to be pathogenic to *Eucalyptus* species in pathogenicity trails, although they have not been found occurring on *Eucalyptus* species in plantation situations. Below follows a discussion on *Chr. austroafricana*, *Chr. cubensis, Chr. deuterocubensis*, *Chr. syzygiicola* and *Chr. zambiensis* as these pathogens are the only *Chrysoporthe* species occurring in Africa.

Chrysoporthe austroafricana, Chr. cubensis and *Chr. deuterocubensis* are of economic importance as they cause serious losses to trees in *Eucalyptus* plantations, both reducing the wood quality and causing death of trees (Hodges *et al.* 1979, Wingfield *et al*. 1989, Davison and Coates 1991, Conradie *et al.* 1992, Roux *et al*. 2005). Importantly, *Chr. austroafricana* and *Chr. cubensis* significantly influenced the establishment of clonal forestry in Brazil and South Africa (Conradie *et al*. 1990, Van der Westhuizen *et al.* 1992, Wingfield 2003). The economic importance of *Chr. zambiensis* is not yet known, as it has only been reported from one location in Zambia (Chungu *et al.* 2010).

2.4.1 *Chrysoporthe cubensis* and *Chr. deuterocubensis*

Chrysoporthe cubensis and *Chr. deuterocubensis* result in severe losses to *Eucalyptus* plantations in tropical and sub-tropical areas of the world (Wingfield 2003). Hodges *et al.* (1979) reported that infection rates with *Chr. cubensis* can be as high as 80%, with 20% mortality after three years in *Eucalyptus* plantations. This was found in

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areas of Brazil with high rainfall and an average temperature exceeding 23°C. Besides tree death, infection by *Chr. cubensis* and *Chr. deuterocubensis* also reduces growth rate, coppicing and wood yield (Davison and Coates 1991). *Chrysoporthe deuterocubensis* cankers girdle the trees and results in cracking, swelling and shedding of the bark (FIG. 3a) that form "skirts" of dead bark at the bases of older trees (Wingfield *et al.* 1989, Nakabonge *et al*. 2006b).

Chrysoporthe cubensis and *Chr. deuterocubensis* form cankers both at the bases and higher up on the trunks of mature *Eucalyptus* trees and both give rise to target-shaped stem cankers around branch stubs (Hodges *et al.* 1979, Sharma *et al.* 1985, Florence *et al.* 1986, Ferreira 1989, Wingfield 2003). Cankers are typically covered with pycnidia and perithecia (Wingfield 2003). These cankers may result in tree death as the pathogens kill the cambium and restricts the upward flow of nutrients (Hodges *et al.* 1979, Sharma *et al*. 1985).

Large scale vegetative propagation of *Eucalyptus* species in the tropics and Southern Hemisphere was initiated because of the canker disease caused by *Chr. cubensis* and *Chr. deuterocubensis* (Wingfield 2003). Prior to the appearance of the disease of eucalypts caused by *Chr. cubensis* in Brazil, the local forestry industry relied largely on the planting of *E. grandis* and *E. saligna* (Wingfield 2003). Both these *Eucalyptus* species were, however, severely damaged by *Chr. cubensis*, which threatened forestry operations. Natural hybrids of *E. grandis* and *E. urophylla* were, however, noted to be free of disease. These trees were propagated vegetatively to produce *Chrysoporthe*-resistant trees, resulting in the development of a clonal *Eucalyptus* industry in Brazil and disease resistance screening programmes (Wingfield 2003).

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Infections on *Miconia* species by *Chr. cubensis* in Colombia are similar to those of *Eucalyptus* species. Infections are recognized by die-back, cankers on branches and trunks and often tree death (Rodas *et al.* 2005, Gryzenhout *et al.* 2006d). Cankers are also covered with perithecia and pycnidia, similar to those found on *Eucalyptus* species (Wingfield 2003). Similarly, on other hosts in the Melastomaceae and Lythraceae, symptoms include die-back and cankering, but usually not mortality (Rodas *et al*. 2005, Gryzenhout *et al*. 2006d).

2.4.2 *Chrysoporthe austroafricana*

Chrysoporthe austroafricana kills the cambium of infected *Eucalyptus* species. The resultant cankers girdle the trees and are characterized by cracking, swelling and shedding of the bark (FIG. 3a) resulting in "skirts" of dead bark at the bases of older trees (Wingfield *et al.* 1989). These cankers lead to trees collapsing in strong wind storms and also gradual death of trees (Wingfield 2003). Infection of younger trees results in stem girdling, wilting and rapid tree death (Wingfield *et al.* 1989, Conradie *et al.* 1990). In South Africa, cankers are normally limited to the bases of the trees (Wingfield *et al.* 1989, Gryzenhout *et al.* 2004), but further north in Malawi, and occasionally in South Africa, target-shaped cankers higher up on the stems of trees are also found (Nakabonge *et al.* 2006b). In South Africa, pycnidia are commonly present on *Eucalyptus* cankers but perithecia are seldom seen (Van Heerden and Wingfield 2001). However, in other African countries both pycnidia and perithecia are common (Nakabonge *et al.* 2006b).

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Chrysoporthe austroafricana is considered to be one of the most important pathogens of plantation-grown *Eucalyptus* species in South Africa (Wingfield *et al.* 1989, Conradie *et al.* 1992, Wingfield 2003). The discovery of *Chr. austroafricana* in South Africa was due to the death of a uniformly susceptible clone of *E. grandis,* known for its excellent timber qualities and which was initially planted on a large scale (Wingfield 2003). The use of resistant clones has been shown to be an effective means of managing *Chr. cubensis* in Brazil (Wingfield 2003). In South Africa, Conradie *et al*. (1990) suggested screening species, hybrids and clones for resistance to *Chr. austroafricana.* Thus, in 1992 a program was established by Van der Westhuizen *et al.* (1992) to select for tolerance of *E. grandis* clones to *Chr. austroafricana.* Due to the successful use of tolerant clones in South Africa, this disease is now present only in old seedling stands, or in trial plots (Wingfield and Roux 2002).

Symptoms of *Chr. austroafricana* infections on trees other than *Eucalyptus* species can be very difficult to detect. Infection on *Syzygium* species and *Tibouchina* species is characterized by dying branches and in some cases stem cankers, especially on *Tibouchina* species (Myburg *et al.* 2002a, Heath *et al*. 2006, Nakabonge *et al.* 2006b). Often, however, infection of native *Syzygium* trees is visible only on a single branch or around wounds (J. Roux pers. comm.). Tree death has not been observed for native African *Syzygium* species (Heath *et al*. 2006, Nakabonge *et al.* 2006b).

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2.4.3 *Chrysoporthe syzygiicola* and *Chrysoporthe zambiensis*

Chrysoporthe zambiensis has to date only been reported to infect *E. grandis* trees in a plantation in Zambia and infection is characterised by swollen cankers and cracking at the bases of trees. *Chrysoporthe syzygiicola* is associated with stem cankers and dying branches on *S. guineense* trees. Pathogenicity trails on *E. grandis* saplings showed that *Chr. syzygiicola* is more virulent than *Chr. austroafricana. Chrysoporthe zambiensis* was also shown to be more virulent than an isolate of *Chr. austroafricana* from Zambia, but less virulent than isolates from South Africa. Both of these pathogens, therefore, pose a potential threat to forestry based on *Eucalyptus* species in Zambia and other countries (Chungu *et al*. 2010).

2.5 Disease Management

Two main management strategies have been used with success against pathogens in the Cryphonectriaceae. In native systems, fungal viruses have been employed successfully (Heiniger and Rigling 1994), while in plantation systems the selection and breeding of tolerant planting stock has proven highly feasible (Van der Westhuizen *et al.* 1992). Both, however, rely on maintaining a relatively low population diversity of the pathogen to ensure success.

2.5.1 Tolerant clones

The most effective means to reduce the impact of Chrysoporthe canker of *Eucalyptus* species is by planting disease tolerant hybrids and clones of *Eucalyptus* species

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(Alfenas *et al.* 1983, Van der Westhuizen *et al.* 1992, Wingfield and Roux 2002). To identify disease tolerant clones, natural screening and artificial inoculation trials have been used (Alfenas *et al.* 1983, Wingfield *et al.* 1991, Van der Westhuizen *et al.* 1992, Van Heerden and Wingfield 2002, Van Heerden *et al.* 2005). During artificial inoculation trials, the level of callus formation after inoculation can reflect susceptibility or resistance, e.g. the greater the formation of callus the more tolerant the clone (Van Zyl and Wingfield 1999). Van Heerden and Wingfield (2002) also measured the lesion lengths formed by *Chr. austroafricana* to determine which clones are most resistant to infection. The larger the lesions formed, the less tolerant the clone (Van Heerden and Wingfield 2002).

It is important to note that clones that are resistant to one *Chrysoporthe* species might not necessarily be resistant to another *Chrysoporthe* species. Inoculation trails have suggested that *Chr. austroafricana* is more virulent than *Chr. cubensis* (Roux *et al.* 2003, Rodas *et al*. 2005) and a study by Vermeulen (2006), showed that a *Chr. austroafricana* isolate was more virulent than that of *Chr. deuterocubensis* on different pre-commercial *Eucalyptus* clones tested. These results were, however, based on inoculation trails done in greenhouses and larger trails conducted under field conditions, including a greater number of clones and isolates of *Chr. austroafricana* and *Chr. deuterocubensis*, are needed to confirm these results.

Artificial inoculations in South Africa with a single isolate of *Chr. austroafricana* (CMW 2113), shown to be the most virulent of an extensive collection of isolates (culture collection of the Forestry and Agricultural Biotechnology Institute), have been used to screen *Eucalyptus* clones for resistance (Van Heerden *et al*. 2005). Van

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Heerden *et al.* (2005), however, showed that this is not sufficient as there is a correlation between the clone and the isolate used under field conditions. In the future, using only one isolate might not be sufficient as a specific clone might be more resistant to one isolate than it is to another, giving a false impression that the clone is resistant to a specific species rather than only to a specific isolate (Van Heerden *et al.* 2005).

The use of molecular techniques to enhance polygalacturanase-inhibiting proteins (PGIP's) for control of pathogens in commercially important *Eucalyptus* species has been considered. Polygalacturanase proteins play a role in the nectrotrophic stage of pathogenesis and host plants produce PGIP’s in response to halt the progress of disease in an infected plant. This approach will, however, not be viable for control of *Chr. austroafricana*, as pathogenicity of this fungus is not altered or correlated to the production of Polygalacturanase proteins (Chimwamurombe 2002, Chimwamurombe *et al.* 2001, 2002).

2.5.2 Biological control using hypoviruses

Hypoviruses are viruses that can reduce the pathogenicity of infected fungal strains. Different hypoviruses, classified in the family *Hypoviridae* (Day *et al.* 1997, Nuss 1992, Hillman *et al.* 2000), have been shown to infect and cause hypovirulence in *C. parasitica* (Grente 1965, Grente and Sauret 1969, Day *et al.* 1977). These hypoviruses are dsRNA viruses, four of which have been shown to infect *C. parasitica.* These hypoviruses are referred to as *Cryphonectria hypovirus* (CHV) 1 (Shapira *et al*. 1991), CHV-2 (Hillman *et al*. 1994), CHV-3 (Smart *et al.* 1999) and CHV-4 (Hillman *et al.*

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2000). CHV-4 is not used as a biological control agent as it has no effect on the virulence of *C. parasitica* (Hillman *et al*. 2000). CHV-1 is normally used and has been shown to reduce the virulence of *C. parasitica*, reducing the development of cankers and allowing cankers to heal (Grente 1965, Day *et al.* 1977, Nuss 1992).

Cryphonectria parasitica infections on *Castanea dentata* are limited in Europe and Michigan (USA) due to the use of hypovirulence. Control is achieved by natural infection of *C. parasitica* with hypoviruses or by inoculation of existing cankers on trees with hypovirulent strains (Heiniger and Rigling 1994). Control has, however, failed almost completely in eastern North America (Heiniger and Rigling 1994, Milgroom and Cortesi 2004) as hypovirulence is not easily spread naturally. This is because hypoviruses are transferred via hyphal anastomosis from infected to uninfected fungal strains of the same VCG (Anagnostakis 1997, Nuss 1996). In populations with high levels of genetic diversity, such as those in the USA, viruses are not easily transferred. This is in contrast to those in Europe where diversity is low (Heiniger and Rigling 1994).

Cryphonectria parasitica is closely related to *Chrysoporthe* species (Myburg *et al.* 2004). Hypoviruses have thus been considered as possible biological control agents for both *Chr. austroafricana* and *Chr. cubensis* (then both still recognized as *Cryphonectria cubensis*) (Van Heerden *et al.* 2001) and, therefore, also possibly for *Chr. deuterocubensis* (previously synonymous to *Chr. cubensis*). An isolate of *Chr. austroafricana* (CMW2113) was infected with a full-length coding strand hypovirus transcript, CHV1-EP713, of *C. parasitica* to determine its potential usefulness as a biocontrol agent (Van Heerden *et al.* 2001). *Chrysoporthe cubensis* had also previously

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been successfully transfected by Chen *et al.* (1994, 1996), although these authors did not include any South African isolates (thus *Chr. austroafricana*) in their study. The hypovirus transcript, CHV1-EP713 of *C. parasitica,* induced hypovirulence in *Chr. austroafricana* and results showed that virulence of *Chr. austroafricana* was significantly reduced in vitro (Van Heerden *et al.* 2001).

Van Heerden *et al.* (2001) found that the barrier posed by VCGs to virus transmission in *C. parasitica* and *Chr. cubensis* (Van Zyl *et al.* 1999) does not occur in *Chr. austroafricana*. The low barrier of VCG's to virus transmission in *Chr. austroafricana* might make it possible to use hypovirulence as a control method for *Chr. austroafricana* (Van Heerden *et al.* 2001).Van Heerden *et al.* (2001) paired the *Chr. austroafricana* isolate CMW2113, transfected with CHV1-EP713 RNA, with different *Chr. austroafricana* VCG's. From a population of 23 VCG's, the hypovirus was transmitted to 14 *Chr. austroafricana* isolates residing in 11 different VCG's (Van Heerden *et al.* 2001).

Hypoviruses need to be present in the spores of the fungus to allow for effective natural spread and thus successful biological control (Van Heerden *et al.* 2001). Virus transmission, however, does not occur through conidia in *Chr. cubensis* (Chen *et al.* 1996) or *Chr. austroafricana* (Van Heerden *et al*. 2001). There are still some opportunities for biological control of Chrysoporthe canker using hypovirulence but substantially more research is needed before this can become a reality (Van Heerden *et al.* 2001).

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2.5.3 Biological control using mitoviruses

Members of the genus *Mitovirus* in the family *Narnaviridae* (Wickner *et al*. 2000) have been considered for possible biological control of *C. parasitica*. Mitoviruses are naked dsRNA viruses containing one open reading frame (Wickner *et al.* 2000), reducing the virulence of the fungus they infect. Mitoviruses have also been shown to occur in *Chr. austroafricana* (Van Heerden 2003).

The *C. parasitica* mitovirus can be transmitted through host populations via three pathways; anastomosis, asexual conidia and sexual ascospores (Polashock *et al.* 1997). This provides mitoviruses with a better chance to spread through a fungal population than hypoviruses (Van Heerden 2003). Isolates of *C. parasitica* naturally infected with a dsRNA Mitovirus showed altered culture morphology and reduction in virulence, suggesting hypovirulence. The hypovirulence induced by the Mitoviruses was, however, not as effective as that caused by viruses in the family *Hypoviridae* (Polashock *et al.* 1997), but the Mitovirus could still possibly be used as a biological control agent for *C. parasitica.*

Van Heerden (2003) screened isolates of *Chr. austroafricana* from *Eucalyptus* species in South Africa for the presence of mitoviruses. Two mitoviruses were found, namely *Cryphonectria cubensis mitovirus 1* or CcMV1, and CcMV2. Isolates containing mitoviruses and virus-free isolates were then used in pathogenicity tests to determine if mitoviruses had an effect on the virulence of *Chr. austroafricana* isolates. Isolates of *Chr. austroafricana* infected with mitoviruses were equally pathogenic as the virus-free

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isolates. Mitoviruses thus do not represent a suitable opportunity for biological control of *Chr. austroafricana* in South Africa (Van Heerden 2003).

3 *HOLOCRYPHIA*

3.1 TAXONOMY AND HISTORY OF *HOLOCRYPHIA*

For many years the *Eucalyptus* canker pathogen *Holocryphia eucalypti* (M. Venter & M. J. Wingf.) Gryzenh. & M.J. Wingf. was confused with the pin oak blight pathogen *Endothia gyrosa* (Schwein.: Fr.) Fr. (Shear *et al.* 1917, Stipes and Phipps 1971, Walker *et al.* 1985, Van der Westhuizen *et al*. 1993). This was because the size and shape of perithecia, asci, ascospores, conidiogenous cells and conidia of *H. eucalypti* are identical to those of *E. gyrosa* (Walker *et al*. 1985). Walker *et al*. (1985), however, noted morphological differences between specimens of *H. eucalypti* from *E. saligna* in Australia and specimens of *E. gyrosa* from the United States of America (USA). The stromata on the Australian herbarium specimens were slightly less developed and generally contained fewer perithecia than those from the USA specimens. For the Australian specimens, the entire perithecial body is seated in the bark, whereas in the USA specimens the perithecial body is seated in fungal tissue. These morphological differences led to further studies of *H. eucalypti*, and to its current taxonomic status.

Morphological differences between *E. gyrosa* and the *Eucalyptus* fungus known today as *H. eucalypti* have in recent years been supported by molecular and more in depth morphological studies (Walker *et al.* 1985, Venter *et al.* 2001, Venter *et al.* 2002). Polymerase chain reaction (PCR) based restriction fragment length polymorphisms (RFLP) (Venter *et al*. 2001,

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2002) and phylogenetic analyses based on sequence data for the ITS and β-tubulin gene regions (Venter *et al.* 2002, Myburg *et al.* 2004) showed that the fungus from *Eucalyptus* species in Australia and South Africa is distinct from that on oaks in the USA. Phylogenetic data and earlier observations by Walker *et al*. (1985) were also supported by differences in culture morphology, the production of pigments and the structures of *H. eucalypti* and *E. gyrosa* on host tissue (Gryzenhout *et al*. 2009). Cultures of *E. gyrosa* on malt extract agar (MEA) and potato dextrose agar (PDA) are fuscous black to buff to cinnamon with fluffy or a sectored appearance, whereas those of *H. eucalypti* are white, fluffy and sometimes have straw yellow patches (Gryzenhout *et al*. 2009). Isolates of *E. gyrosa* also often colour the growth medium purple (Gryzenhout *et al*. 2009). The stromatal structures of *E. gyrosa* are large, erumpent and mostly superficial, whereas those in *H. eucalypti* are semi-immersed and smaller (Venter *et al*. 2002, Gryzenhout *et al*. 2009). Based on these findings, Australian and South African isolates were described as the new species *Cryphonectria eucalypti* M. Venter & M.J. Wingf. (Venter *et al*. 2002).

Cryphonectria eucalypti was described in *Cryphonectria* as opposed to *Endothia*, the only two related genera with orange stromatic stromata at the time, based on the fact that its perithecia occur beneath the bark surface and they have a valsoid appearance near the periphery. This is typical morphology for species of *Cryphonectria* (Micales and Stipes 1987). Furthermore, *Cryphonectria* has semi-immersed stromata consisting primarily of prosenchyma and distinct ectostromatal and entostromatal discs (Micales and Stipes 1987). *Endothia* species are characterized by perithecia that generally occur above the bark surface in a diatrypoid configuration. The stromata are strongly developed, widely erumpent, sub-globose and consist primarily of pseudoparenchyma and continuous entostromata and ectostromata (Venter *et al*. 2002). There was, however, an irregularity in these observations,

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as the South African and Australian species had non-septate and cylindrical to allantoid ascospores (Venter *et al*. 2002), typical of the genus *Endothia* (Roane 1986, Shear *et al*. 1917), and thus different from the single septate ascospores of *Cryphonectria*. Despite these similarities with *Endothia, C. eucalypti* was described in *Cryphonectria* based on morphological similarities with *Cryphonectria* as well as phylogenetic analyses that grouped *C. eucalypti* closest to species in *Cryphonectria* (Venter *et al.* 2002, Myburg *et al.* 2004).

Most recently, phylogenetic studies including newly described genera and species have shown that *C. eucalypti* groups separately from other *Cryphonectria* species (Gryzenhout *et al.* 2006a) (FIG. 1). This resolved the problem that the *Eucalyptus* pathogen has single-celled ascospores as opposed to two-celled ascospores present in other *Cryphonectria* species. The new genus *Holocryphia* Gryzenh. & M.J. Wingf. was described to include *C. eucalypti,* now known as *H. eucalypti* (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. (Gryzenhout *et al.* 2006a) (FIG. 1). *Holocryphia* currently represents a monotypic genus (Gryzenhout *et al*. 2009).

3.2 DISTRIBUTION AND HOST RANGE

Holocryphia eucalypti has been reported from Australia (Walker *et al*. 1985, Old *et al.* 1986, Davison and Coates 1991), Uganda (Roux and Nakabonge 2009), South Africa (Van der Westhuizen *et al*. 1993) and most recently from New Zealand (Gryzenhout *et al*. 2010b). In South Africa, the pathogen occurs on several *Eucalyptus* species, namely *E. grandis* W. Hill: Maid., *E. nitens* (Deane & Maid.) Maid., *E. urophylla* R.T. Blake, and *Eucalyptus* hybrids of *E. grandis* with *E. camaldulensis* Dehnh. and *E. urophylla* respectively (Van der Westhuizen *et al.* 1993). In continental Australia (Walker *et al*. 1985, Davison and Coates 1991, Venter *et*

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al. 2002) and New Zealand (Gryzenhout *et al.* 2010b), *H. eucalypti* is known to infect numerous *Eucalyptus* species and in Australia it is also known to infect *T. urvilleana* (Heath *et al.* 2007). In Tasmania, it infects unspecified *Eucalyptus* species (Wardlaw 1999, Yuan and Mohammed 1997, 2000), while in Uganda it has only been reported on *E. grandis* (Roux and Nakabonge 2009).

3.3 ORIGIN OF *HOLOCRYPHIA EUCALYPTI*

Nakabonge *et al.* (2007) showed that a South African population of *H. eucalypti* has a very low gene and genotypic diversity, compared to that of an Australian population. *Holocryphia eucalypti* is, therefore, thought to be native to *Eucalyptus* species in Australia (TABLE 4) as the low diversity of the South African population is indicative of an introduced pathogen (Nakabonge *et al.* 2007). This supports previous observations that *H. eucalypti* occurs widely in native eucalypt forests in Australia (Walker *et al.* 1985, Old *et al.* 1986). More than 50% of the alleles observed in the South African population were unique, indicating that *H. eucalypti* was introduced into South Africa from a region other than those from which isolates could be obtained for the study by Nakabonge *et al.* (2007).

3.4 SYMPTOMS AND IMPACT

In South Africa and Uganda, cankers caused by *H. eucalypti* on eucalypts are generally recognized by cracked and slightly swollen areas of the bark (FIG. 3b) with additional die-back symptoms in South Africa (Van der Westhuizen *et al.* 1993, Wingfield and Roux 2002, Roux and Nakabonge 2009). These cankers are generally more superficial than cankers

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caused by *Chr. austroafricana* and they occur over the entire surface of the boles of trees (Van der Westhuizen *et al.* 1993). Severe cankers have, however, been reported where trees were under environmental stress such as those subjected to drought (Gryzenhout *et al.* 2003). Pathogenicity tests using several different *Eucalyptus* clones have shown that *H. eucalypti* could cause considerable damage (Gryzenhout *et al*. 2003).

Symptoms associated with *H. eucalypti* in Australia include bark cracks, cankers, die-back of coppice shoots and in severe cases tree death (Walker *et al.* 1985, Old *et al.* 1986, Wardlaw 1999). Cankers on trees in eastern Australia are covered with both asexual and sexual structures, whereas in Western Australia only the asexual state is present (Walker *et al.* 1985, Davison and Coates 1991). Surveys by Yuan and Mohammed (1997) also showed that the majority of trees infected by *H. eucalypti* appeared to be stressed.

Holocryphia eucalypti is considered to be an opportunistic pathogen in Australia, causing disease in situations where trees have been stressed by other factors, such as defoliating insects (Old *et al.* 1986, Old *et al.* 1990). It is also known as a wound associated pathogen (White and Kile 1993). In Australia, *H. eucalypti* is considered a more serious problem in *E. nitens* plantations, showing the potential to become a more important constraint to *Eucalyptus* plantations in future (Wardlaw 1999).

3.5 Management

As *H. eucalypti* is considered to be an opportunistic pathogen (Old *et al.* 1986, 1990, Yuan and Mohammed 2000), stress situations and marginal sites should be avoided for the planting of *Eucalyptus* species. Control measures could be difficult due to the unpredictability of *H.*

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eucalypti, as indicated by differences in pathogenicity among isolates as well as pathogen interaction with stress factors and host (Yuan and Mohammed 2000, Gryzenhout *et al.* 2003). For instance, isolates that might not be virulent in one particular environment could be highly pathogenic elsewhere (Gryzenhout *et al*. 2003). Gryzenhout *et al.* (2003) showed that different clones tested exhibit varying levels of tolerance to *H. eucalypti*. Breeding and selection of tolerant clones have been shown to be highly effective with other canker pathogens such as *Chr. austroafricana* (Wingfield and Roux 2002). It might thus be possible to develop a selection and breeding program to select trees tolerant to *H. eucalypti* (Gryzenhout *et al.* 2003).

4 CELOPORTHE

4.1 TAXONOMY AND HISTORY OF CELOPORTHE

The genus *Celoporthe* was first described from South Africa in 2006 and currently includes five known species, namely *Celoporthe dispersa* Nakab., Gryzenh., Jol. Roux & M.J. Wingf. from South Africa (Nakabonge *et al*. 2006a), *Cel. eucalypti* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou, *Cel. guangdongensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou and *Cel. syzygii* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from China (Chen *et al.* 2011) and *Cel. indonesiensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from Indonesia (Chen *et al*. 2011). *Celoporthe dispersa* forms three distinct sub-clades representing isolates from native South African *Heteropyxis canescens* Oliv., *S. cordatum* and non-native *T. granulosa* trees. These three sub-clades could represent three distinct species but have not been described formally. This was because no morphological differences could be found among

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the sub-clades and limited specimens were available for morphological comparison (Nakabonge *et al.* 2006a).

Celoporthe is morphologically similar to *Chrysoporthe* when observed macroscopically in the field, as species of both genera have black conidiomata of similar shape (Nakabonge *et al.* 2006a). There are, however, morphological differences that support the distinct nature of these genera. *Celoporthe* can be differentiated from *Chrysoporthe* based on the occurrence of short perithecial necks that are not always distinctly fuscous black, conidia that are oblong to cylindrical or ovoid, the presence of pseudoparenchymatous stromatic tissue in the conidiomatal bases and short necks, and cultures that are white with grey to chestnut-coloured patches (Nakabonge *et al.* 2006a, Gryzenhout *et al.* 2009). On the contrary, *Chrysoporthe* species have long perithecial necks that are black, conidia that are uniform and oblong, the stromatic tissue of the conidiomatal base is of *textura globulosa* and the neck of *textura porrecta*, and cultures are white to cinnamon-coloured (Nakabonge *et al*. 2006a, Gryzenhout *et al.* 2009). DNA sequence comparisons also clearly distinguish between *Celoporthe* and *Chrysoporthe* (Nakabonge *et al*. 2006a).

Celoporthe species can be distinguished from each other based on characteristics of the asci, conidiomata, conidiophores, conidia, paraphyses and growth in culture. *Celoporthe dispersa* has an optimal growth temperature of 25°C whereas the species from Asia have an optimum growth temperature of 30°C. *Celoporthe syzygii* is morphologically distinguishable from *Cel. dispersa* by having longer asci and conidia that are shorter than those of other *Celoporthe* species (Chen *et al.* 2011). *Celoporthe eucalypti* is morphologically similar to *Cel. guangdongensis*, but can be distinguished by having shorter paraphyses than *Cel. guangdongensis* and longer paraphyses than *Cel. dispersa*, *Cel. indonesiensis* and *Cel.*

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syzygii. *Celoporthe guangdongensis* has longer paraphyses than the other *Celoporthe* species while *Cel. indonesiensis* has longer conidia and shorter paraphyses than the other Asian *Celoporthe* species, and lastly it has shorter conidia and longer paraphyses than *Cel. dispersa* (Nakabonge *et al*. 2006a, Chen *et al*. 2011).

4.2 DISTRIBUTION AND HOST RANGE

Celoporthe dispersa has only been reported from South Africa, occurring on native *H. canescens* (Heteropyxidaceae, Myrtales), *S. cordatum* (Myrtaceae, Myrtales) and non-native *Tibouchina granulosa* (Melastomataceae, Mytales) (Nakabonge *et al.* 2006a). Artificial inoculations have shown that the fungus is able to form lesions on *Eucalyptus* species even though it has not been found occurring naturally on *Eucalyptus* species (Nakabonge *et al.* 2006a). *Celoporthe indonesiensis* is known from *S. aromaticum* in Indonesia (Myburg *et al.* 2003, Chen *et al*. 2011) and the Chinese species, *Cel. eucalypti* and *Cel. guangdongensis* are known from non-native *Eucalyptus* species, while *Cel. syzygii* is known from non-native *S. cumini* (L.) Skeels in China (Chen *et al*. 2011).

It is possible that *Cel. dispersa, Cel.* e*ucalypti, Cel. guangdongensis, Cel. indonesiensis* and *Cel. syzygii* have a wider distribution than is currently known and that these fungi were overlooked due to their morphological similarities to *Chrysoporthe* species (Nakabonge *et al.* 2006a, Chen *et al*. 2010). A morphologically similar fungus has, for instance, been found on clove in Zanzibar (Myburg *et al.* 2003), although its identity could not be confirmed. *Celoporthe indonesiensis* present on clove in Indonesia has also largely been overlooked due to its co-occurrence with *Chr. cubensis* on clove and the morphological similarities between

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these genera (Myburg *et al.* 2003, Nakabonge *et al.* 2006a). Clearly, further surveys are required to better understand the host range and taxonomy of these fungi.

4.3 ORIGIN OF *CELOPORTHE*

Nakabonge *et al.* (2006a) hypothesized that *Cel. dispersa* is native to southern Africa. They based this hypothesis on the presence of *Cel. dispersa* on native hosts and the fact that as far it is known, it occurs only from southern Africa (Nakabonge *et al*. 2006a). Chen *et al*. (2011), hypothesized that *Celoporthe* species that were identified from non-native trees in Asia could have originated from native Myrtales in southern or Southeast Asia based on the species diversity of *Celoporthe* in the area and the ability of these species to infect *Syzygium* species native to Asia. No molecular or other studies have, however, been done to support these views and additional surveys are needed to expand the host and geographic ranges of *Celoporthe* species to obtain a better indication of their possible origin.

4.4 SYMPTOMS AND IMPACT

Celoporthe dispersa is associated with cankers on native *Het. canescens* and *S. cordatum,* and non-native *T. granulosa* trees, as well as branch die-back on *Het. canescens* and *T. granulosa* (FIG. 3c). Cankers on *Het. canescens* from which the fungus was first collected were severe with some trees dying. It was, however, not shown that *Cel. dispersa* is responsible for the death of these trees, as pathogenicity tests could not be performed on *Het. canescens* (Nakabonge *et al.* 2006a). Pathogenicity tests have shown that *Cel. dispersa* is pathogenic to *E. grandis* and it could thus become important on commercially grown *Eucalyptus* species in South Africa. It is also possible that it has previously been overlooked

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on *Eucalyptus* species because of its morphological similarity to *Chrysoporthe* species (Nakabonge *et al*. 2006a).

Symptoms associated with infection of *Eucalyptus* and *Syzygium* trees by *Cel*. *eucalypti, Cel. guangdongensis* and *Cel*. *syzygii* include cracking of the bark and the formation of girdling stem cankers. It is not clear whether these symptoms are associated with *Celoporthe* species only or if symptoms are caused by *Chr. deuterocubensis* or a combination of both as they co-occur on these trees (Chen *et al*. 2010, 2011). Pathogenicity tests by Chen *et al*. (2011), however, showed that Chinese *Celoporthe* species from *Syzygium* and *Eucalyptus* are pathogenic to various *Eucalyptus* genotypes and *S. cumini* trees and that *Eucalyptus* genotypes differ in susceptibility to infection by *Celoporthe* species. This implies that it will be possible to select *Eucalyptus* planting stock with tolerance to infection by the Chinese *Celoporthe* species.

5 *AURIFILUM*

5.1 TAXONOMY AND HISTORY OF *AURIFILUM*

Aurifilum is a recently described genus in the Cryphonectriaceae represented by a single species, *A. marmelostoma* Begoude, Gryzenh. & Jol. Roux, which was isolated from *Terminalia* species in Cameroon (Begoude *et al.* 2010). The specimens collected from Cameroon closely resembled specimens of Cryphonectriaceae obtained from *T. ivorensis* in Ghana and Kenya (Gryzenhout *et al.* 2005c, Begoude *et al.* 2010). Whether these specimens belong to the same genus could, however, not be determined as long sterile cells, or paraphyses, present in Cameroonian specimens were not observed in specimens from Ghana and Kenya, and molecular data is not available (Begoude *et al.* 2010).

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Aurifilum is morphologically similar to several taxa in the Cryphonectriaceae, such as *Amphilogia* and *Rostraureum* (Gryzenhout *et al*. 2005a, 2005c, 2009, Begoude *et al.* 2010). There is, however, several unique morphological characters that distinguish it from other genera in the family. *Aurifilum marmelostoma* has a darkened ostiolar opening at the apex of the conidiomata that has not been observed in other Cryphonectriaceae, and the conidiomata are broadly convex and thus wider than those of *Amphilogia* and *Rostraureum* (Begoude *et al*. 2010).

5.2 Distribution and host range

Aurifilum marmelostoma has to date only been positively identified from cankers on native *T. ivorensis* and non-native *T. mantaly* in Cameroon. As the identity of the specimens collected from *T. ivorensis* in Ghana and Kenya is not known (Begoude *et al*. 2009) and *Terminalia* species are wide spread in Africa, it is possible that the distribution of *A. marmelostoma* could be more extensive in Africa. *Terminalia* species belong to the Myrtaceae and it is also possible that *A. marmelostoma* has a broader host range in the Myrtaceae, as has been seen with other Cryphonectriaceae such as *Cel. dispersa* (Nakabonge *et al.* 2006a).

5.3 Origin of *Aurifilum*

It is currently not possible to determine the origin of *A. marmelostoma* as no molecular data is available and only a limited number of isolates have been collected. Similar to the situation of *Cel. dispersa* and *Chr. austroafricana*, the presence of *A. marmelostoma* on a native host could, however, indicate that it is native to Africa. This view is further supported by the fact that *A. marmelostoma* has only been collected from Africa.

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5.4 SYMPTOMS AND IMPACT

Native *Terminalia* species play an important role in the forestry industry of western Africa, and *A. marmelostoma* could pose a threat as it has been shown to be pathogenic to non-native *T. mantaly* during nursery inoculations. Disease symptoms caused by *A. marmelostoma* on *T. mantaly* consists of cankers covered with fruiting structures on the trunks of dead trees and senescing branches. On *T. ivorensis*, *A. marmelostoma* caused cankers on tree trunks, cracked bark containing yellow to orange fruiting structures, and necrotic cambium. The impact of *A. marmelostoma* on *T. ivorensis* is, however, not clear as pathogenicity tests have not yet been conducted on these trees (Begoude *et al*. 2010).

6 ECOLOGY AND MODE OF INFECTION OF THE CRYPHONECTRIACEAE

Species in the genera *Aurifilum, Celoporthe, Chrysoporthe* and *Holocryphia* are all canker causing pathogens. A canker is defined as a necrotic, often sunken lesion on a stem, main root, branch or twig of a tree (Sinclair and Lyon 2005, Agrios 2005). These cankers form due to the disintegration of tissues outside the xylem (Sinclair and Lyon 2005). Fungi that are associated with cankers generally require wounds for infection or may infect through stromata and lenticels (Hodges *et al.* 1979, Michailides 1991, Agrios 2005).

Limited information is available on the disease development and ecology of species in *Chrysoporthe*, *Holocryphia* and especially *Aurifilum* and *Celoporthe*. This is important because understanding the life cycle of a pathogen typically promotes opportunities to control it. Extensive studies have been done on *C. parasitica*, which is also known to be one of the world's most serious tree canker pathogens (Anagnostakis 1987). The infection mode is most

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likely the same, or very similar, to that of *Aurifilum, Celoporthe, Chrysoporthe* and *Holocryphia* species and this knowledge could be applied to learn more about these genera, while keeping in mind that the climate and environment in which these fungi occur are different from where *C. parasitica* occurs.

Similar to *Aurifilum, Celoporthe, Chrysoporthe* and *Holocryphia,* conidia ooze out of *C. parasitica* conidiomata as long orange tendrils during cool, moist weather. Conidia of *C. parasitica* are spread by birds, mammals (Heald and Studhalter 1914, Scharf and DePalma 1981) and flying or crawling insects (Craighead 1912, Studhalter and Ruggles 1915, Russion *et al*. 1984) or splashing rain (Elliston 1981, Griffin 1986, Newhouse 1990). Conidia can also survive in soil for extended periods of time, from where they might be dispersed by wind-born dust (Heald and Gardner 1914). The ascospores are forcefully shot into the air from perithecia (Mickleborough 1909, Elliston 1981, Newhouse 1990) and spread by wind over long distances (Anderson 1914, Heald *et al.* 1915, Shear *et al.* 1917, Griffin 1986). Ascospores of *Chr. cubensis* are also known to be dispersed by wind (Bruner 1917), similar to those of *C. parasitica*.

For *C. parasitica,* spore dispersal and infection occur throughout the year during mild weather (White and Kile 1993). *Cryphonectria parasitica* is known to infect through wounds (White and Kile 1993) Similarly, *H. eucalypti* has also been reported to be associated with wounds (White and Kile 1993) and the same could be true for *Chrysoporthe* species. Once *C. parasitica* infects the tree it grows into the inner bark and cambium (Agrios 2005, Sinclair and Lyon 2005) and over-winters in colonized bark and lesions and later produce perithecia and pycnidia to continue its life cycle.

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Research is needed to better understand the development and ecology of species in *Aurifilum, Celoporthe, Chrysoporthe*, and *Holocryphia.* If these fungi do, for instance, need wounds to infect their hosts, preventing wounding of trees during forestry practices might lower the incidence of disease. Similarly, if the role of insects in the life cycles of these fungi is better understood, it might also provide more effective management strategies. It is also not known whether these fungi can exist as endophytes in healthy plant tissue, in which case disease could be caused when trees are under stress, such as off site planting or drought. This would also greatly impact on the movement of germplasm, as endophytic infections are more difficult to recognize, allowing for the spread of pathogens over long distances through trade.

7 CONCLUSIONS

The Cryphonectriaceae represents a recently described family and includes thirteen teleomorph and two associated anamorph genera. Four of these genera occur on the African continent, namely *Aurifilum, Celoporthe, Chrysoporthe* and *Holocryphia.* The host and geographical distributions of these genera are still incompletely known in Africa and extended surveys are needed to expand our knowledge of their distribution.

Chrysoporthe species cause serious canker diseases on *Eucalyptus* species world-wide. Five species of *Chrysoporthe* occur on the African continent namely *Chr. austroafricana*, *Chr. cubensis, Chr. deuterocubensis, Chr. syzygiicola* and *Chr. zambiensis*. *Chrysoporthe austroafricana, Chr. cubensis* and *Chr. zambiensis* have been associated with disease of eucalypts, while *Chr. syzygiicola* shows the potential to be an important pathogen of eucalypts in greenhouse inoculations.

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Both *Cel. dispersa* and *H. eucalypti* have the potential to cause serious disease on *Eucalyptus* species currently planted in South Africa. Even though symptoms associated with *H. eucalypti* observed in South Africa and Uganda include only superficial cankers, it is considered a serious threat in Australia. *Celoporthe dispersa* has not been found on *Eucalyptus* species, but has been shown to be pathogenic to *Eucalyptus* during artificial inoculation trails.

Aurifilum marmelostoma has been shown to be pathogenic to non-native *T. mantaly*, and poses a potential threat to forestry based on *Terminalia* species in West Africa. It is currently only known from Cameroon on native *T. ivorensis* and non-native *T. mantaly*. The origin of *Aurifilum* is still unknown, but its presence on native *T. ivorensis* and absence from other countries indicates it might originate from Africa.

Further surveys are needed to expand our knowledge on the ecology, distribution and other possible hosts of the Cryphonectriaceae in Africa. Further research is also needed to improve our understanding of the disease cycle of pathogens residing in these genera. It is also likely that new species and genera of the Cryphonectriaceae will be discovered in Africa, similar to newly found taxa in other areas of the world. The origin of *Chrysoporthe*, *Celoporthe* and *Aurifilum* species is still unclear and population-level studies, including expanded collections of isolates, will be required to fully understand the centre of origin of these species.

The aim of this thesis is to provide studies that will lead to a better understanding of the origin, distribution and host range of the Cryphonectriaceae in Africa. This will be achieved through collections from native Myrtales in Southern Africa as well as by determining the population diversity of the most common species collected. A second aim will be to expand

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previous studies on the *Eucalyptus* canker pathogen *Chr. austroafricana* by comparing population data from a Namibian population with that of populations in neighboring Malawi Mozambique, South Africa and Zambia.

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TABLE I. Differences between *Chrysoporthe* species (Gryzenhout *et al*. 2004, 2005b, 2006d, 2009, Chungu *et al*. 2010, Van der Merwe *et al.* 2010).

	Chr. austroafricana	***Chr. cubensis***	***Chr. deuterocubensis***	***Chr. hodgesiana***	***Chr. inopina***	***Chr. doradensis***	***Chr. zambiensis***	***Chr. syzygiicola***
Optimal growth temperature	30°C	30°C	30°C	25°C	25°C	30°C	30°C	30°C
Teleomorph	Present	Present	Present	Not known	Present	Present	Not known	Not known
Ascospores	With tapered apices. Ascospore width less than 3µm	With rounded apices Ascospore width less than 3µm	With tapered apices. Ascospore up to 3µm in width	n/a	With rounded apices Ascospore width more than 3µm	With tapered apices Ascospore width less than 3µm	n/a	n/a
Ascus size	Asci longer than 28 µm	Asci shorter than 28 µm	Asci up to 28 µm long	n/a	Asci longer than 28 µm	Asci shorter than 28 µm	n/a	n/a
Conidial morphology	Oblong to ovoid	Oblong to ovoid	Oblong	Oblong to ovoid	Oblong	Variably in shape, cylindrical to oblong to ovoid, and occasionally allantoids	Oblong	Oblong
Spore mass	Bright luteous (yellow)	Bright luteous (yellow)	Bright luteous (yellow)	Bright luteous (yellow).	Bright luteous (yellow)	Pale luteous (Cream)	Pale luteous (Cream)	Bright luteous (yellow)
Conidia	Hyaline, non-septate, oblong and shorter than 4.5 µm	Hyaline, non-septate, oblong and shorter than 5 µm	Hyaline, non-septate, oblong and up to 5 µm long	Hyaline, non-septate, oblong and shorter than 5.5 µm	Hyaline, non-septate, oblong and shorter than 4 µm	Hyaline, non-septate, oblong to ovoid and shorter than 6.5 µm	Hyaline, non-septate, oblong and shorter than 4 µm	Hyaline, non-septate, oblong to ovoid and shorter than 4 µm

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TABLE II. Distribution and host ranges of *Chrysoporthe* species in Africa.

Pathogen	Host	Distribution	Reference
Chr. austroafricana	*Eucalyptus* spp.	Malawi	Nakabonge *et al.* 2006b
		Mozambique	Nakabonge *et al.* 2006b
		South Africa	Wingfield *et al.* 1989
		Zambia	Nakabonge *et al.* 2006b
	Syzygium cordatum	Malawi	Nakabonge *et al.* 2006b
		Mozambique	Nakabonge *et al.* 2006b
		South Africa	Heath *et al*. 2006
		Zambia	Nakabonge *et al.* 2006b
	S. guineense	South Africa	Heath *et al.* 2006
	Tibouchina granulosa	South Africa	Myburg *et al.* 2002a
Chr. cubensis	*Eucalyptus* spp.	Cameroon	Gibson 1981
		Democratic Republic of Congo	Hodges *et al.* 1986, Micales *et al.* 1987
		Ghana	Roux and Apetorgbor 2009
		Republic of Congo	Roux *et al.* 2003
Chr. deuterocubensis	*Eucalyptus* spp.	Kenya	Nakabonge *et al.* 2006b
		Malawi	Nakabonge *et al.* 2006b
		Mozambique	Nakabonge *et al.* 2006b
	S. aromaticum	Zanzibar, Tanzania	Nutman and Roberts 1952
Chr. syzygiicola	*S. guineense*	Zambia	Chungu *et al*. 2010
Chr. zambiensis	*E. grandis*	Zambia	Chungu *et al.* 2010

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TABLE III. VCG data obtained from population studies of *Chrysoporthe cubensis, Chrysoporthe deuterocubensis* and *Chrysoporthe austroafricana.*

	Host	Country	No. of isolates	Ĝ	Reference
Chr. austroafricana	*Eucalyptus*	South Africa	34	0.4%	Heath 2004
	Eucalyptus	South Africa	100	0.095%	Van Heerden 2003
	Syzygium	South Africa	62	26%	Heath 2004
	Syzygium	Namibia	18	36.7%	Vermeulen 2006
	Tibouchina	South Africa	37	22%	Heath 2004
Chr. cubensis	*Eucalyptus*	Colombia	31	37%	Van der Merwe 2000
	Eucalyptus	Venezuela	52	46%	Van der Merwe 2000
Chr. deuterocubensis	*Eucalyptus*	Indonesia	29	28.4%	Van der Merwe 2000

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TABLE IV. Microsatellite/simple sequence repeats data showing the population diversity of *Chrysoporthe austroafricana, Chr. deuterocubensis* and *Holocryphia eucalypti*.

	Host	Country	No. of isolates	Ĝ	Reference
Chr. austroafricana	*Eucalyptus*	South Africa	100	45%	Heath 2004
	Eucalyptus	South Africa	34	11.5%	Van der Merwe 2000
	Syzygium	South Africa	38	5%	Heath 2004
	Tibouchina	South Africa	64	33%	Heath 2004
Chr. deuterocubensis	*Eucalyptus*	Kenya	10	17.2%	Nakabonge 2006b
	Eucalyptus	Malawi	51	5.4%	Nakabonge 2006b
H. eucalypti	*Corymbia calophylla*	Western Australia	30	63.2%	Nakabonge *et al*. 2007
	Eucalyptus	South Africa	72	3.6%	Nakabonge *et al*. 2007
	Eucalyptus	Western Australia	23	55.7%	Nakabonge *et al*. 2007
	Eucalyptus	Eastern Australia	20	43.7%	Nakabonge *et al*. 2007

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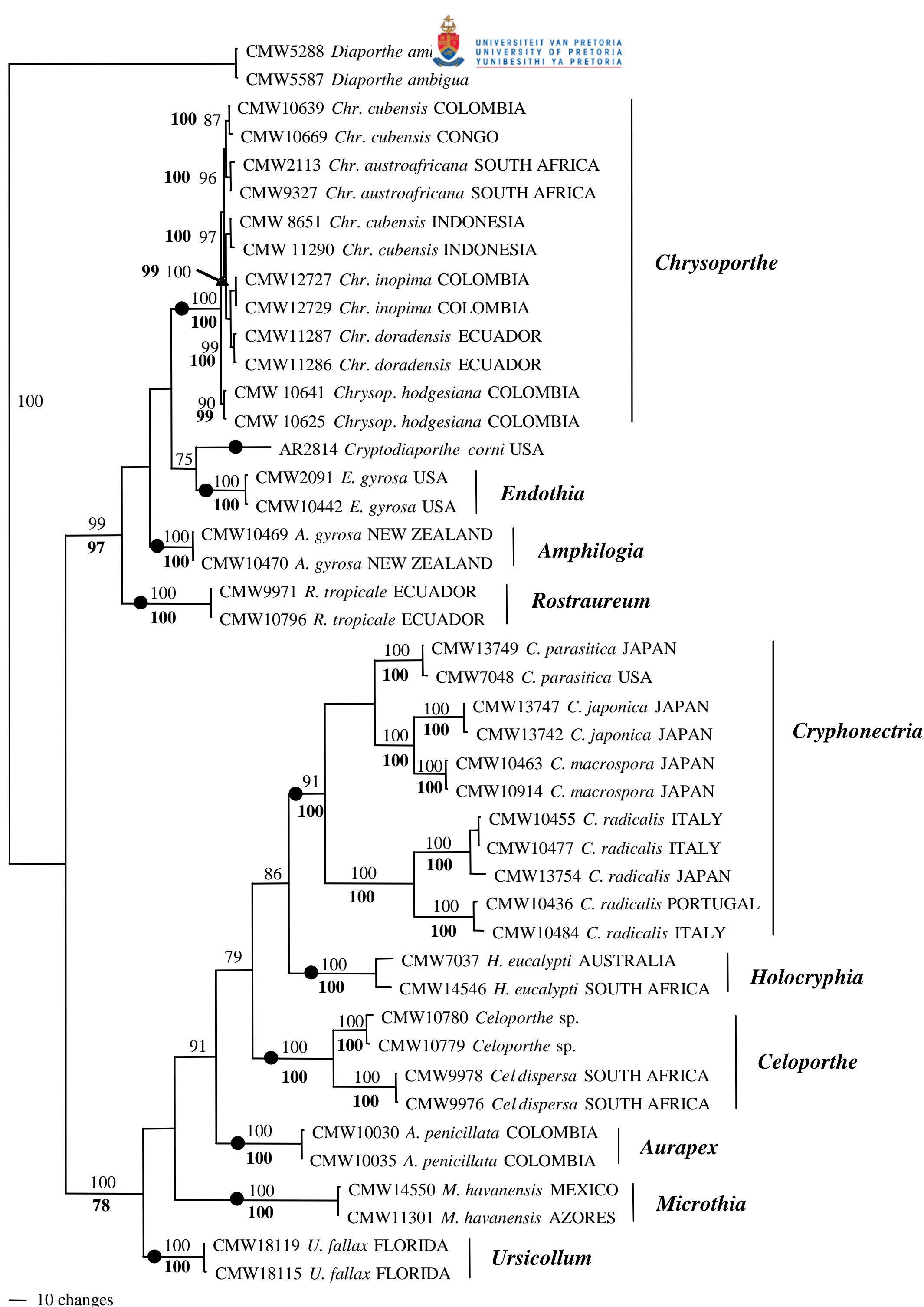

FIGURE 1. Phylogenetic tree indicating the various generic groupings in Cryphonectriaceae and obtained with parsimony from a combined DNA sequence dataset of the ITS1, 5.8S rRNA gene and ITS2 regions of the ribosomal operon, and β-tubulin genes (Gryzenhout *et al*. 2009). Bootstrap confidence levels (> 70 %) and posterior probabilities (in bold typeface) are indicated on the branches, and those branches representing genera are marked with a dot. The outgroup taxon used in the tree is *Diaporthe ambigua*. This tree does not include the most recent genera and species, namely *Aurifilum marmelostoma*, *Cel. eucalypti, Cel. guangdongensis, Cel. indonesiensis*, *Cel. syzygii* and *Chr. deuterocubensis*, as no published trees are available that include all these species (Begoude *et al*. 2010, Chen *et al.* 2011, Van der Merwe *et al*. 2011).

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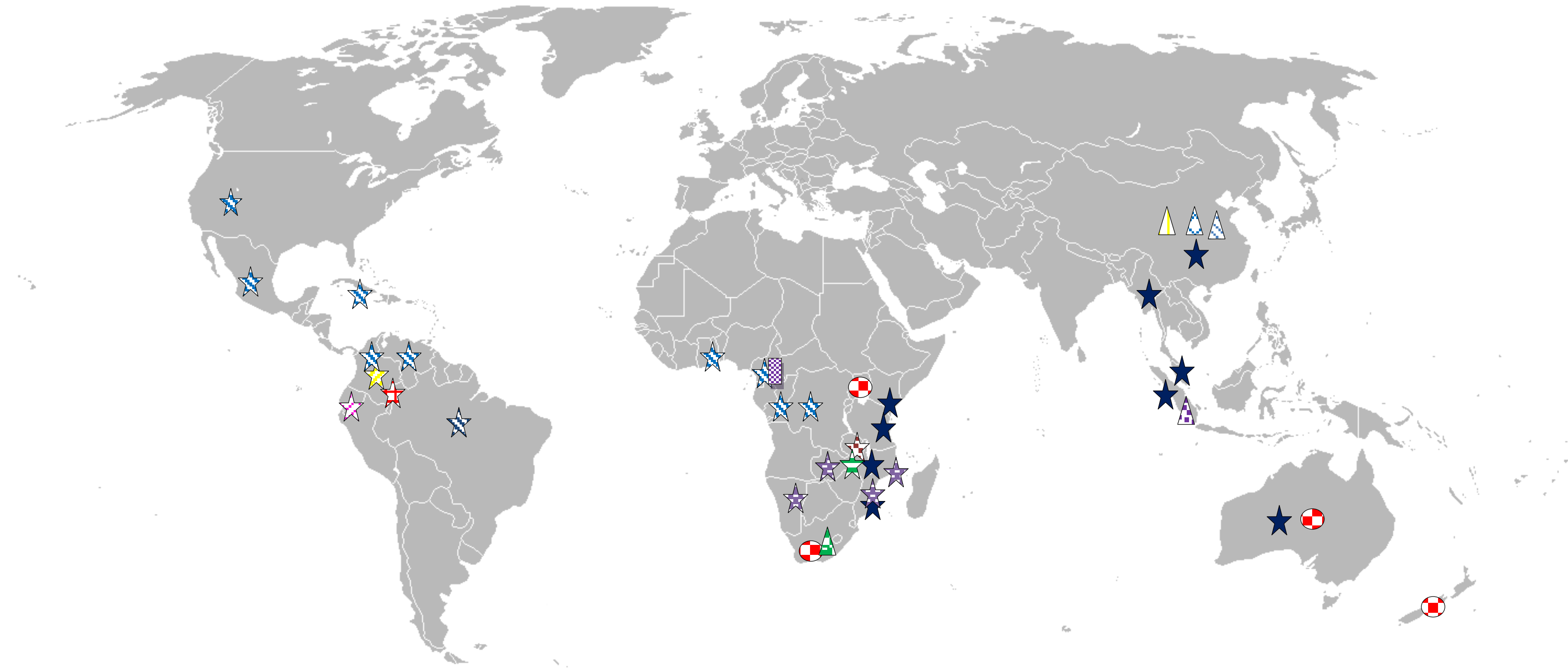

Chrysoporthe cubensis	*Chrysoporthe deuterocubensis*	*Chrysoporthe austroafricana*
Chrysoporthe hodgesiana	*Chrysoporthe inopina*	*Chrysoporthe doradensis*
Chrysoporthe zambiensis	*Chrysoporthe syzygiicola*	*Holocryphia eucalypti*
Celoporthe dispersa	*Celeporthe eucalypti*	*Celeporthe guandongensis*
Celeporthe indonesiensis	*Celeporthe syzygii*	*Aurifilum marmelostoma*

FIGURE 2. World map showing location of *Chrysoporthe* species, *Celoporthe* species, *Holocryphia eucalypti* and *Aurifilum marmelostoma.*

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FIGURE 3. (a) *Chrysoporthe austroafricana* infection on a *Eucalyptus grandis* in South Africa showing typical cracked and flaring bark at the base of the tree. (Figure provided by Prof. J. Roux, FABI). (b) *Holocryphia eucalypti* on a *Eucalyptus* species (Figure provided by Prof. J. Roux, FABI). (c) *Celoporthe dispersa* on *Heteropyxis canescens* (Nakabonge *et al.* 2006a).

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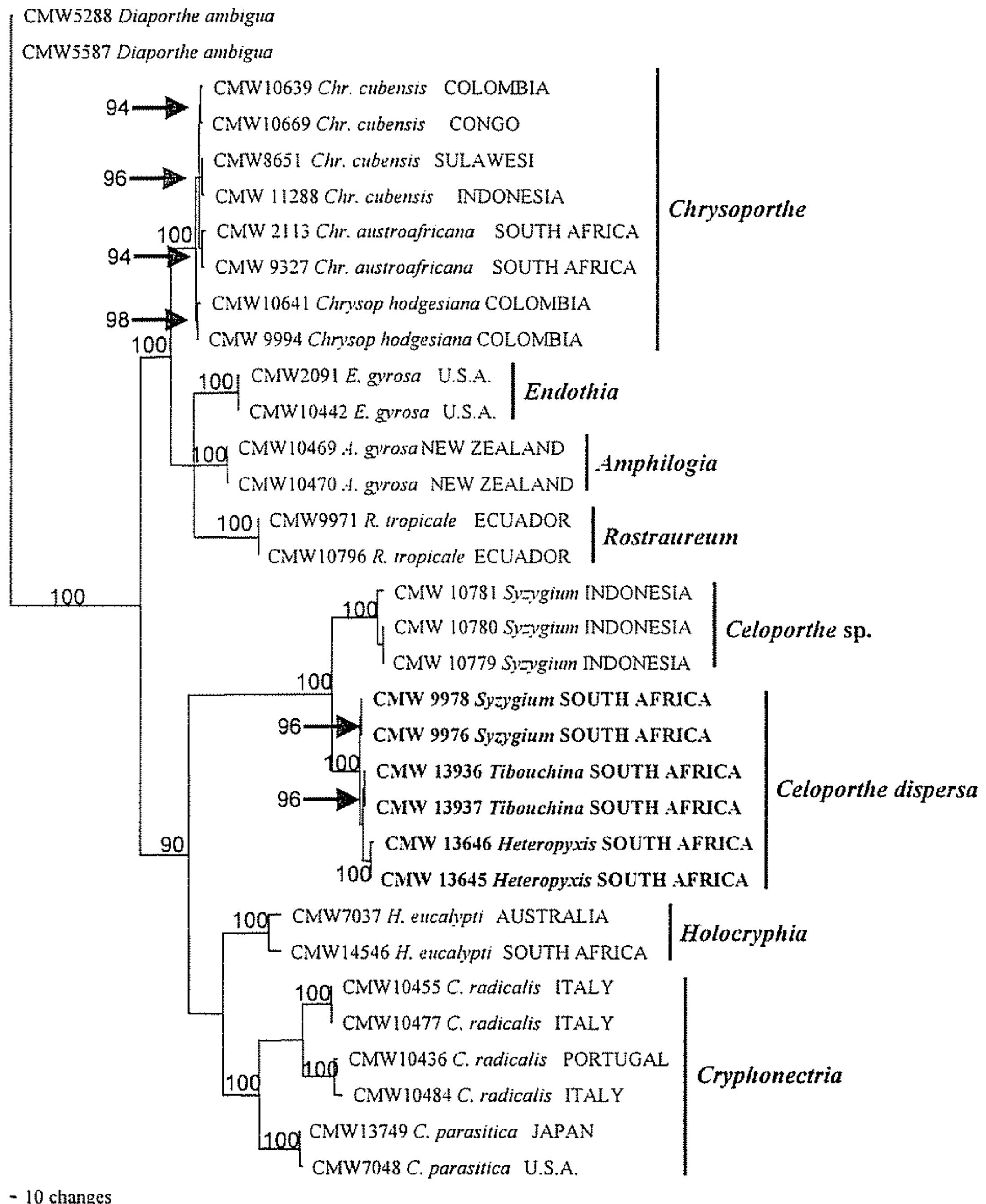

FIGURE 4. A phylogenetic tree showing the groupings within *Celoporthe dispersa* and generated from combined sequence data of the ITS ribosomal DNA and β-tubulin gene sequence data (Nakabonge *et al.* 2006a) and generated from heuristic searches performed on the combined data set (tree length of 1725, CI of 0.737 and RI of 0.922). Bootstrap values (1000 replicates) above 50 % are indicated on the branches. Isolates sequenced in this study are in bold. *Diaporthe ambigua* sequences were used as outgroup.

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Chapter 2

New records of the Cryphonectriaceae from southern Africa including *Latruncellus aurorae* gen. sp. nov.

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ABSTRACT

The Cryphonectriaceae accommodates some of the world's most important tree pathogens, including four genera known from native and introduced Myrtales in Africa. Surveys in the past three years in southern Africa have led to the discovery of cankers with fruiting structures resembling those of the Cryphonectriaceae on trees in the Myrtales in Namibia, South Africa, Swaziland and Zambia. These fungi were identified using morphological characteristics and DNA sequence data. For the first time, we report *Chrysoporthe austroafricana* from Namibia and on *Syzygium guineense* and *Holocryphia eucalypti* in Swaziland on a *Eucalyptus grandis* clone. The host and geographic ranges of *Celoporthe dispersa* are expanded to include *S. legatti* in South Africa and *S. guineense* in Zambia. In addition, a monotypic genus, *Latruncellus aurorae* gen. sp. nov., is described from *Galpinia transvaalica* (Lythraceae, Myrtales) in Swaziland. The present, and other recent studies, clearly emphasize the limited understanding of the diversity and distribution of fungi in the Cryphonectriaceae in Africa.

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1 INTRODUCTION

The Cryphonectriaceae includes fungi previously treated broadly in the *Cryphonectria-Endothia* complex (Gryzenhout *et al*. 2009) and currently accommodates twelve genera (Gryzenhout *et al*. 2009, 2010a, Begoude *et al*. 2010, Chungu *et al*. 2010). The family includes important tree pathogens such as *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf., *Chr. cubensis* (Bruner) Gryzenh. & M.J. Wingf., *Cryphonectria parasitica* (Murrill) M.E. Barr and *Endothia gyrosa* (Schwein.: Fr.) Fr. (Gryzenhout *et al*. 2009). Of these, the best known is *C. parasitica,* the causal agent of chestnut blight that devastated native populations of chestnut trees [*Castanea dentata* (Marsh.) Borkh. and *Castanea sativa* Mill. (Fagaceae)] in North America and Europe (Anagnostakis 1987, Heiniger and Rigling 1994). *Chrysoporthe austroafricana* and *Chr. cubensis* are important canker pathogens of plantation-grown *Eucalyptus* trees, resulting in considerable economic losses to forestry enterprises in Africa and South America (Hodges *et al*. 1979, Wingfield 2003). In Africa, four genera of the Cryphonectriaceae are known, including *Aurifilum* (Begoude *et al.* 2010), *Celoporthe* (Nakabonge *et al.* 2006a), *Chrysoporthe* (Gryzenhout *et al*. 2004) and *Holocryphia* (Gryzenhout *et al.* 2006a), all of which include species that are tree pathogens.

Species of *Chrysoporthe* have a wide distribution in tropical and sub-tropical areas of the world (Gryzenhout *et al*. 2009). Five species are known in Africa, including *Chr. cubensis*, *Chr. austroafricana* (Gryzenhout *et al.* 2009), *Chr. deuterocubensis* Gryzenh. &M.J. Wingf. (Van der Merwe et al. 2011), *Chr. syzygiicola* Chungu, Gryzenh. & Jol. Roux and *Chr. zambiensis* Chungu, Gryzenh. & Jol. Roux (Chungu *et al*. 2010). *Chrysoporthe cubensis* is known from Cameroon (Gibson 1981), the Democratic Republic of Congo (Hodges *et al.* 1986) and Ghana (Roux and Apetorgbor 2009), while *Chr. deuterocubensis* is known from

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Kenya, Malawi, Mozambique (Nakabonge *et al*. 2006b) and Republic of Congo (Roux *et al*. 2003). *Chrysoporthe austroafricana* has been found only in eastern and southern African countries including Malawi, Mozambique, South Africa and Zambia (Nakabonge *et al.* 2006b), while *Chr. syzygiicola* and *Chr. zambiensis* was recently discovered as new species from Zambia (Chungu *et al*. 2010). Hosts of these species include native and introduced trees in the Myrtales, namely *Eucalyptus* spp. (Myrtaceae), *Syzygium* spp. (Myrtaceae) and *Tibouchina granulosa* Cogn. ex Britton (Melastomataceae) (Gryzenhout *et al*. 2009, Chungu *et al*. 2010). *Chrysoporthe cubensis* and *Chr. deuterocubensis* are considered to have been introduced respectively into Africa from South America and southeastern Asia (Wingfield 203, Nakabonge *et al*. 2006b, 2007, Van der Merwe *et al*. 2011). In contrast, the widespread presence of *Chr. austroafricana* on native hosts and their absence from other continents suggests that *Chr. austroafricana* is an African fungus (Heath *et al*. 2006, Nakabonge *et al.* 2006b).

Holocryphia eucalypti (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. is a stress-associated pathogen of *Eucalyptus* spp. and is of relatively minor importance in South Africa (Van der Westhuizen *et al*. 1993, Gryzenhout *et al*. 2003). In Australia, however, it has been reported as a pathogen of some concern, resulting in death of trees (Yuan and Mohammed 2000). Its only other known host is *Tibouchina urvilleana* (DC.) Cogn. (Melastomataceae) (Heath *et al*. 2007) and it evidently has undergone a host shift (Slippers *et al*. 2005) to this tree. Although previously known only from Australia and South Africa, it was recently reported from *E. grandis* Hill. ex Maid. in Uganda (Roux and Nakabonge 2009) and *Eucalyptus* spp. in New Zealand (Gryzenhout *et al.* 2010b). *Holocryphia eucalypti* is considered to have been introduced into South Africa, because the South African population

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has little genetic diversity compared to that of Australian populations (Nakabonge *et al*. 2007).

Aurifilum marmelostoma Begoude, Gryzenh. & Jol. Roux (Begoude *et al*. 2010) and *Celoporthe dispersa* Nakab., Gryzenh., Jol. Roux & M.J. Wingf. (Nakabonge *et al*. 2006a) are two recently described monotypic genera in the Cryphonectriaceae that occur in Africa. *Celoporthe dispersa* has been reported only from South Africa, where it infects native *Heteropyxis canescens* Oliv. (Heteropyxidaceae, Myrtales) and *S. cordatum* trees, and non-native *T. granulosa* trees (Nakabonge *et al.* 2006a). *Celoporthe dispersa* is thought to be native to southern Africa (Nakabonge *et al*. 2006a). *Aurifilum marmelostoma* was described from cankers on native *Terminalia ivorensis* (Combretaceae, Myrtales) A. Chev. and non-native *T. mantaly* H. Perrier in Cameroon (Begoude *et al.* 2010).

The economic importance of fungi in the Cryphonectriaceae, their ecological impact, seemingly wide host ranges in the Myrtales and their ability to cross-infect trees (Gryzenhout *et al*. 2009), make these fungi important to plantation industries as well as to biodiversity and conservation programs in Africa. The aim of this study was to expand on surveys for the Cryphonectriaceae on Myrtales in southern Africa and to improve the base of knowledge regarding their species diversity and distribution in the sub-region.

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2 MATERIALS AND METHODS

2.1 FUNGAL ISOLATES

Native and non-native trees belonging to the Myrtales, especially trees from which members of the Cryphonectriaceae have been reported (Gryzenhout *et al*. 2009), were inspected for stem cankers and fruiting bodies resembling those of the Cryphonectriaceae. Surveys were conducted during 2006–2008 in four southern African countries (Namibia, South Africa, Swaziland and Zambia) where scientific collaborations have been established to promote the study of plantation forestry diseases. Isolations were made from pieces of bark bearing fruiting structures resembling those of the Cryphonectriaceae with techniques described by Gryzenhout *et al*. (2009). Resulting cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (TABLE I) and duplicates of isolates representing a new genus were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (TABLE I). Herbarium specimens of fruiting structures on bark representing the new genus were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa (TABLE I).

2.2 IDENTIFICATION

2.2.1 DNA sequence comparisons

DNA was extracted from the mycelium of cultures grown on 2% Malt Extract Agar (MEA). Mycelium was scraped from the surfaces of the MEA plates and freeze-dried. Freeze-dried mycelium was ground to a fine powder with 2 mm-diameter metal beads in a Retsch cell

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disrupter (Retsch Gmbh, Germany) after which the protocol described by Möller *et al.* (1992) was followed for DNA extraction. DNA concentrations were determined on a NanoDrop 3.1.0 (ND-1000 uv/Vis spectrometer, NanoDrop Technologies, Wilmington, DE, USA).

Sequences for two gene regions previously shown as appropriate to identify species in the Cryphonectriaceae (Gryzenhout *et al.* 2009), were amplified with the Polymerase Chain Reaction (PCR). The internal transcribed spacer (ITS) regions (ITS1, ITS2), and the conserved 5.8S gene of the ribosomal RNA operon were amplified with the primer pair ITS1 and ITS4 (White *et al*. 1990). The β-tubulin 1 and β-tubulin 2 regions of the β-tubulin gene (BT) were amplified respectively with primer pairs BT1a, BT1b and BT2a, BT2b (Glass and Donaldson 1995). A third gene region, a part of the Large Subunit (LSU) nuclear ribosomal DNA, shown to be appropriate to differentiate between genera in the Cryphonectriaceae, was also used. The LSU region was amplified with primer pairs LR0R and LR7 (Vilgalys and Hester 1990, Rehner and Samuels 1994). Reactions for ITS and BT were performed in a total volume of 25 µl composed of 40-60 ng DNA template, 0.25 µl (0.5 µM) of each primer, 2.5 µl dNTP's (0.2 mM of each dNTP), 0.25 µl (0.5 U) Super-therm polymerase Taq, 2.5 µl (10 X) dilution buffer, 2.5 µl $MgCl_2$ (Southern Cross Biotechnology, Cape Town, South Africa) and sterile distilled water (12.25µl). LSU reactions were performed in a total volume of 50 µl as described by Castleburg *et al*. (2002). PCR reactions were carried out on a thermal cycler (Master cycle® Perkin Elmer Corporation, Massachusetts, USA). The program included an initial denaturation step at 94 °C for 3 min, followed by 40 amplification cycles consisting of 30 sec at 94 °C, 45 sec of annealing at 55°C for ITS, BT 1 and LSU, 65 °C for BT 2 and 1 min at 72 °C, followed by a final step of 4 min at 72 °C. PCR products were viewed with UV light on 1 % agarose gels containing ethidium bromide. PCR products were cleaned with 0.06

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g/ml Sephadex G-50 (SIGMA-ALDRICH, Amersham Biosciences Limited, Sweden) according to the manufacturer's instructions.

DNA fragments were sequenced with the same primer pairs used in the PCR amplification reactions. Sequencing reactions were performed as described by Begoude *et al.* (2010). The products were sequenced in both directions with the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism™ 3100 DNA sequencer (Applied Biosystems). Sequences obtained were viewed and edited with VECTOR NTI ADVANCED 10 (Vector NTI® Software, 1600 Faraday Avenue, Carlsbad California, 92008 USA). Sequences were aligned with those published for fungi in the Cryphonectriaceae (Gryzenhout *et al.* 2009, 2010a, 2010b, Begoude *et al.* 2010), with the web interface (http://timpani.genome.adjp/%7Emafft/server/) of the alignment program MAFFT Version 5.8 (Katoh *et al.* 2002). The alignments were deposited at TREEBASE (www.treebase.org) (Submission no 10804).

Phylogenetic analyses were performed with the software package Phylogenetic Analysis Using Parsimony (PAUP) Version 4.01b10 (Swofford 2000). Phylogenetic analyses were done for each gene region separately with maximum parsimony (MP) (heuristic search with 100 random sequence additions). A 1000 replicate partition homogeneity test (PHT) was performed to examine the null hypotheses that the BT and ITS gene datasets were homologous and could be combined for further analyses (Farris *et al.* 1994). This was done after the exclusion of uninformative sites, with a heuristic search with 100 random sequence additions, tree bisection-reconnection (TBR) branch swapping and MAXTREES set to 5000 to allow completion of analysis. A 1000 bootstrap replication was performed to determine the support of branches for the most parsimonious tree for datasets representing each gene region

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(Felsenstein 1985). *Diaporthe ambigua* Nitschke was used as the outgroup taxon (Gryzenhout *et al*. 2009). The same analyses were repeated (for PHT MAXTREES set to auto increase) for the conserved BT exon data supplemented with DNA sequences of the LSU (Gryzenhout *et al.* 2009) in order to ascertain placement of genera within the greater Cryphonectriaceae. The LSU and BT exon dataset was midpoint rooted.

Additional phylogenetic analyses were conducted based on maximum likelihood (ML) and Bayesian analyses for the combined data of the ITS and BT gene regions and for the LSU and BT exon gene regions respectively. The correct model for the datasets was identified with JMODELTEST version 0.0.1 (Posada 2008). The TrN+I+G model (Tamura and Nei 1993) was shown to be appropriate for the ITS and BT dataset and the TIM2+I+G model (Posada 2008) for the LSU and BT exon dataset. Maximum likelihood analyses were performed with PHYML V3. (Guindon and Gascuel 2003). A 1000 replicate bootstrap analysis was done to assess the confidence levels of the branch nodes in the phylogenetic trees. The Bayesian analyses were performed on the two datasets with the Markov chain Monte Carlo (MCMC) algorithm in the program MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). The number of generations was set to 3 000 000 and sample frequency set to 100. Burn–in was at 8000 for the ITS and BT dataset and 6000 for the LSU and BT exon dataset.

2.2.2 Morphology

Fruiting structures representing an apparently undescribed species in the Cryphonectriaceae on original bark material of *Galpinia transvaalica* N.E. Brown, were cut from the bark under a dissection microscope and its morphology studied with methods described by Gryzenhout *et al.* (2009). Fifty measurements of asci, ascospores, conidia, conidiophores and

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conidiogenous cells were taken for the holotype specimen (PREM 60348) and 20 measurements from other specimens (PREM 60349, PREM 60347). Measurements are presented as (min–)(average-S.D.) – (average+S.D.)(–max) mm. Digital images were captured with a HRc Axiocam digital camera, and measurements were computed using AXIOVISION 3.1 software (Carl Zeiss Ltd., Germany). Characteristics of fruiting bodies were compared with those of other genera and species in the Cryphonectriaceae (Gryzenhout *et al*. 2009, 2010a, Begoude *et al.* 2010, Chungu *et al*. 2010).

Cultural characteristics of the apparently undescribed species were determined on two representative isolates (CMW28276, CMW30633). Discs were taken from the edges of actively growing cultures on 2% MEA and transferred to the centers of 90 mm Petri dishes containing 2% MEA. Five plates per isolate were placed in the dark in incubators set at temperatures ranging from 15 to 35 °C at five degree intervals. Two measurements of diameter, perpendicular to each other, were taken of each culture until the fastest growing culture had covered the surface of the plate. Growth data were analyzed in Microsoft Excel to determine the optimum temperature for growth. Colony colours were described with the charts of Rayner (1970).

2.3 PATHOGENICITY TEST

Pathogenicity experiments were conducted with two isolates (CMW28276, CMW30633) from *G. transvaalica* on ten *G. transvaalica* trees and ten two-year-old plants of the *Eucalyptus* (species) clone ZG14, which had been shown to be highly susceptible to infection by both *Cel. dispersa* and *Chr. austroafricana* (Van Heerden and Wingfield 2001, Nakabonge *et al.* 2006a). Trees were maintained at 25 °C in a greenhouse under natural

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day/night conditions for two weeks to acclimatize, after which they were inoculated. For inoculations the bark was removed from trees with a 5 mm cork-borer, and a plug of mycelium was placed into the wound with the mycelium facing the cambium. Wounds were sealed with a strip of Parafilm to prevent desiccation and cross contamination.

Six weeks after inoculation the bark associated with the inoculation sites was removed and the lengths of lesions on the cambium were measured. Pieces of necrotic tissue were transferred to MEA to re-isolate the inoculated fungus. Variation in lesion lengths was assessed in Excel, using one-way analysis of variance (ANOVA) with a 95% level of significance.

3 RESULTS

3.1 FUNGAL ISOLATES

Fruiting structures resembling those of the Cryphonectriaceae were found on *G. transvaalica* (FIG. 1d-f) and an *E. grandis* clone in Swaziland, *S. guineense* and *S. legatti* in South Africa, *S. guineense* in the Caprivi region of Namibia (Katima Mulilo and Popa Falls) and *S. guineense* in Zambia (FIG. 1d-f). In Katima Mulilo infections were found on the roots (FIG. 1d-e) of *S. guineense* trees that are submerged by the Zambezi River at least two months each year, as well as on branch stubs and stem cankers on these trees. Infections on *S. guineense* in Zambia and on *S. guineense* and *S. legatti* in South Africa were relatively inconspicuous and were found on branch cankers and branch stubs. Infections on *G. transvaalica* were associated with obvious branch and stem cankers (FIG. 1b). Fruiting structures were present both on the surface and beneath the bark of *G. transvaalica* trees, unlike those on the

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Syzygium and *Eucalyptus* trees that were found only on the surfaces of cankers or on necrotic areas. No fruiting structures were observed on the stems of native *Dissotis* sp. (Melastomataceae), a woody shrub related to *Tibouchina*, growing in riverine areas of South Africa and Zambia, or on *E. camaldulensis* Dehnh. trees growing in woodlots in the Caprivi.

3.2 IDENTIFICATION

3.2.1 DNA sequence comparisons

DNA for two Cryphonectriaceae isolates from Namibia, six from South Africa, six from Swaziland, and two from Zambia were amplified and sequenced. The datasets for the ITS and BT gene regions consisted of 61 taxa each and for the LSU and BT exon gene region of 27 taxa each. Alignment lengths of different gene regions were 547–928 bp (TABLE II).

Results of the PHT showed that datasets for the ITS and BT gene regions were homologous (P = 0.045) and thus could be combined (Cummings *et al.* 1995). Results of the PHT for the more conserved analysis based on LSU and BT exon data showed that these datasets also could be combined (P = 0.02) (Cummings *et al*. 1995). This was supported by the trees for each gene region that essentially had the same topology and support for the various genera.

Based on the ITS and BT datasets individually (data not shown), as well as the combined dataset, isolates collected in this study grouped in four genera of the Cryphonectriaceae, namely *Celoporthe, Chrysoporthe*, *Holocryphia* and a group closely related to *Aurifilum*. Results of the parsimony analyses correlated with results from Maximum likelihood and Bayesian analyses (FIG. 2). These trees displayed the clades observed previously for

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Chrysoporthe spp. in Africa with *Chr. austroafricana* (ML, Bootstrap confidence level (BS)/ Posterior probabilities (BPP), 100/90), *Chr. zambiensis* (94/100), *Chr. syzygiicola* (88/100), *Chr. cubensis* from South America (93/98), and *Chr. cubensis* from Southeast Asia (100/100) grouping separately (Gryzenhout *et al*. 2004, Nakabonge *et al*. 2006b). In both trees, isolates from *S. guineense* in Namibia and Soutpansberg grouped with the *Chr. austroafricana* isolates and separately from other *Chrysoporthe* spp.

The three sub-clades 3–5 previously observed for *Cel. dispersa* isolates from different hosts (Nakabonge *et al*. 2006a) were also observed in this study (FIG. 2). These included isolates from *T. granulosa* from Durban in the KwaZulu-Natal Province (96/97), *S. guineense* from Tzaneen in the Limpopo Province (100/100), and *H. canescens* from Buffelskloof Nature Reserve in the Mpumalanga Province (100/100). Isolates from *S. cordatum* and *S. legatti* in the Soutpansberg (94/100) and Zambia (97/100) formed two additional, distinct sub-clades (Sub-clades 4, 5) within *Celoporthe* (FIG. 2). Isolates from the *E. grandis* clone in Swaziland grouped with *H. eucalypti* (100/100).

Based on ITS and BT datasets isolates from *G. transvaalica* grouped close to *A. marmelostoma*. The isolates from *G. transvaalica* however formed a distinct sub-clade (100/100), suggesting a second species in this recently described genus or on the other hand a distinct genus (FIG. 2). The same groupings for genera in the Cryphonectriaceae were observed in the more conserved dataset containing BT sequence data, excluding the introns, and combined with the LSU data suggesting a previously undescribed taxon among isolates collected in this study (FIG. 3). Although there was only 1 bp difference based on LSU data between *A. marmelostoma* and the fungus from *G. transvaalica*, BT exon data showed a clear

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and highly supported distinction (MP, BS=97) with 12 fixed base pair differences between the two taxa.

3.2.2 Morphology

Fruiting structures found on *G. transvaalica* resembled those of members of the Cryphonectriaceae, with distinct orange stromatic tissue (Gryzenhout *et al*. 2009) that turns purple in the presence of 3% KOH and yellow in lactic acid (Castlebury *et al*. 2002). Teleomorph structures were scarce, which is consistent with observations for other Cryphonectriaceae such as *Cel. dispersa* (Nakabonge *et al.* 2006a). Teleomorph structures were orange, semi-emerged with pseudoparenchymatous to prosenchymatous tissue, similar to genera in the Cryphonectriaceae (Gryzenhout *et al.* 2009). Ascospores resembled those of other Cryphonectriaceae ranging from fusoid to ellipsoid with a single septum (Gryzenhout *et al*. 2009). Conidiomatal structures were more abundant on specimens examined, occurring both on the surface and on the underside of bark peeling loose from the cambium. Conidiomata were orange, semi-immersed, with uni-to multilocular conidial locules, and conidia were cylindrical with sizes similar to those of species in the Cryphonectriaceae (Gryzenhout *et al*. 2009).

The fungus on *G. transvaalica* was similar to genera and species in the Cryphonectriaceae that have uniformly orange sexual and asexual states (TABLE III), in contrast to genera that have black fruiting bodies or those that are partially black (Gryzenhout *et al*. 2009). It nonetheless could be distinguished from the taxa with uniformly orange sexual and asexual states (TABLE III) based on a number of characteristics. The fungus has long paraphyses (<90 µm) between conidiophores (FIG. 4i), similar to those in conidiomata of *Aurifilum* (Begoude

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et al. 2010), *Holocryphia* and *Microthia* (Gryzenhout *et al.* 2009). The anamorph structures of this fungus are conical, thus similar to those of *Aurifilum*, *Amphilogia* and *Rostraureum*. However it could, be distinguished from all of these genera due to the presence of distinct conidiomatal necks (FIG. 4e), and more important the unique, constricted shape of the neck, which makes fruiting structures similar to the shape of pawns chess (FIG. 4e).

Sequence data showed that the unknown fungus from *G. transvaalica* is most closely related to the monotypic genus *Aurifilum.* Several important differences, however, separate the unknown fungus from *A. marmelostoma*. The most important of these is the morphology of the conidiomata. Conidiomata of *A. marmelostoma* are broadly convex without necks, while those of the unidentified fungus are conical with distinctly constricted, fattened necks (FIG. 4e). The ostioles in the unidentified fungus are not obviously darkened as they are in those of conidiomata of *A. marmelostoma* (TABLE III). The unidentified fungus has fusoid to oval ascospores that are constricted at median to off-median septa (FIG. 4d), whereas those of *A. marmelostoma* are fusoid to ellipsoid with a medium septum without constrictions. The conidiophores of *A. marmelostoma* are cylindrical with inflated bases, while those of the unidentified fungus are subulate. In addition, conidia of *A. marmelostoma* are cylindrical to allantoid (FIG. 4h), while those of the undescribed fungus are only cylindrical (Begoude *et al.* 2010). Lastly, the unidentified fungus grows optimally at 25 °C in contrast to *A. marmelostoma* that has an optimum at 30 °C.

3.2.3 Taxonomy

Comparisons of DNA sequence data of the unknown fungus from *G. transvaalica* with genera residing in the Cryphonectriaceae showed that it is related most closely to *A.*

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marmelostoma, possibly representing a second species in this genus (FIGS. 2-3). However, anamorph structures of the unknown fungus are significantly different from those of *A. marmelostoma*, or from any other genus in the Cryphonectriaceae. For example, anamorph structures of the unknown fungus are uniformly orange with constricted, fattened necks with a shape similar to that of a chess pawn while those of *A. marmelostoma* do not have necks and have blackened ostiolar openings. These differences are consistent with the magnitude of morphological differences used in the past to described new genera in the Cryphonectriaceae (Gryzenhout *et al*. 2009). Other differences probably represent those at the level of species, such as those in optimal growth temperature and shape of ascospores, conidiophores and conidia. We thus propose that the fungus from *G. transvaalica* most appropriately belongs in a new genus described as follows:

Latruncellus M. Verm., Gryzenh. & Jol. Roux. gen. nov.

Mycobank MB518285

Etymology: The name refers to the shape of the conidiomata which have inflated heads that resemble chess pawns. The Romans did not know Chess, but played a similar battle game. The smallest and least valuable piece in the game was a latrunculus, meaning "robber, brigand, bandit", and this piece was apparently the analogue of a Chess pawn; -cellus refers to the small size of the fruiting structures.

Latin: *Ascostromata* subimmersa pulvinata aurantiaca. *Asci* fusoidei vel ellipsoidei. *Ascosporae* uniseptatae hyalinae. *Conidiomata* conica collis distinctis complanatis, latrunculiformia subimmersa unilocularia vel multilocularia, marginibus pseudoparenchymatis intus prosenchymata, aurantiaca, collo textura globulosa. *Paraphyses* adsunt. *Conidia* minuta hyalina non septata, pro guttulis cirrhisque aurantiacis exsudatis.

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Ascostromata: semi-immersed, pulvinate, upper region eustromatic, lower region pseudostromatic, edges pseudoparenchymatous with prosenchymatous tissue inside, orange. *Perithecia* embedded in host tissue at base of stroma, *textura porrecta*, perithecial bases hyaline when young. *Asci* fusoid to ellipsoid. *Ascospores* fusoid to oval, single septate, septum median to off-median, hyaline.

Conidiomata: conical with distinct, constricted necks, pawn-shaped, semi-immersed, uni- to multilocular, convoluted, part of ascomata as conidial locules or as solitary structures, edges of conidiomata pseudoparenchymatous and inside prosenchymatous, orange, necks with *textura globulosa*. *Conidiophores* subulate to flask-shaped, aseptate and septate with attenuated apex, branched and unbranched, hyaline. *Paraphyses* present. *Conidia* minute, hyaline, cylindrical, aseptate, exuded as orange droplets and tendrils.

Latruncellus aurorae M. Verm., Gryzenh. & Jol. Roux. sp. nov.

Mycobank MB518286 FIG.4

Etymology: aurorae (Latin) "of the dawn" referring to the orange color of the fruiting structures.

Latin: subimmersa globosa aurantiaca 131-343 µm supra corticem crescentia, 337-827 µm diametro. *Perithecia* in contextu hospitis in basi stromatis immersa, e textura porrecta composita, bases peritheciorum paucae (1-3), juventute hyalinae, collis 45-119 µm longis 58-134 µm latis, solum perjuvenia visa. *Asci* fusoidei vel ellipsoidei (31.5-)34.5-46.5(-49.5) x (5-)6.5-10(-12) µm. *Ascosporae* fusoideae vel ovales, uniseptatae, septo medio vel prope medium, sporae saepe in septo constrictae, apice obtusae, hyalinae, (8.5-)9.5-11(-12.5) x (2.5-)3-3.5-(4.5) µm.

Conidiomata: conica latrunculiformes subimmersa uniloculares vel multiloculares, solitaria vel loculi conidiales pro partes ascomatum, margines conidiomatum pseudoparenchymatae,

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intus prosenchymata, aurantiaca, supra corticem 51-199 µm alta, 63-129 µm lata, collis distinctiscomplanatis e textura globulosa formatis, 34-84 µm longis, maxime 52-98 µm latis. Conidiophorae subulatae vel ampulliformes, septatae vel non, apice attenuato, ramosae vel non, hyalinae, (7-)8.5-15.5(-22) µm x (1.5-)1.5-2(-2.5)µm. Paraphyses cylindricae apice obtusae, non septatae (22-)24.5-56.5(-76.5) x (1.5-)1.5-2.5(-3) µm. Conidia hyalina cylindrica non septata, 3-4(-4.5) x 1-1.5(-2) µm, pro guttulis cirrhisque aurantiacis exsudatis.

Ascostromata: semi-immersed, pulvinate, upper region eustromatic, lower region pseudostromatic, edges pseudoparenchymatous with prosenchyma inside, orange, extended 131-343 µm above the bark, 337-827 µm in diam. *Perithecia* embedded in host tissue at base of stroma, *textura porrecta*, perithecial bases few (1-3), perithecial bases hyaline when immature, necks extending above level of ascostroma, 45-119 µm long and 58-134 µm wide. *Asci* fusoid to ellipsoid, (31.5-)34.5-46.5(-49.5) x (5-)6.5-10(-12) µm. *Ascospores* fusoid to oval, single septate, septum median to off-median, often constricted at septum, apex obtuse, hyaline, (8.5-)9.5-11(-12.5) x (2.5-)3-3.5-(4.5) µm.

Conidiomata: conical, pawn-shaped, semi immersed, uni- to multilocular, convoluted, part of ascomata as conidial locules or as solitary structures, edges of conidiomata pseudoparenchymatous and inside prosenchymatous, orange, 51-199 µm high above the bark and 63-129 µm wide. Necks distinct, constricted, of *textura globulosa*, 34-84 µm long, 52-98 µm wide at widest point. *Conidiophores* subulate to flask-shaped, aseptate to septate, with attenuated apex, branched and unbranched, hyaline, (7-)8.5-15.5(-22) µm x (1.5-)1.5-2(-2.5)µm. *Paraphyses* cylindrical with obtuse apex, aseptate, (22-)24.5-56.5(-76.5) x (1.5-)1.5-2.5(-3) µm. *Conidia* hyaline, cylindrical, aseptate, exuded as orange droplets and tendrils, 3-4(-4.5) x 1-1.5(-2) µm.

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Culture characteristics: On MEA luteous to pale luteous, covering a 90 mm plate in seven days at an optimum temperature of 25 C. Minimal growth was observed at 15 C and no growth was observed at 35 C.

Specimens examined: SWAZILAND, LUBOMBO: close to the South Africa and Mozambique border. Isolated from bark of *G. transvaalica,* 2008, J. Roux, (HOLOTYPE, PREM 60348, ex-type culture CMW28276/CBS 125526, PARATYPES, PREM 60349, PREM 60347, living cultures CMW28274/CBS124904, CMW30633/CBS124905).

The following key, adapted from Begoude *et al.* (2010) and based on anamorph and teleomorph characteristics, are presented to aid in morphological identifications.

1a. Orange conidiomata……………………………………………………………………2

1b. Conidiomata uniformly black to black with orange necks……………………………11

2a. Conidiomata pulvinate to globose, ascospores septate or aseptate……………………3

2b. Conidiomata conical or rostrate or pyriform or convex, with or without a neck, ascospores septate……………………………………………………………………7

3a. Ascospores septate……………………………………………………………………4

3b. Ascospores aseptate……………………………………………………………………6

4a. Semi-immersed, usually no paraphyses…………………………………………………5

4b. Semi-immersed to superficial, paraphyses present…………………………***Microthia***

5a. Conidiomata usually larger than 350 µm, uni- to multilocular, ascospores with median septum……………………………………………………………***Cryphonectria***

5b. Conidiomata usually smaller than 350 µm in diam, unilocular, ascospores with median to sub-median septum…………………………………………***Cryptometrion***

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6a. Stromata strongly developed, large, erumpent, mostly superficial, numerous conidial locules, no paraphyses…………………………..…………………….…....***Endothia***

6b. Stromata small to medium, semi-immersed, few conidial locules or one convoluted locule, paraphyses present……..…………………………………….....***Holocryphia***

7a. Conidiomata with necks, ascospores single septate……..………..……………..…….8

7b. Conidiomata without necks, ascospores single to multiple septate…..........................10

8a. Conidiomata with prominent, delimited neck ……………………………......…..9

8b. Conidiomata with neck continuous with base, rostrate, white sheath of tissue surrounding perithecial necks when sectioned longitudinally…...……........***Rostraureum***

9a. Conidiomata rostrate to pyriform with large base, neck attenuated or not, teleomorph still unknown.………….……..………...***Ursicollum***

9b. Conidiomata conical with constricted, fattened neck, shape like a Chess pawn………………………..……………………………………..……***Latruncellus***

10a. Conidiomata conical, uniformly orange, ascospores 1 to 3-septate.............***Amphilogia***

10b. Conidiomata convex, with blackened ostiolar openings, ascospores 1-septate……………………………………………………………………….***Aurifilum***

11a. Conidiomata uniformly black when mature……………………..…………………...11

11b. Conidiomata black with orange neck, teleomorph still unknown ....***Aurapex***

12a. Conidiomata pulvinate to pyriform with attenuated neck, base tissue of *textura globulosa* when sectioned longitudinally, perithecial necks long and covered with dark tissue, emerging from orange stroma.……………………...……….***Chrysoporthe***

12b. Conidiomata pulvinate or conical, occasionally with short necks, base tissue prosenchymatous, apices of conidiomata can be orange to scarlet when young, perithecial necks short and of same color (orange to umber) as stroma……***Celoporthe***

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3.3 PATHOGENICITY TESTS

Six weeks after inoculation with isolates of *L. aurorae* lesions were visible on both *G. transvaalica* (min = 11.96, max = 59.26, st. dev. = 12.84) and the *Eucalyptus* clone ZG14 (min = 12.62, max = 34.70, st. dev.= 6.23). No lesions were observed on trees inoculated with sterile MEA. Statistical analyses showed a significant difference in lesion lengths on trees inoculated with the test fungi and the controls for both *G. transvaalica* and *Eucalyptus* clone ZG14 ($P<0.001$). No statistical differences ($P>0.05$) were found between lesions on *G. transvaalica* and those on *Eucalyptus* clone ZG14. Average lesion lengths on *G. transvaalica* was 28 mm and average lesion length on *Eucalyptus* was 23 mm. *Latruncellus aurorae* was re-isolated from lesions on both *G. transvaalica* and *Eucalyptus*.

4 DISCUSSION

Results of this study considerably expand available knowledge regarding the distribution and host range of the Cryphonectriaceae in southern Africa (FIG. 5). Previously unknown hosts and areas of occurrence have been discovered for these fungi. For example, *Chr. austroafricana* was found in the Soutpansberg area, outside its previously known range in South Africa, and other members of the Cryphonectriaceae were recorded for the first time from Namibia and Swaziland (FIG. 5). Furthermore, a previously unknown genus in the Cryphonectriaceae was discovered on a member of the Lythraceae in Swaziland. This is the first report of the Cryphonectriaceae infecting Lythraceae in Africa.

The presence and abundance of *Chr. austroafricana* on a native African host in Namibia, and the fact that *S. guineense* appeared to be relatively tolerant to infection, provides added

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support for the view (Heath *et al*. 2006) that *Chr. austroafricana* has an African origin. This is illustrated by the fact that *Chr. austroafricana* was relatively common on native *S. guineense* trees in the Caprivi region. In addition to being found on stems and branches of *S. guineense* at Popa Falls, the fungus also was common on the roots and branches of *S. guineense* at Katima Mulilo. Disease symptoms, such as stem girdling and tree death, known to be associated with *Chrysoporthe* infections on *Eucalyptus* spp., were not observed on *S. guineense*.

An intriguing aspect of the *Chr. austroafricana* infections on *S. guineense* in Katima Mulilo was that they were on roots partially submerged in water at the time of sampling. This could have interesting implications regarding our understanding of the epidemiology of this fungus. Roots of *S. guineense* growing on the banks of the Zambezi River are known to be submerged during the rainy season. This raises the questions as to when and how infection took place. For example, it is not known whether infection takes place after exposure of the roots to water, or whether the fungus survives or even sporulates on the roots while they are submerged. Although it is known that both *Chr. austroafricana* (Wingfield *et al*. 1989) and *Chr. deutrocubensis* (Davison and Coates 1991) can infect roots, the only report of an association of a *Chrysoporthe* sp. with water is the fact that spores of the asexual state are typically rain-splash dispersed. The presence of *Chr. austroafricana* on the roots of *S. guineense* also could be related to the fact that submerged roots are stressed.

Chrysoporthe austroafricana occurs on non-native *Eucalyptus* spp. in Malawi, Mozambique, South Africa and Zambia (Wingfield *et al*. 1989, Nakabonge *et al*. 2006b, Chungu *et al*. 2010). This fungus however was not found on *Eucalyptus* spp. in Namibia and was present only on native *Syzygium* trees in that country. This could be due to the fact that only *E.*

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camaldulensis trees are planted in the Caprivi region while *E. grandis* is planted in most other African countries. The latter species is much more susceptible to infection by *Chr. austroafricana* than is *E. camaldulensis* (Van Heerden *et al.* 2005).

Celoporthe dispersa was first described from native *S. cordatum*, *H. canescens* and non-native *T. granulosa* in South Africa (Nakabonge *et al.* 2006a). Collections of the fungus in the present study greatly expand its geographic and host range. Nakabonge *et al.* (2006a), observed three distinct phylogenetic sub-clades for *Cel. dispersa,* corresponding to the three hosts (*Heteropyxis*, *Syzygium* and *Tibouchina*) and localities (Lydenburg, Tzaneen and Durban) where isolates were collected. The isolates from Soutpansberg in South Africa and Zambia collected in this study belong in two additional sub-clades within *Cel. dispersa*. It is possible that these five groups represent cryptic species. However, herbarium specimens found previously and linked to the groups from *Heteropyxis* and *Tibouchina,* were limited and had only anamorph structures (Nakabonge *et al*. 2006a). Similarly, infected plant tissue from the current study had few fruiting structures, making comprehensive comparisons among the collections difficult. Additional collections must be made in order to resolve the taxonomy of these isolates.

Holocryphia eucalypti was known previously only from South Africa and Australia and most recently has been found in Uganda (Roux and Nakabonge 2009) and New Zealand (Gryzenhout *et al.* 2010b). The presence of *H. eucalypti* in Swaziland is not unexpected because Swaziland shares most of its border with South Africa, and it has similar climatic conditions. There is also a lack of quarantine and control of movement of raw material between South Africa and Swaziland. The fungus is not a particularly important pathogen in South Africa (Van der Westhuizen *et al*. 1993, Gryzenhout *et al*. 2003) and the report from

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Uganda (Roux and Nakabonge 2009) also indicates minimal impact on trees. *Holocryphia eucalypti*, however, can cause severe damage under stress conditions (Old *et al*. 1986, Wardlaw 1999, Gryzenhout *et al.* 2003) and this could have implications for its role as a plantation pathogen in Swaziland and other African countries, especially under conditions of climate change (Van Staden *et al*. 2004, Desprez-Loustau *et al.* 2007).

Based on phylogeny, the newly described genus *Latruncellus* might more appropriately represent a new species of *Aurifilum.* This, however, is not supported by morphology. Within the Cryphonectriaceae, shape and colour of anamorph structures represent the most important distinctive characteristic for the various described genera, and these are supported with 100% bootstrap and posterior probability values in phylogenetic studies (Gryzenhout *et al*. 2009, 2010a, Begoude *et al*. 2010). A number of genera thus have been described in the Cryphonectriaceae following the approach of supplementing phylogenetic groupings with anamorph morphology. Many of these genera are monotypic, but genera such as *Chrysoporthe* and *Cryphonectria* consist of species strongly supported by morphological characteristics and phylogenetic data.

The alternative to having the various phylogenetic groupings in the Cryphonectriaceae representing separate genera is for all species in the Cryphonectriaceae belong to either a single genus or in two genera representing the two larger groupings observed in the Cryphonectriaceae (FIGS. 2-3 clade A and clade B). Describing the phylogenetic clades in one genus is unfeasible because species shown to be closely related based on DNA sequence data are often morphologically vastly different. For instance, *Rostraureum* and *Chrysoporthe*, and *Microthia* and *Aurapex,* group closely based on DNA sequence data (FIGS. 2-3), but *Rostraureum* and *Microthia* have orange conidiomata while *Chrysoporthe* and *Aurapex* have

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black conidiomata. Similarly, accepting only two genera in the Cryphonectriaceae, based on the two larger phylogenetic clades, includes inconsistent morphological differences between species in the two phylogenetic groups. For instance, fungi with orange and dark conidiomata, and septate as well as aseptate ascospores are found in both groups. Based on currently available data, describing the various phylogenetic groups, united by a defined suite of morphological features as distinct genera, appears to be the most logical means to treat the taxonomy of the Cryphonectriaceae (Gryzenhout *et al*. 2009). Isolates from *G. transvaalica* were described therefore in the new genus *Latruncellus* as opposed to being treated as a new species in the genus *Aurifilum*.

Little is known regarding the distribution and host range of *A. marmelostoma* and *L. aurorae*. *Aurifilum marmelostoma* is known only from Cameroon where it is associated with stem cankers on *T. ivorensis* and *T. mantaly* (Combretaceae). In contrast, *L. aurorae* is known only from Swaziland, on *G. transvaalica* (*Lythraceae*). All the tree genera on which these fungi are found belong in the Myrtales, and it seems likely that *Aurifilum* and *Latruncellus* have wider host and geographical ranges than those currently known for them.

While this study has greatly increased the base of knowledge regarding the distribution and taxonomy of the Cryphonectriaceae in Africa, it also has highlighted the fact that the host and geographic range data for these fungi in Africa remains incomplete. To control the movement of pathogens between countries in Africa and to anticipate potentially detrimental host jumps (Slippers *et al*. 2005) to other tree species it will be important to have an expanded knowledge regarding the host range and distribution of pathogens. This is especially true for pathogens of native trees for which very limited information is available.

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Tree pathogens are typically not highly destructive on their native hosts in their areas of origin but they can cause significant damage when they are moved to new areas and exposed to related trees that have evolved in their absence (Anagnostakis 1987, Wingfield *et al*. 2008). In the Cryphonectriaceae, the devastation caused by *C. parasitica* in Europe and North America provides an apt example (Anagnostakis 1987, Gryzenhout *et al*. 2009). Thus pathogens occurring on seemingly unimportant hosts, such as *L. aurorae* on the native tree *G. transvaalica* have the potential to cause serious disease problems if they are moved to new areas. Inoculations in this study showed that *L. aurorae* is pathogenic to both *G. transvaalica* and the *Eucalyptus* clone tested. Although *L. aurorae* has not been found on *Eucalyptus* spp., the results of this study show that it has the potential to cause disease on these trees. This and similar pathogens should thus also be considered when formulating quarantine standards.

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Yuan ZQ, Mohammed C. 2000. The pathogenicity of isolates of *Endothia gyrosa* to *Eucalyptus nitens* and *E. globules*. Australas Plant Pathol 29: 29–35.

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TABLE I. Isolates used in DNA sequence comparisons and pathogenicity trials[1]

Identity	Isolate number			Host	Country and Region	Collector	GenBank accession numbers [D]
	CMW [A]	CBS [B]	PREM [C]				
Aurifilum marmelostoma	28285	124929	60257	*T. mantaly*	Cameroon, Centre	D. Begoude	HQ171215[4]
"	28288	124930		*T. ivorensis*	Cameroon, Centre	D. Begoude	HQ171216[4]
Celoporthe dispersa	29378			*S. legatti*	South Africa, Limpopo	J Roux, M Vermeulen	GU726942[2], GU726954[3]
	29898			"	"	J Roux, M Vermeulen	GU726943[2], GU726955[3]
	29900			"	"	J Roux, M Vermeulen	GU726944[2], GU726956[3]
"	29375			*S. guineense*	Zambia, Copperbelt	J Roux, M Vermeulen	GU726940[2], GU726952[3]
"	29376			*S. guineense*	Zambia, Copperbelt	J Roux, M Vermeulen	GU726941[2], GU726953[3]
"	29905			*S. cordatum*	South Africa, Limpopo	J Roux, M Vermeulen	GU726945[2], GU726957[3]
Chrysoporthe austroafricana	22760			*S. guineense*	Namibia, Caprivi	J Roux	GU726949[2], GU726961[3]
"	22751			"	"	J Roux	GU726948[2], GU726960[3]
"	32954			"	South Africa, Limpopo	J Roux, M Vermeulen	GU726951[2], GU726963[3]
"	29904			"	"	J Roux, M Vermeulen	GU726950[2], GU726962[3]
Cryptometrion aestuescens	18790	124008	60249		Indonesia	MJ Wingfield	HQ171211[4]
	18793	124007			Indonesia	MJ Wingfield	HQ171212[4]
Holocryphia eucalypti	11690			*E. grandis* clone	Swaziland	J Roux	GU726938[2], GU726936[3]
"	11689			".	"	J Roux	GU726939[2], GU726937[3]
Latruncellus aurorae	28274	124904	60349	*G. transvaalica*	'	J. Roux	GU726946[2], GU726958[3], HQ171213[4]
	28275			"	"	J. Roux	HQ171209[2], HQ171207[3], HQ171214[4]

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“	28276[1]	125526	60348	“	”,	J. Roux	GU726947[2], GU726959[3]
“	30633[1]	124905	60347	“	“	J. Roux	HQ171210[2], HQ171208[3]

A Culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

B CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

C PREM, Agricultural Research Council, Pretoria, South Africa.

D GeneBank accession numbers for sequence data of the ITS[2] (primers ITS1/2), BT[3] 1 and 2 (primers Bt1a/1b and Bt21/2b) and LSU[4] (primers LR0R and LR7).

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TABLE II. Statistics resulting from Maximum parsimony analyses

Statistic	ITS	BT	LSU	BT exon	Combined ITS and BT	Combined LSU and BT exon
Aligned characters	567	928	636	547	1495	1183
Constant characters	350	510	607	442	860	1032
parsimony-uninformative characters	20	11	7	16	31	26
parsimony-informative characters	197	407	22	89	604	125
Tree length	453	1041	45	177	1514	258
Consistence index (CI)	0.664	0.598	0.689	0.655	0.610	0.655
Retention Index (RI)	0.894	0.882	0.837	0.765	0.881	0.757
Rescaled consistency index (RC)	0.594	0.527	0.577	0.502	0.537	0.496

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TABLE III. Morphological characteristics of genera in the Cryphonectriaceae having uniformly orange fruiting bodies compared with those of *Latruncellus aurorae* (adapted from Begoude *et al*. 2010).

	Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*	*Latruncellus*
Teleomorph	**Structure of ascostroma**	pulvinate, erumpent, slightly immersed to superficial	Large, pulvinate, erumpent, semi-immersed	Large, pulvinate to clavate, erumpent, superficial	pulvinate, semi-immersed	Large, pulvinate, erumpent, semi-immersed	pulvinate, erumpent, immersed to semi-immersed	Not known	Large, pulvinate to pyriform, semi-immersed	**Pulvinate, semi-immersed**
	Ascospore shape	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Hyaline, cylindrical	Hyaline, cylindrical	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Not known	Hyaline, ellipsoidal to fusoid	**Hyaline, fusoid to oval**
	Ascospore septation	1-3 septate	One septate	Aseptate	Aseptate	One septate	One septate	Not known	One septate	**One septate**
Anamorph	**Structure of conidiomata**	Conical to pyriform superficial	Pulvinate, semi-immersed, erumpent	Pulvinate, superficial, erumpent	pulvinate, erumpent, semi-immersed	Pulvinate, semi-immersed	Clavate to rostrate	Pyriform or rostrate, superficial	Broadly convex	**Conical with fattened neck**
	Conidiomatal neck	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent, ostiolar opening darkened	**Present**
	Conidiomatal stromatic tissue	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Of different textura type	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	**Prosenchyma and pseudoparenchyma**
	Paraphyses	Absent	Absent	Absent	Present	Present	Absent	Absent	Present	**Present**

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Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*	*Latruncellus*
Conidia	Variable size, hyaline, oblong to slightly curved, aseptate	Minute, hyaline, cylindrical, aseptate	Minute, hyaline, cylindrical, aseptate	Hyaline, cylindrical, aseptate	Hyaline, cylindrical, aseptate	Uniform capitalshyaline, cylindrical, aseptate	Hyaline, cylindrical, aseptate	Minute, hyaline, cylindrical to allantoid, aseptate,	**Minute, hyaline, cylindrical, aseptate,**

(Gryzenhout *et al.* 2009, Begoude *et al.* 2010)

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FIGURE 1. Hosts and symptoms associated with infection by Cryphonectriaceae (A) *Galpinia transvaalica* (B) Canker on *G. transvaalica* (C) Berries of *G. transvaalica* (D) *Syzygium guineense* (E) Canker with fruiting structures of *Chrysoporthe austroafricana* on a root of *S. guineense* in Katima Mulilo (F) Berries of *S. guineense.*

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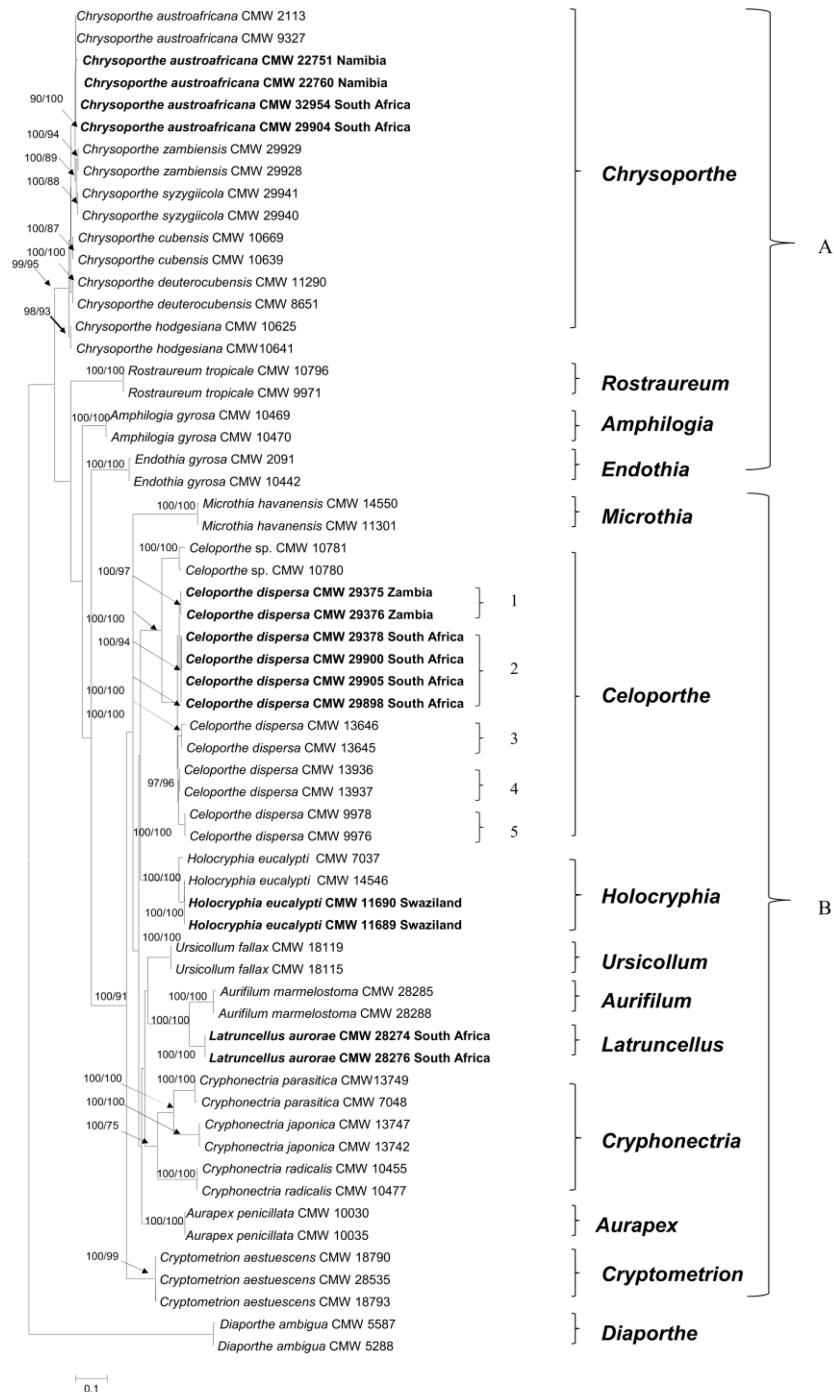

FIGURE 2. Phylogram obtained from the combined datasets of the ITS and BT gene sequences. The phylogram was obtained with maximum likelihood analyses using the TrN+I+G parameter model. Confidence levels >70% of the tree branch nodes, determined by posterior probabilities (BPP) and ML, 1000 replicate bootstrap analysis (BS) and are indicated on tree branches (BPP/BS). Isolates sequenced in this study are in bold. Five clades of *Celoporthe dispersa* are marked 1 to 5. *Diaporthe ambigua* was defined as the outgroup taxon.

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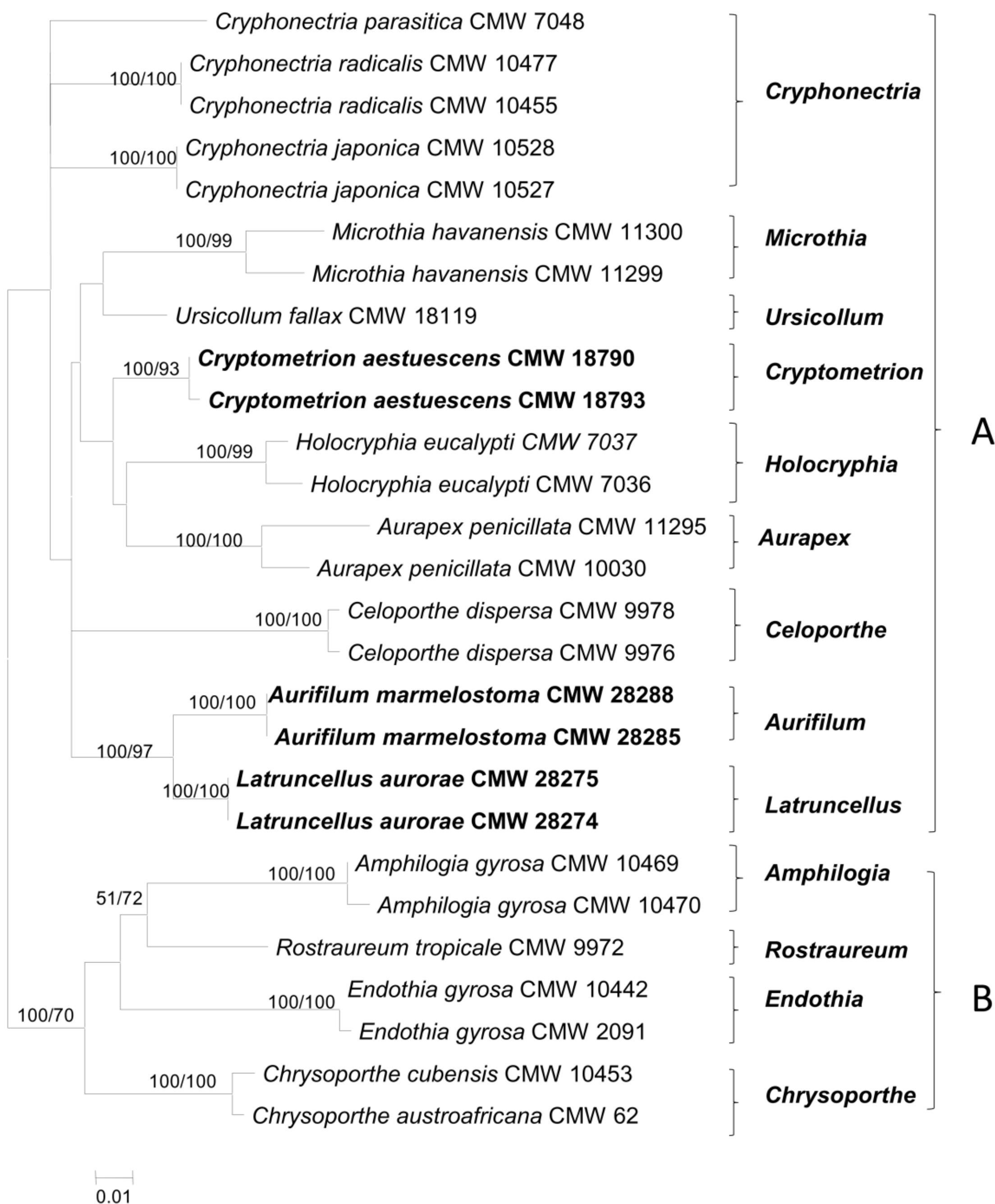

FIGURE 3. Phylogram obtained from the combined datasets of the LSU and BT exon gene sequences. The phylogram was obtained with maximum likelihood using the TIM2+I+G parameter model. Confidence levels >70% of the tree branch nodes, determined by posterior probabilities (BPP) and ML,1000 replicate bootstrap analysis (BS) are indicated (BPP/BS). Isolates sequenced in this study are in bold. The tree was midpoint rooted.

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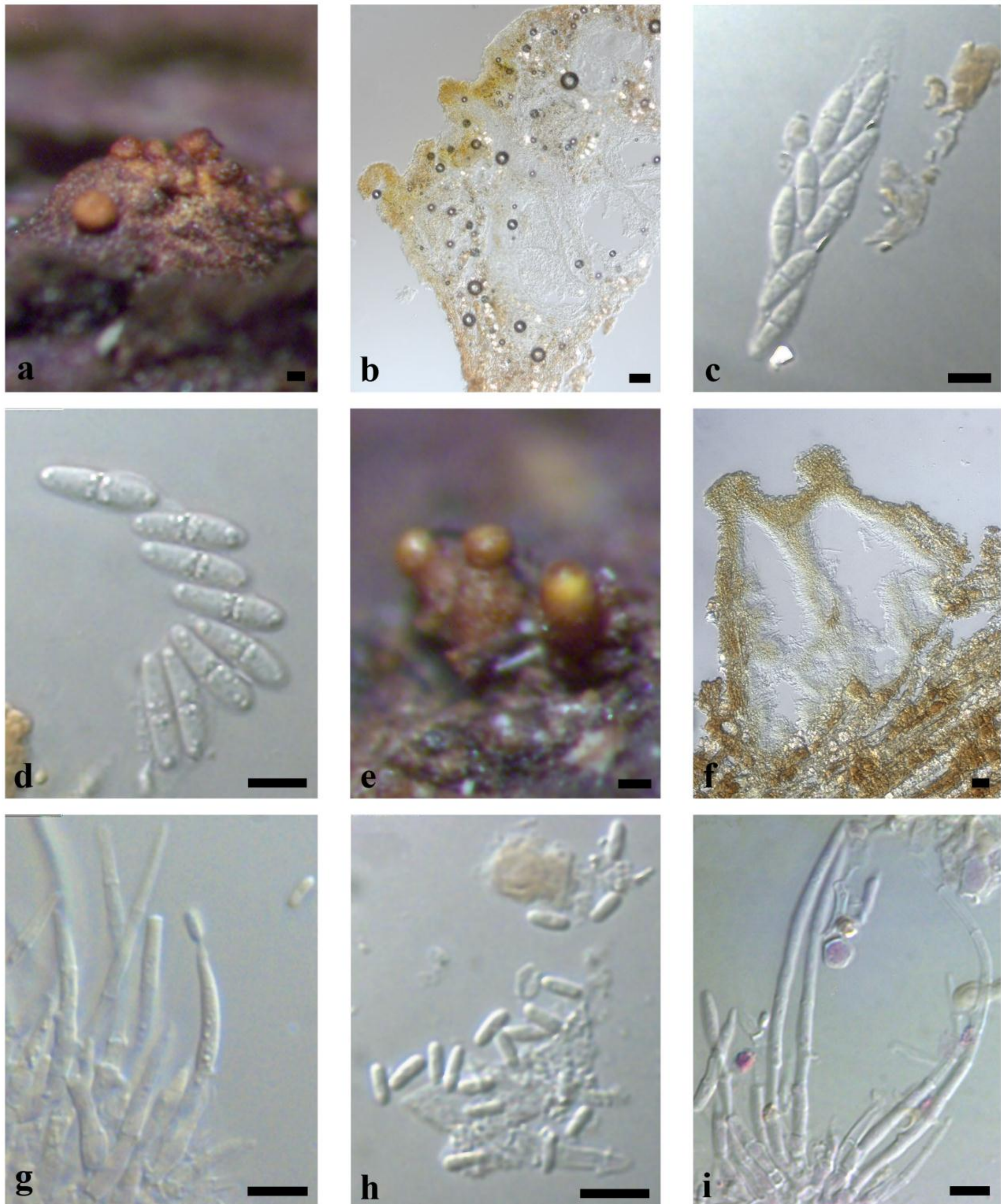

FIGURE 4. Fruiting structures of *Latruncellus aurorae* (A) Ascostroma on bark. (B) Longitudinal section through ascostroma. (C) Ascus (D) Ascospores (E) Conidiomata on bark. (F) Longitudinal section through conidioma. (G) Conidiogenous cells (H) Conidia (I) Paraphyses (Scale bar a, b, e, f = 50µm; c, d, g, h, i = 5 µm).

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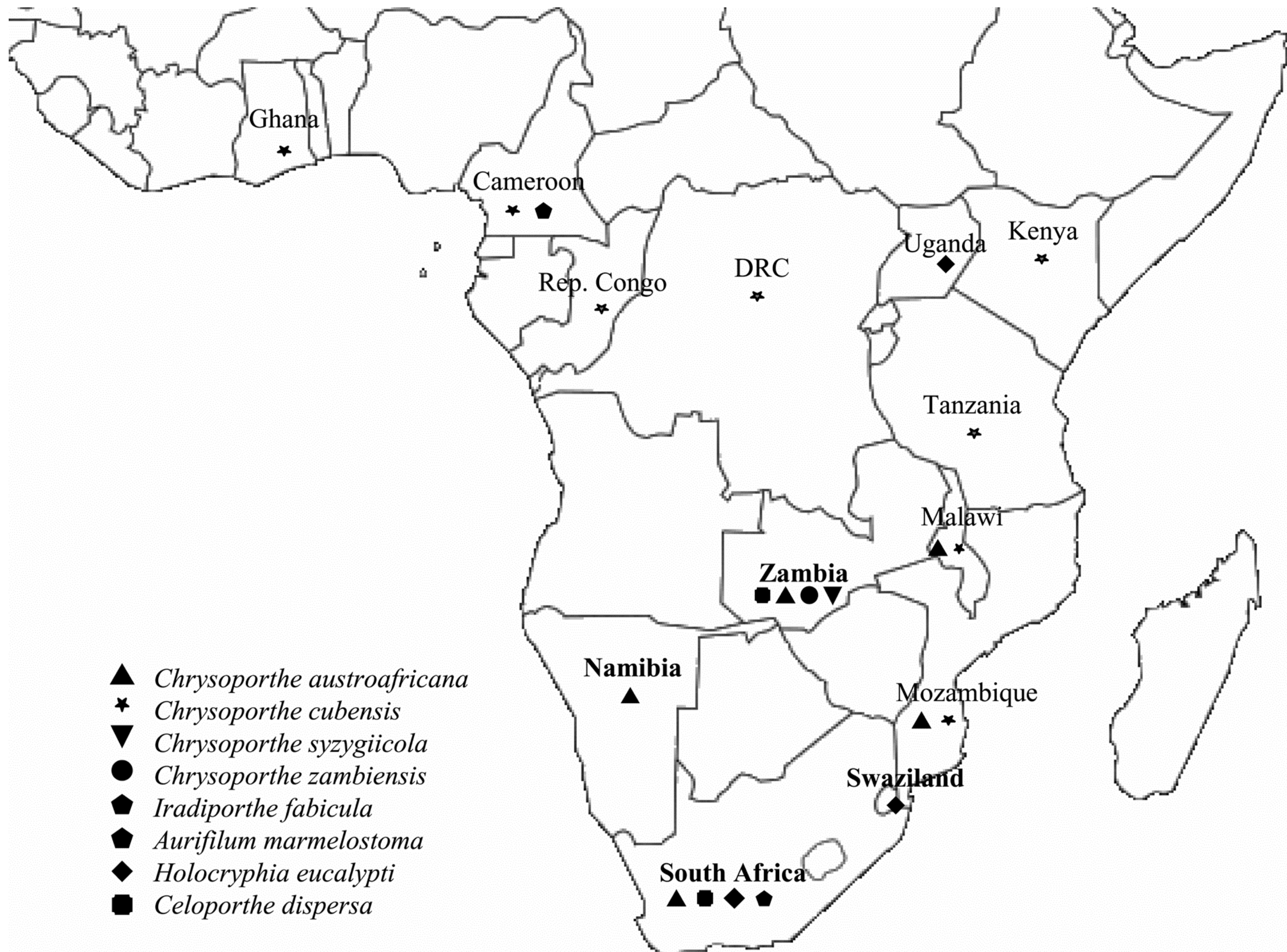

FIGURE 5. Map showing current distribution of Cryphonectriaceae in Africa (markers are only an indication of countries where Cryphonectriaceae occur and not specific locations), Countries in boldface represent new reports.

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Chapter 3

Population structure of *Chrysoporthe austroafricana* in southern Africa determined using Vegetative Compatibility Groupings (VCGs)

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ABSTRACT

Chrysoporthe austroafricana is one of the most damaging pathogens of *Eucalyptus* trees in southern Africa. It is also known to occur on ornamental, non-native, *Tibouchina granulosa* trees and native *Syzygium* species. Previous population studies on this pathogen have focused only on South Africa and were mostly based on vegetative compatibility groups (VCGs). Isolates from more recent surveys have not been included in such studies. It is unclear if there are specific routes of movement of the fungus between countries in southern Africa, or how diverse the populations in these countries are. The aim of this study was to use VCGs to consider the diversity in populations of isolates collected in various countries in southern Africa (Malawi, Mozambique, Namibia, South Africa and Zambia) and from different hosts. Results showed a high diversity amongst isolates from different countries and hosts, and suggested little movement of VCGs among countries or hosts based on the available isolates.

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1 INTRODUCTION

Chrysoporthe austroafricana Gryzenh. & M.J. Wingf. is a well known fungal pathogen of plantation-grown *Eucalyptus* species in southern and eastern Africa (Wingfield *et al.* 1989, Conradie *et al.* 1990, Gryzenhout *et al.* 2004, Roux *et al.* 2005, Nakabonge *et al.* 2006). It was first reported as *Cryphonectria cubensis* (Bruner) Gryzenh. & M.J. Wingf. in 1989 (Wingfield *et al.* 1989), causing disease and death of *Eucalyptus* trees in plantations in South Africa. *Chrysoporthe austroafricana* has subsequently been reported from Malawi, Mozambique, Zambia (Nakabonge *et al.* 2006) and Namibia (Vermeulen *et al.* 2011 – Chapter 2 of this thesis), infecting non-native *Eucalyptus* species (Roux *et al*. 2005, Nakabonge *et al.* 2006), native *Syzygium cordatum* Hachst., *Syzygium guineense* (CD.) (Heath *et al.* 2006, Nakabonge *et al*. 2006, Vermeulen *et al*. 2011) and non-native *Tibouchina granulosa* Cogn.: Britton (Myburg *et al.* 2002).

There is substantial evidence to suggest that *Chr. austroafricana* is native to Africa. This is based on its wide-spread presence on native *S. cordatum* and *S. guineense* in southern African countries (Heath *et al.* 2006, Nakabonge *et al*. 2006, Vermeulen *et al*. 2011), and pathogenicity trials showing that native *S. cordatum* is more tolerant to infection by this pathogen than non-native *Eucalyptus* clones (Heath *et al*. 2006). Symptoms on native *S. cordatum,* and particularly *S. guineense,* are also less severe than those observed on *Eucalyptus* species, and death of these native trees due to infection by *Chr. austroafricana* has not been observed (Heath *et al*. 2006, Nakabonge *et al.* 2006, Vermeulen *et al*. 2011). Despite extensive collections from other eucalypt growing regions of the world, *Chr. austroafricana* has not been detected elsewhere. Van Heerden and Wingfield (2001), believed that *Chr. austroafricana* was introduced into South Africa based on the low

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diversity observed with VCG's for a population from non-native *Eucalyptus* spp. in South Africa, and the misconception that *Chr. austroafricana* was synonymous to *Chr. cubensis* (Van Heerden and Wingfield 2001). Heath (2005), later showed that *Chr. austroafricana* has a high level of genetic diversity in South Africa based on microsatellite data, as would be expected of a native pathogen (Tsutsui *et al*. 2000, Liu and Milgroom 2007, Stukenbrock and McDonald 2008, Linde *et al.* 2009).

No information is available on the movement of *Chr. austroafricana* among countries in southern Africa. *Chrysoporthe austroafricana* is able to cross-infect non-native *Eucalyptus* species and *T. granulosa,* presumably from native Myrtales (Heath *et al.* 2006) illustrating a host shift (Slippers *et al*. 2005). For instance, Heath (2005) showed that there are shared VCGs between populations from *Syzygium* and *Eucalyptus* species (5 VCGs) and populations from *Syzygium* species and *T. granulosa* (1 VCG) in South Africa. This information is not available for VCGs shared among different countries, or among hosts within other countries in southern Africa. It is also unknown whether the VCGs previously characterised in South Africa occur elsewhere. The aim of this study was to determine the population structure and diversity of populations of *Chr. austroafricana* from Malawi, Mozambique, Namibia and Zambia based on VCG diversity. Furthermore, we wished to determine whether there are shared VCGs among the different countries and hosts in southern Africa. Results from this study also lead to the development of tester VCG strains of *Chr. austroafricana* for future use.

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2 MATERIALS AND METHODS

2.1 FUNGAL ISOLATES

Chrysoporthe austroafricana isolates were collected from *S. guineense* trees in Namibia and deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (TABLE 1). These isolates were collected from three localities in the Caprivi region of the country in 2007 and 2008. Samples of bark from the roots, stems and branches of trees growing along the banks of the Zambezi and Kavango rivers were collected for isolations made as described in Vermeulen *et al.* (2011).

Additional isolates from Malawi, Mozambique and Zambia (Nakabonge *et al.* 2006) and those representing previously identified *Chr. austroafricana* VCGs from *Eucalyptus* species in South Africa (Van Heerden and Wingfield 2001, Heath 2005) were obtained from the CMW culture collection (TABLE 1). The identities of the newly collected isolates from Namibia were confirmed as *Chr. austroafricana* using a PCR-RFLP (restriction fragment length polymorphisms) fingerprinting technique developed by Van der Merwe *et al.* (2010). This was done to ensure that only *Chr. austroafricana* isolates were included in this study since *Chr. cubensis* and *Chr. deuterocubensis,* that are morphologically similar to *Chr. austroafricana,* are also known from Africa and co-occur with *Chr. austroafricana* in some countries (Nakabonge *et al.* 2006, Vermeulen *et al.* 2011).

For the PCR-RFLP assays, DNA was extracted from mycelium grown on 2% Malt extract agar (MEA) and the β-tubulin one (BT1) region was amplified using the primer pair BT1a

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and BT1b (Glass and Donaldson 1995), as described in Vermeulen *et al.* (2011). Amplicons were digested with the restriction enzymes *Ava*I and *Hind*III (Van der Merwe *et al.* 2010). Restriction fragments were separated on a 2% Agarose gel and the gel was stained with GelRed^{TM} Nucleic Acid Gel Stain (Biotium, Hayward, CA, U.S.A.) following the instructions of the suppliers.

2.2 VEGETATIVE COMPATIBILITY STUDIES

Vegetative Compatibility Groups (VCGs) were determined for all isolates from Malawi, Mozambique, Namibia and Zambia. To determine VCGs, mycelial plugs where transferred from the edges of actively growing cultures onto oatmeal agar (30g rolled oats, 20g agar and 1L dH_2O). Two isolates were placed 2 cm apart on 6.5 cm diameter Petri dishes. A single isolate from each tree was tested against all other isolates in all possible combinations. Plates were sealed with Parafilm and incubated at 25°C for two weeks. VCGs were then identified based on the ability of different isolates to merge and form confluent mycelium, or to form a barrage reaction along the line of contact (Anagnostakis 1997). Reactions were assessed after two weeks and reactions were scored as vegetatively compatible or vegetatively incompatible. Where a barrage formed between two isolates at the line of contact, it was scored as incompatible and where two isolates merged to form confluent mycelium it was scored as compatible. Representative VCGs of Malawi, Mozambique, Namibia and South Africa (Heath 2005, Van Heerden and Wingfield 2001) were then compared with each other as described above to determine whether there were shared VCGs in the different countries of southern Africa. All VCG tests were repeated once to confirm the results.

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2.4 Statistical analyses of VCG data

Genotypic diversity (G) was determined for larger populations from Mozambique and Namibia using two different statistical parameters. The first parameter was Stoddart and Taylor's (1988) genotypic diversity (G). To be able to compare diversity levels between populations from different areas, the genotypic diversity (G) was divided by the sample size (N) to obtain maximum percentage of genotypic diversity ($\hat{G}$) (Stoddart and Taylor 1988, McDonald *et al*. 1994). The second parameter used was the Shannon Index (SI) (Bowman *et al*. 1971, Groth and Roelfs 1986). SI takes into account the frequency and evenness of the distribution of a particular phenotype. SI was converted into normalized Shannon diversity index (H_s). H_s was used to compare populations of different sizes and as an indication of phenotypic diversity based on VCGs (Sheldon 1969).

3 RESULTS

3.1 Fungal Isolates

Twenty-seven isolates resembling *Chrysoporthe* species were obtained from *S. guineense* in the Caprivi region of Namibia (Katima Mulilo, Island View and Popa Falls). One hundred and five additional isolates were obtained from the CMW culture collection, including eight isolates from *Eucalyptus* species and one isolate from *S. cordatum* in Malawi, fourteen isolates from *Eucalyptus* species and twenty-three isolates from *S. cordatum* in Mozambique and three isolates from *Eucalyptus* species in Zambia. The additional fifty-six isolates were from South Africa (Table 1), representing isolates of VCGs previously identified by Heath

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(2005) from *S. cordatum* (26 isolates) and *T. granulosa* (10 isolates), and Van Heerden and Wingfield (2001) from *Eucalyptus* species. (20 isolates).

All the isolates from Namibia were positively identified as *Chr. austroafricana*, matching the banding patterns described by Van der Merwe *et al*. (2010) for *Chr. austroafricana*. Digests of the BT1 PCR products with *Ava*I produced three bands (data not shown), characteristic of *Chr. austroafricana* and *Chr. deuterocubensis*. To further distinguish between *Chr. austroafricana* and *Chr. deuterocubensis,* BT1 PCR products were digested with *Hind* III, and produced only one band characteristic of *Chr. austroafricana* (Van der Merwe *et al*. 2010).

3.2 VEGETATIVE COMPATIBILITY STUDIES

Chrysoporthe austroafricana isolates from Malawi (8 isolates / 8 VCGs), Mozambique (37 isolates / 30 VCGs), Namibia (27 isolates / 21 VCGs) and Zambia (3 isolates / 2 VCGs) represented 61 VCGs from different hosts (TABLE 1 and TABLE 2). Very few VCGs were shared among different hosts (TABLE 3) and countries (TABLE 4) in southern Africa. VCG "networks" or "clusters" (Cortesi *et al*. 1996) were observed among populations; i.e. two isolates that are incompatible with each other (2 VCGs) but each compatible with a third isolate (TABLE 1).

3.4 STATISTICAL ANALYSES OF VCG DATA

A high diversity was observed for the Namibian ($\hat{G}$ = 53%, H_s = 20%) and the Mozambican ($\hat{G}$ = 65%, H_s = 28%) population. For the population from Mozambique the diversity was

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high for both the populations from *Eucalyptus* ($\hat{G}$ = 50%, H_s = 9%) and *S. cordatum* ($\hat{G}$ = 79%, H_s = 19%) and higher than observed in previous studies for populations from South Africa (Van Heerden and Wingfield 2001, Heath 2005) (TABLE 5). Limited numbers of isolates were available from Malawi and Zambia and no meaningful statistical analyses could be conducted for these countries. All of the isolates from Malawi, however, represented unique VCGs, while the three isolates from Zambia represented two unique VCGs (TABLE 2).

4 DISCUSSION

The high population diversity observed for *Chr. austroafricana* in southern Africa supports the view that it is native to Africa (Heath *et al.* 2006). The genetic diversity of Mozambican and Namibian populations based on VCGs was higher than that observed for the South African populations studied by Heath (2005) and Van Heerden and Wingfield (2001). This suggests that the centre of diversity of *Chr. austroafricana* is most likely in a country other than South Africa. This is further supported by the high diversity for the Mozambique population from both native *S. cordatum* and non-native *Eucalyptus* species, and although inadequate population samples exist for Malawi and Zambia, the isolates obtained for this study all belonged to different VCGs. This is comparable with the number of VCGs seen per population for the closely related fungus *C. parasitica* in its native range (China 64 isolates / 54 VCGs and Japan 79 isolates / 71 VCGs) (Liu and Milgroom 2007).

Although the population sizes of *Chr. austroafricana* from the various countries of southern Africa were not all optimal, the available evidence suggests little movement of *Chr. austroafricana* among countries in southern Africa. A very limited number of shared VCGs were observed among the different countries for which isolates were available. This suggests

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that these populations have been present in these countries over a long period with little introduction of new phenotypes from the outside. The same is true for movement of phenotypes among hosts of *Chr. austroafricana* in southern Africa. It is believed that *Chr. austroafricana* underwent a host jump from native Myrtales to non-native Myrtales in South Africa (Heath *et al.* 2006, Slippers *et al*. 2005). The limited number of shared VCGs between native and non-native hosts is indicative that the host jump was not recent and that the founder population has not yet been sampled.

Forestry in South Africa is based on a clonal program where resistance was established to a single, highly virulent isolate of *Chr. austroafricana.* Currently, breeding programmes rely on natural infection of clones in trials to obtain information on disease tolerance of future planting material. It has been shown that different VCGs can differ in their pathogenicity to hosts (Van Heerden and Wingfield 2001, Tsror Lahkim and Levin 2003, Elmer *et al.* 1999). Although pathogenicity has not been linked to VCG types in this study, our results showed that a high diversity of VCGs exists outside South Africa. It is thus possible that the high diversity of VCG types also indicate diverse levels of pathogenicity and that introduction of such genotypes would pose a threat to the existing trees planted in South Africa.

Pathogen populations that are more diverse are able to better adapt to changes in host resistance than pathogen populations that are genetically uniform (McDonald *et al*. 1989, Delmotte *et al*. 1999, McDonald and McDermott 1993). This implies that more diverse populations will be able to more quickly overcome the resistance of clones selected for their tolerance to specific pathogens. *Eucalyptus* plantations in southern Africa, and other areas of the world, depend on planting disease tolerant hybrids and clones of *Eucalyptus* species to reduce the impact of Chrysoporthe canker (Alfenas *et al.* 1983, Van der Westhuizen *et al.*

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1992, Van Heerden and Wingfield 2001, Wingfield and Roux 2002). It is thus important to understand the diversity of *Chr. austroafricana* in southern Africa to help insure continued control of this pathogen.

The high diversity demonstrated by the VCG data in our study confirms that *Chr. austroafricana* is a fungus native to Africa. This is despite the fact that it is best known to be highly damaging on a non-native host. A significant amount of sampling would thus be necessary to provide sufficient resolution to saturate the diversity found, and to adequately characterise the distributions of the various VCGs. Such in depth sampling is, however, difficult in the various African countries already sampled.

The VCG tester strains developed in this study enables investigation of some level of population diversity. It also allows a relatively cheap and easy system to obtain at least basic information on this pathogen without the use of expensive molecular tools. A system of VCG tester strains (EU-1 to EU-74) have been developed for the related pathogen *C. parasitica* that was introduced into North America and Europe from Japan and China (Cortesi *et al*. 1998, Robin *et al*. 2000). In these countries, the database is useful to trace the history and origin of introductions and movements among areas. They also provide information on the reproduction of *C. parasitica* in these areas, and to evaluate the possible success of biological control programs using hypovirulence, which is highly dependent on the clonality of the pathogen population (Gurer *et al*. 2001, Milgroom and Cortesi 1999, Milgroom *et al.* 2008, Adamcikova *et al*. 2009, Jankovsky *et al*. 2010). The situation for *Chr. austroafricana* is, however, different because this is a native pathogen of which the representative population diversity has not yet been fully sampled and new VCGs are continuously produced. In this regard, developing a VCG tester database with the same functionality as that available for

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C. parasitica is challenging.

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TABLE I. Origin, hosts and Vegetative Compatibility Groups (VCG) of isolates of *Chrysoporthe austroafricana* used in this study.

ORIGEN	HOST	ISOLATE NR	VCG	
			Country	**Southern Africa**
Malawi	*Eucalyptus* sp.	CMW17105	ME1	SA1
Malawi	*Eucalyptus* sp.	17108	ME2	SA2
Malawi	*Eucalyptus* sp.	17109	ME3	SA3
Malawi	*Eucalyptus* sp.	17115	ME4	SA4
Malawi	*Eucalyptus* sp.	17118	ME5	SA5
Malawi	*Eucalyptus* sp.	17132	ME6	SA6
Malawi	*Eucalyptus* sp.	17133	ME7	SA7
Malawi	*S. cordatum*	17098	MS1	SA8
Mozambique	*Eucalyptus* sp.	13878	MOE1	SA9
Mozambique	*Eucalyptus* sp.	13881	MOE2	SA10
Mozambique	*Eucalyptus* sp.	13882	MOE3	SA11
Mozambique	*Eucalyptus* sp.	13886	MOE4	SA12
Mozambique	*Eucalyptus* sp.	13887	MOE2	SA10/ SA66/ SA70
Mozambique	*Eucalyptus* sp.	13888	MOE2	SA10
Mozambique	*Eucalyptus* sp.	13889	MOE2	SA10
Mozambique	*Eucalyptus* sp.	13916	MOE5	SA13
Mozambique	*Eucalyptus* sp.	13918	MOE6	SA14
Mozambique	*Eucalyptus* sp.	13930	MOE7	SA15
Mozambique	*Eucalyptus* sp.	13931	MOE7	SA15
Mozambique	*Eucalyptus* sp.	17084	MOE8	SA16

ORIGEN	HOST	ISOLATE NR	VCG	
			Country	**Southern Africa**
Mozambique	*Eucalyptus* sp.	17087	MOE9	SA17
Mozambique	*Eucalyptus* sp.	17094	MOE10	SA18
Mozambique	*S. cordatum*	13874	MOS1	SA19
Mozambique	*S. cordatum*	13875	MOS2	SA20
Mozambique	*S. cordatum*	13876	MOS3	SA21
Mozambique	*S. cordatum*	13877	MOS4	SA22
Mozambique	*S. cordatum*	13890	MOS5	SA23
Mozambique	*S. cordatum*	13891	MOS6	SA24
Mozambique	*S. cordatum*	13892	MOS7	SA25
Mozambique	*S. cordatum*	13893	MOS8	SA26
Mozambique	*S. cordatum*	13894	MOS9	SA27
Mozambique	*S. cordatum*	13895	MOS10	SA28
Mozambique	*S. cordatum*	13897	MOS11	SA29
Mozambique	*S. cordatum*	13900	MOS12	SA30
Mozambique	*S. cordatum*	13904	MOS13	SA31
Mozambique	*S. cordatum*	13907	MOS14	SA32
Mozambique	*S. cordatum*	13908	MOS15	SA33
Mozambique	*S. cordatum*	13909	MOS16	SA34
Mozambique	*S. cordatum*	13921	MOS17	SA35
Mozambique	*S. cordatum*	13922	MOS3	SA22
Mozambique	*S. cordatum*	13925	MOS18	SA36
Mozambique	*S. cordatum*	13926	MOS6	SA24/ SA37
Mozambique	*S. cordatum*	13927	MOS2	SA20
Mozambique	*S. cordatum*	13932	MOS19	SA38

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ORIGEN	HOST	ISOLATE NR	VCG	
			Country	Southern Africa
Mozambique	*S. cordatum*	13935	MOS20	SA39
Namibia	*S. guineense*	(123)23707	NS1	SA40
Namibia	*S. guineense*	(131)24268	NS2	SA41
Namibia	*S. guineense*	(128)24269	NS3	SA42
Namibia	*S. guineense*	(126)24272	NS4	SA43
Namibia	*S. guineense*	(129)24273	NS5	SA44
Namibia	*S. guineense*	(135)24276	NS6	SA45
Namibia	*S. guineense*	(130)24278	NS5	SA44
Namibia	*S. guineense*	(133)24281	NS7	SA46
Namibia	*S. guineense*	(118)24282	NS8	SA47
Namibia	*S. guineense*	(119)24285	NS8	SA47
Namibia	*S. guineense*	(121)24291	NS8	SA47
Namibia	*S. guineense*	(36)28240	NS9	SA48
Namibia	*S. guineense*	(39)28241	NS10	SA49
Namibia	*S. guineense*	(42)28244	NS11	SA50
Namibia	*S. guineense*	(46)28247	NS8	SA47
Namibia	*S. guineense*	(48)28249	NS8	SA47
Namibia	*S. guineense*	(57)28255	NS12	SA51
Namibia	*S. guineense*	(63)28259	NS13	SA52
Namibia	*S. guineense*	(64)28260	NS14	SA53
Namibia	*S. guineense*	(69)28263	NS15	SA54
Namibia	*S. guineense*	(72)28265	NS16	SA55
Namibia	*S. guineense*	(73)28266	NS13	SA52
Namibia	*S. guineense*	(79)28269	NS17	SA56

ORIGEN	HOST	ISOLATE NR	VCG	
			Country	Southern Africa
Namibia	*S. guineense*	(82)28270	NS18	SA57
Namibia	*S. guineense*	(33)28271	NS19	SA58
Namibia	*S. guineense*	(76)28371	NS20	SA59
Namibia	*S. guineense*	(30)32953	NS21	SA12
South Africa	*E. grandis*	11318	SAE1	SA60
South Africa	*E. grandis*	11319	SAE2	SA61
South Africa	*E. grandis*	11320	SAE3	SA62
South Africa	*E. grandis*	11321	SAE4	SA63
South Africa	*E. grandis*	11324	SAE5	SA64
South Africa	*E. grandis*	11326	SAE6	SA65
South Africa	*E. grandis*	11327	SAE7	SA66
South Africa	*E. grandis*	11330	SAE8	SA67
South Africa	*E. grandis*	11331	SAE9	SA68
South Africa	*E. grandis*	11334	SAE10	SA69
South Africa	*E. grandis*	11335	SAE11	SA70
South Africa	*E. grandis*	11337	SAE12	SA71
South Africa	*E. grandis*	11339	SAE13	SA72
South Africa	*E. grandis*	11340	SAE14	SA73
South Africa	*E. grandis*	11341	SAE15	SA74
South Africa	*E. grandis*	11342	SAE16	SA75
South Africa	*E. grandis*	11344	SAE17	SA76
South Africa	*E. grandis*	11345	SAE18	SA77
South Africa	*E. grandis*	11346	SAE19	SA78
South Africa	*E. grandis*	11347	SAE20	SA79

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ORIGEN	HOST	ISOLATE NR	VCG	
			Country	Southern Africa
South Africa	*Syzygium* spp.	10036	SAS1	SA80
South Africa	*Syzygium* spp.	10038	SAS2	SA81
South Africa	*Syzygium* spp.	10039	SAS3	SA82
South Africa	*Syzygium* spp.	10040	SAS4	SA83
South Africa	*Syzygium* spp.	10047	SAS5	SA84
South Africa	*Syzygium* spp.	10050	SAS6	SA85
South Africa	*Syzygium* spp.	10051	SAS7	SA86
South Africa	*Syzygium* spp.	10052	SAS8	SA87
South Africa	*Syzygium* spp.	10053	SAS9	SA88
South Africa	*Syzygium* spp.	10059	SAS10	SA89
South Africa	*Syzygium* spp.	10060	SAS11	SA90
South Africa	*Syzygium* spp.	10061	SAS12	SA91
South Africa	*Syzygium* spp.	10062	SAS13	SA92
South Africa	*Syzygium* spp.	10063	SAS14	SA33
South Africa	*Syzygium* spp.	10064	SAS15	SA93
South Africa	*Syzygium* spp.	10066	SAS16	SA94
South Africa	*Syzygium* spp.	10067	SAS17	SA74
South Africa	*Syzygium* spp.	10071	SAS18	SA95
South Africa	*Syzygium* spp.	10072	SAS19	SA96
South Africa	*Syzygium* spp.	10075	SAS20	SA97
South Africa	*Syzygium* spp.	10080	SAS21	SA98
South Africa	*Syzygium* spp.	10081	SAS22	SA99
South Africa	*Syzygium* spp.	10082	SAS23	SA100
South Africa	*Syzygium* spp.	10086	SAS24	SA101

ORIGEN	HOST	ISOLATE NR	VCG	
			Country	Southern Africa
South Africa	*Syzygium* spp.	10087	SAS25	SA102
South Africa	*Syzygium* spp.	10193	SAS26	SA62
South Africa	*T. granulosa*	9327	SAT1	SA72/ SA81
South Africa	*T. granulosa*	9339	SAT2	SA63
South Africa	*T. granulosa*	9341	SAT3	SA37/ SA39
South Africa	*T. granulosa*	9345	SAT4	SA103
South Africa	*T. granulosa*	9348	SAT5	SA104
South Africa	*T. granulosa*	9349	SAT6	SA105
South Africa	*T. granulosa*	9350	SAT7	SA60
South Africa	*T. granulosa*	9359	SAT8	SA91
South Africa	*T. granulosa*	9364	SAT9	SA106
South Africa	*T. granulosa*	9370	SAT10	SA107
Zambia	*Eucalyptus* sp.	13966	ZE1	SA89
Zambia	*Eucalyptus* sp.	13970	ZE2	SA108
Zambia	*Eucalyptus* sp.	13975	ZE3	SA108

ME=Malawi *Eucalyptus* VCG
MS=Malawi *Syzygium* VCG
MOE=Mozambique *Eucalyptus* VCG
MOS=Mozambique *Syzygium* VCG
NS=Namibia *Syzygium* VCG
SAE=South African *Eucalyptus* VCG
SAS=South Africa *Syzygium* VCG
SAT=South Africa *Tibouchina* VCG
ZA=Zambia *Eucalyptus* VCG
SA=southern Africa VCG

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TABLE II. Number of VCGs identified for *Chrysoporthe austroafricana* population in southern Africa

Country	Host	No. of isolates	No. of VCGs
Malawi	*Eucalyptus* spp.	7	7
	S. cordatum	1	1
Mozambique	*Eucalyptus* spp.	14	10
	S. cordatum	23	20
Namibia	*S. guineense*	27	21
Zambia	*Eucalyptus* spp.	3	2
South Africa	*Eucalyptus* spp. [a]	100	23
	Syzygium[b]	62	32
	Tibouchina[b]	37	10

[a] Van Heerden and Wingfield (2001)

[b] Heath (2005)

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TABLE III. VCGs of *Chrysoporthe austroafricana* shared between hosts in southern Africa, including data from this study and those published by Heath (2005) and Van Heerden and Wingfield (2001).

Host	*Tibouchina*	*Eucalyptus* spp.	*Syzygium* spp.
Tibouchina	10	3	4
Eucalyptus spp.		39	4
Syzygium spp.			68

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TABLE IV. VCGs of *Chrysoporthe austroafricana* shared between different countries in southern Africa.

Distribution	Malawi	Mozambique	Namibia	South Africa	Zambia
Malawi	8	0	0	0	0
Mozambique		30	1	5	0
Namibia			21	1	0
South Africa				50	1
Zambia					2

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TABLE VI. Diversity based on VCGs for populations from southern Africa

Country	Host	No. of isolates	Diversity	
			$\hat{G}$[c]	Hs[d]
Mozambique	*Eucalyptus* spp.	14	50	9
	S. cordatum	23	79	19
Namibia	*S. guineense*	27	53	20
South Africa	[a]*E. grandis*	100	0.4	55
	[b]*Syzygium* spp.	62	26	36
	[b]*T. granulosa*	37	22	24

[a] Van Heerden and Wingfield (2001)

[b] Heath (2005)

c Maximum % of genotypic diversity (Stoddard and Taylor 1988)

d Normalized Shannon diversity index (Sheldon 1969)

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Chapter 4

Species delineation of the tree pathogen genus *Celoporthe* (Cryphonectriaceae) in southern Africa

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ABSTRACT

The genus *Celoporthe* was first described when *Cel. dispersa* was discovered in South Africa associated with die-back and cankers on native *Syzygium cordatum* and *Heteropyxis canescens* and the non-native ornamental *Tibouchina granulosa,* all residing in the Myrtales. Recently, four additional species have been described in the genus, namely *Cel. eucalypti, Cel. guangdongensis* and *Cel. syzygii* from *Eucalyptus* species and *Syzygium cumini* in China, and *Cel. indonesiensis* from *S. aromaticum* and *Eucalyptus* in Indonesia. Inoculation trials have shown that all *Celoporthe* species, including those that have not been found on *Eucalyptus* species in nature, are pathogenic to *Eucalyptus* and they are thus potentially threatening to commercial *Eucalyptus* forestry. New isolates, morphologically similar to *Celoporthe* have been collected from *S. legatti* in South Africa and *S. guineense* in Zambia. Multigene phylogenetic analyses based on DNA sequences of the ITS region of the ribosomal operon, Translation Elongation Factor 1α gene and two areas in the β-tubulin gene revealed three cryptic species in *Celoporthe*. Phylogenetic data were supported by morphological differences. These resulted in the description of two previously unknown species of *Celoporthe*, namely *Cel. fontana* and *Cel. woodiana*, for two of these cryptic groups, while the third group represented *Cel. dispersa*. These species can all readily infect *Eucalyptus* as well as several species of *Syzygium*, the latter of which are native to Africa.

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1 INTRODUCTION

The genus *Celoporthe* (Cryphonectriaceae) was first described in 2006 and currently includes five species. These include *Celoporthe dispersa* Nakab., Gryzenh., Jol. Roux & M.J. Wingf. from South Africa and Zambia (Nakabonge *et al*. 2006a, Vermeulen *et al*. 2011), *Cel. eucalypti* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou, *Cel. guangdongensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou and *Cel. syzygii* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from China (Chen *et al.* 2011) and *Cel. indonesiensis* S.F. Chen, Gryzenh., M.J. Wingf. & X.D. Zhou from Indonesia (Chen *et al*. 2011). *Celoporthe* species are known only from hosts in the Myrtales (Nakabonge *et al*. 2006a, Chen *et al*. 2011, Vermeulen *et al*. 2011). *Celoporthe dispersa* has only been reported from South Africa and Zambia, occurring on native *Heteropyxis canescens* Oliv. (Heteropyxidaceae, Myrtales), *Syzygium cordatum* Oliv., *S. guineense* (CD.) and *S. legatti* Burtt Davy & Greenway (Myrtaceae, Myrtales) and non-native *Tibouchina granulosa* Cogn. ex Britton (Melastomataceae, Myrtales) (Nakabonge *et al.* 2006a, Vermeulen *et al.* 2011). *Celoporthe indonesiensis* occurs on *S. aromaticum* (L.) Merr & Perry in Indonesia (Myburg *et al.* 2003, Chen *et al*. 2011) while the Chinese species *Cel. eucalypti* and *Cel. guangdongensis* were collected on non-native *Eucalyptus* species, and *Cel. syzygii* on-native *S. cumini* (L.) Skeels (Chen *et al*. 2011).

Celoporthe dispersa is associated with cankers and branch die-back on *S. cordatum* and *T. granulosa* (Nakabonge *et al*. 2006a). Cankers on *Het. Canescens,* from which *Cel. dispersa* was first collected, were severe with some trees dying. It was, however, not shown that *Cel. dispersa* was responsible for the death of these trees, as pathogenicity tests could not be performed on *Het. canescens. Celoporthe dispersa* also poses a potential threat to *Eucalyptus*

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forestry as inoculation trials showed that the fungus is pathogenic to *Eucalyptus* species even though it has not been seen on these trees in nature (Nakabonge *et al*. 2006a).

Infections of *Eucalyptus* and *Syzygium* trees with *Cel. eucalypti, Cel. guangdongensis* and *Cel. syzygii* are associated with cracking of the bark and the formation of girdling stem cankers. It is, however, not clear whether these symptoms are associated with *Celoporthe* species only, or if symptoms are caused by *Chr. deuterocubensis,* a well-known *Eucalyptus* pathogen (Van der Merwe *et al*. 2010) known to occur in China (Chen *et al*. 2010), or a combination of both, as they co-occur on these hosts in China (Chen *et al*. 2011). Pathogenicity tests (Chen *et al*. 2011) showed that Chinese *Celoporthe* species from *Syzygium* and *Eucalyptus* are as pathogenic to various *Eucalyptus* genotypes and *S. cumini* trees as *Chr. deuterocubensis* (Chen *et al*. 2010). *Eucalyptus* genotypes used in that study differed in susceptibility to infection by *Celoporthe* species, and selection of *Eucalyptus* planting stock with tolerance to infection by *Celoporthe* species should be possible.

Nakabonge *et al*. (2006a), observed three distinct phylogenetic sub-clades for *Cel. dispersa* isolates from South Africa (FIG 1). These represented isolates from three different hosts and locations, namely *Het. canescens* (Lydenburg, Mpumalanga Province), *S. cordatum* (Tzaneen, Limpopo Province), and *T. granulosa* (Durban, KwaZulu-Natal Province). Isolates residing in these sub-clades were not described as distinct species because there were no obvious morphological differences observed between structures on the limited herbarium material available, and no teleomorph structures were available for isolates from *Het. canescens* and *T. granulosa*. Vermeulen *et al*. (2011), observed two additional sub-clades, representing isolates from *S. cordatum* and *S. legatti* in South Africa (Soutpansberg, Limpopo Province) and from *S. guineense* in Zambia (Ikelenge, North Western Province) (FIG 1). Due

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to the limited availability of specimens for the five sub-clades from Africa, comprehensive morphological comparisons between the collections were not possible at the time (Vermeulen *et al*. 2011).

The five distinct sub-clades in *Celoporthe* from Africa observed by Nakabonge *et al*. (2006a) and Vermeulen *et al*. (2011) could represent five cryptic species similar to the species described from China and Indonesia (Chen *et al*. 2011). The aim of this study was to determine whether more than one species is present in Africa based on newly available collections. Use was made of multi-locus DNA sequence data and morphological comparisons to determine this. Furthermore, we considered whether they differ in pathogenicity on a *Eucalyptus* clone and *S. cordatum* seedlings in the greenhouse.

2 MATERIALS AND METHODS

2.1 FUNGAL ISOLATES

Isolates of *Cel. dispersa* (Nakabonge *et al*. 2006a) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (TABLE 1). Additional samples were obtained from surveys of native Myrtales conducted in Soutpansberg (Limpopo Province) and Lydenburg (Mpumalanga Province), South Africa. Isolations were made from pieces of bark bearing fruiting structures resembling those of *Celoporthe* species using techniques previously described by Gryzenhout *et al*. (2009). The cultures obtained were deposited in the CMW culture collection (TABLE 1) and duplicates of selected isolates were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Herbarium specimens of fruiting

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structures on bark of selected fungi were also deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

2.2 IDENTIFICATION

2.2.1 DNA sequence comparisons

DNA was extracted from mycelium grown on 2% Malt extract agar (MEA) as described in Vermeulen *et al*. (2011). DNA concentrations were determined using a NanoDrop version 3.1.0 ND-1000 uv/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Four gene regions were amplified using the Polymerase Chain Reaction (PCR). The β-tubulin 1 and β-tubulin 2 regions of the β-tubulin gene (BT) were amplified using the primer pairs BT1a, BT1b and BT2a, BT2b, respectively (Glass & Donaldson 1995). The internal transcribed spacer (ITS) regions (ITS1, ITS2), and the conserved 5.8S gene of the ribosomal RNA (rDNA) operon were amplified using the primer pair ITS1 and ITS4 (White *et al.* 1990). A portion of the Elongation factor 1 α (TEF1α) gene was amplified using primers EF1-728 and EF986R (Carbone and Kohn 1999).

PCR reactions were performed in a total volume of 25 µl comprising of 40 ng DNA template, 0.5 µM of each primer, 0.2 mM of each dNTP, 0.5 U Super-term polymerase Taq (Southern Cross Biotechnology, Cape Town, South Africa), 10 X dilution buffer, 1 µl $MgCl_2$ (Southern Cross Biotechnology, Cape Town, South Africa) and sterile distilled water (18 µl). PCR were carried out on a thermal cycler (Master cycle® Perkin Elmer Corporation, Massachusetts, United States) and included an initial denaturation step at 94 °C for 3 min, followed by 40 amplification cycles consisting of 30 sec at 94 °C, 45 sec of annealing at 55 °C for βT1, ITS

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and TEF1α, 65 °C for BT 2, and 1 min at 72 °C followed by 4 min at 72 °C to ensure complete elongation of the fragments. PCR products were visualized with UV light on 1 % agarose gels containing ethidium bromide to determine the presence or absence of bands. PCR products were cleaned using 0.06 g/ml of Sephadex G-50 (SIGMA-ALDRICH, Amersham Biosciences Limited, Sweden) according to the manufacturer's instructions.

DNA fragments were sequenced using the same primer pairs used in the PCR amplification reactions. Sequencing reactions were performed in a volume of 10 µl consisting of 5X dilution buffer, 4 µl H_2O, DNA, 10X reaction mix and 2pmol / µl of primer. PCR sequencing products were cleaned as described above using Sephadex G-50 columns. The products were sequenced in both directions using the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism™ 3100 DNA sequencer (Applied Biosystems).

The presence of a mononucleotide repeat (T) upstream of the ITS primer binding site in several isolates (TABLE 1), caused slipped-strand mispairing (Fazekas *et al.* 2010). This resulted in the formation of stutter products, after which the quality of sequence data is greatly reduced. In order to obtain these sequences, PCR products were cloned into pGEM®-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* JM109 High efficiency competent cells (Promega) following the supplier's instructions. Plasmid DNA was prepared using the alkaline lyses method (Sambrook and Russel 2006) and sequenced using primer pair SP6 and T7 (Promega) as described above. A second mononucleotide (A) repeat was present upstream of the Mononucleotide (T) repeat in ITS, and there are also mononucleotide repeats in the BT (C) and TEF1α (T) regions. For these gene regions sequence data could be obtained from both primer binding sites up to the repeat, and forward and reverse sequences were repeated in order to validate the sequence data.

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Gene sequences were visualized and edited using CLC MAIN WORKBENCH, CLC BIO version 5.5 (CLC bio A/S, Science Park Aarhus, Finlandsgade 10-12, 8200 Aarhus N, Denmark). The National Centre for Biotechnology Information (NCBI) database was accessed and preliminary identifications were obtained for sequences using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were then aligned with published sequences of closely related fungal species (TABLE 1) using the web interface (http://timpani.genome.adjp/%7Emafft/server/) of the alignment program MAFFT Version 5.8 (Katoh *et al.* 2002).

Phylogenetic analyses were performed using the software package Phylogenetic Analysis Using Parsimony (PAUP) Version 4.01b10 (Swofford 2000). Phylogenetic analyses were done for each gene region separately using maximum parsimony (heuristic search with 100 random sequence additions). A 1000 bootstrap replication was also performed, to determine the support of branches for the most parsimonious tree for datasets representing each of the gene regions (Felsenstein 1985). A 1000 replicate partition homogeneity test (PHT) was performed to test the null hypotheses that the BT, ITS and TEF1α gene datasets were homologous and could be combined for further analyses (Farris *et al*. 1994). PHT was done after the exclusion of uninformative sites, using a heuristic search with 100 random sequence additions, tree bisection-reconnection (TBR) branch swapping and MAXTREES set to 5000 in order to allow completion of analysis. A 1000 bootstrap replication was performed to determine the support of branches for the most parsimonious tree for datasets representing each gene region (Felsenstein 1985).

A second phylogenetic analysis was done based on maximum likelihood (ML) for each gene region separately, and for the combined data set. The correct models for the datasets were

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identified using JMODELTEST version 0.0.1 (Posada 2008). The TrNef+I model (Posada 2008) was shown to be appropriate for the ITS, the TIM3+G model (Tavaré 1986) for BT, the HKY+I model (Hasegawa *et al.* 1985) for TEF1α and the TIM2+G model (Tavaré 1986) for the combined data set. Maximum likelihood analyses were performed using PHYML V3 (Guindon and Gascuel 2003). A 1000 replicate bootstrap analysis was done to assess the confidence levels of the branch nodes in the phylogenetic trees.

2.2.2 Morphology

To study the morphology of isolates for which herbarium specimens were not available, representative isolates for each phylogenetic clade (TABLE 1) were inoculated into sterile *Eucalyptus* clone ZG14 and *S. cordatum* stem sections on water agar (Gryzenhout *et al.* 2009), as well as on oatmeal agar and *S. cordatum* seedlings to induce sporulation. Stems of *Eucalyptus* clone ZG 14 and *S. cordatum* seedlings were cut into 6cm sections and autoclaved. The bark stem sections were removed with a 5mm cork borer to expose the cambium layer and mycelium plugs from seven-day-old cultures were placed, mycelium facing downwards, into the wounds. The stem sections were then placed onto water agar and incubated at 25°C for 6 weeks. Mycelium plugs from seven-day-old cultures were also placed on oatmeal agar and incubated at 25°C for 6 weeks. *S. cordatum* seedlings were inoculated (five repeats per isolate, per method) as described below in the pathogenicity section, and kept at 25°C for 6 weeks.

Fruiting structures formed on *Eucalyptus* clone ZG14 and *S. cordatum* stem sections, oatmeal agar and *S. cordatum* seedlings, as well as those on original bark material collected from the field, were cut from the bark under a dissection microscope. Thin sections of resultant

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fruiting structures were made by hand and crushed on microscope slides in 3% potassium hydroxide (KOH) to observe conidia, conidiophores and conidiogenous cells (Gryzenhout *et al.* 2009). Twenty measurements of each of the above mentioned structures were taken and are presented as (min–)(average-S.D.) – (average+S.D.)(–max) mm. Fifty measurements of asci, ascospores, conidia, conidiophores and conidiogenous cells were taken for fruiting structures on bark from natural infections. Digital images were captured with a HRc Axiocam digital camera and measurements were computed using AXIOVISION 3.1 software (Carl Zeiss Ltd., Germany). Characteristics of fruiting bodies were compared to characteristics published for *Celoporthe* spp. (Nakabonge *et al.* 2006a, Gryzenhout *et al*. 2009, Chen *et al*. 2011).

The cultural characteristics of representative isolates (TABLE 1) of the sub-clades were assessed using MEA medium. Discs were taken from the edges of actively growing cultures on MEA and plated onto the middle of 90 mm Petri dishes containing MEA. Five plates per isolate were placed in the dark in incubators set at temperatures ranging from 15˚C to 35˚C. Two measurements perpendicular to each other were taken of each plate until the fastest growing culture covered the plate. Colour notations of Rayner (1970) were used to describe cultures.

2.3 PATHOGENICITY TESTS

Pathogenicity experiments were conducted with representative isolates of the sub-clades observed in this study (TABLE 1). Ten two-year-old plants of *Eucalyptus* clone ZG14 that has previously been shown to be highly susceptible to both *Chr. austroafricana* and *Cel. dispersa* (Van Heerden and Wingfield 2001, Nakabonge *et al.* 2006a) and ten *S. cordatum* seedlings were inoculated with each isolate. Trees were kept at 25˚C in a greenhouse under natural

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day/night conditions for two weeks to acclimatize, after which they were inoculated. For inoculations, the bark was removed from trees using a 5 mm cork-borer, and a mycelial plug was placed into the wound, with the mycelium facing the cambium. For controls, sterile MEA plugs were placed into the wounds. Wounds were sealed with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum plugs.

Six weeks after inoculation, the bark associated with the inoculation sites was removed and the lengths of lesions on the cambium were measured. Pieces of necrotic tissue were transferred to MEA to re-isolate the inoculated fungi. The pathogenicity trial was repeated once under the same conditions. Variation in lesion lengths was assessed in SAS® version 8.2 using the general linear model command (PROC GLM) (SAS Institute 1999).

3 RESULTS

3.1 FUNGAL ISOLATES

Ten isolates previously identified as *Cel. dispersa* were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (TABLE 1) and two from herbarium specimens from Zambia (Vermeulen *et al.* 2011). Two additional isolates were made from newly collected herbarium material obtained during field collections from *S. legatti* in Soutpansberg (South Africa), and two from *Het. canescens* in Lydenburg (South Africa) collected from the same area as those from the study by Nakabonge *et al.* (2006a). Infections on *S. legatti* in Soutpansberg and on *Het. canescens* in Lydenburg in South Africa were relatively inconspicuous with infections found on branch

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stubs and branch cankers. Despite continued and widespread surveys, also in other areas of southern Africa, these were the only additional specimens that could be obtained.

3.2 IDENTIFICATION

3.2.1 DNA sequence comparisons

Sequence data sets consisted of 27 to 31 taxa (TABLE 1) and the aligned DNA sequence dataset varied from 268bp for the BT dataset to 1577bp for the combined ITS, BT and TEF1α data set (TABLE 2). Results of the PHT showed that datasets for the three gene regions were homologous (*P* =0.072) and could be combined (Cummings *et al*. 1995). This was also supported by the trees for each gene region that essentially had the same topology and support for the various clades. Based on the ITS, BT and TEF1α data sets individually (FIG 2a–c), as well as the combined dataset (FIG 2d), isolates from Africa grouped in three distinct clades.

The African clades grouped separatey from the four new species described recently from Asia (Chen *et al*. 2011). Isolates from *S. legatti* (Limpopo Province, South Africa) and *Het. canescens* (Mpumalanga Province, South Africa) grouped with the type specimen of *Cel. dispersa* [MP and ML, 1000 replicate bootstrap analysis indicated as (MP/ML), 99/100]. Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa) (99/100) and *S. guineense* (North Western Province, Zambia) (98/100) formed two additional sub-clades (FIG 2d). Results of the parsimony analyses correlated with those for the Maximum Likelihood analyses. The three sub-clades in southern Africa were observed in all gene regions as well as the combined gene data (FIG. 2a–d).

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The different phylogenetic sub-clades observed were associated with uniquely fixed DNA nucleotides (TABLE 3). Several differences in the number of mononucleotide repeats were observed in non-protein coding regions for sequences of the ITS, BT and TEF1α regions. These were excluded from the aligned data sets as the true repeat number could not be confirmed.

3.2.2 Morphology

Most isolates representing the three African sub-clades (FIG 2d) could be induced to sporulate when inoculated into sterilized *Eucalyptus* clone ZG14 and *S. cordatum* stem sections on water agar. Only anamorph structures were, however, observed on inoculated material. Conidiomata resulting from the various types of inoculations differed in shape and position, relative to specimens from bark from natural infections, consistent with previous observations (Chen *et al*. 2011). However, the conidia, conidiogenous cells and paraphyses resulting from inoculations were similar to natural infection, also corresponding to observations by Chen *et al*. (2011).

There were bark specimens for *Het. canescens* in Lydenburg, *S. legatti* in Soutpansberg and *S. guineense* in Zambia. Fruiting structures on bark resembled those of *Cel. dispersa* (Nakabonge *et al.* 2006a, Gryzenhout *et al.* 2009). Only teleomorph structures were present on bark specimens from *Het. canescens* in Lydenburg, and only anamorph structures were present on bark specimens from *S. legatti* in Soutpansberg and *S. guineense* in Zambia. Teleomorph structures on *Het. canescens* from Lydenburg, and anamorph structures from *S. legatti* in Soutpansberg were similar to those previously described for *Cel. dispersa* (TABLE 2). However, anamorph structures from *S. guineense* (Zambia) differed from specimens on

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Het. canescens from Lydenburg and those previously described for *Cel. dispersa* (Nakabonge *et al*. 2006a) because the conidiomata were multilocular, whereas those of *Cel. dispersa* and specimens on *Het. canescens* from Lydenburg and Soutpansberg are typically unilocular. The conidiomatal and ascostromatal morphology of the *T. granulosa* (KwaZulu-Natal Province, South Africa) group of isolates could not be assessed because there were inadequate specimens representing naturally infected tissue.

Artificial inoculations revealed morphological differences corresponding to the three sub-clades from Africa defined with DNA sequence data. These three sub-clades (FIG 2d) could be differentiated based on conidial size, although in general there was an overlap between characteristics of conidia, conidiogenous cells and paraphyses. Isolates representing the *Cel. dispersa* s.s. sub-clade had longer (max 5.5 µm) and wider conidia (max 2.5 µm) than isolates representing the sub-clade from *T. granulosa* (KwaZulu-Natal Province, South Africa) (max 4.5 µm × 2 µm), and wider conidia than isolates from *S. guineense* in Zambia (max 6 µm × 2 µm). Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa) had conidia that were oblong to cylindrical and occasionally allantoid (FIG 3i) while those from *S. guineense* in Zambia had conidia that are oblong to cylindrical and occasionally ovoid (FIG 3f). Additional differences among the sub-clades were observed based on colony characteristics. The optimal temperature for growth of isolates from *S. guineense* (Zambia) was 30°C while those from South Africa grew optimally at 25°C. Colony color and texture were, however, similar.

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3.2.3 Taxonomy

Based on phylogenetic analyses of DNA sequence data for three gene regions, isolates of *Celoporthe* from Africa resided in three different sub-clades, separate from recently described species from China and Indonesia. These three sub-clades can be identified based on uniquely fixed nucleotides (TABLE 3), a combination of morphological characteristics from natural material and artificial inoculations, and geographic separation. Isolates from Soutpansberg and Lydenburg grouped with those of the type specimens for *Cel. dispersa* from *S. cordatum* (Limpopo Province, South Africa). Isolates from *T. granulosa* (KwaZulu-Natal Province, South Africa), however, grouped separately from other isolates and had conidia that are oblong to cylindrical, occasionally allantoid. Isolates from *S. guineense* in Zambia grouped separately based on all three gene regions, are geographically separated from other groups and can be morphologically distinguished from other groups in Africa based on multilocular conidiomata as opposed to unilocular conidiomata, and conidia that are oblong to cylindrical and occasionally ovoid. The African isolates are, therefore, recognized as representing three distinct species, namely *Cel. dispersa* and two previously unrecognized species described as follows:

Celoporthe fontana M. Verm., Gryzenh. & Jol. Roux, sp. nov. prov. nom. (FIG 3a–f)

MycoBank MB563045

Etymology: *fontana* (Latin) “of the spring” referring to the origin of the Zambezi River, which is close to the type locality of this species.

Latin: Teleomorpha ignota. Conidiomata globosa conica vel pulvinata, subimmersa fucosa vel nigra, multilocularia; loculi superficiebus interioris laevis vel convolutis. Textura stromatis pseudoparenchymata, cinnamomea vel brunnea. Conidiophorae hyalinae, basi vel

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supra irregulariter in cellulas cylindricas ramosae, cum vel sine septis. Conidia hyalina non septata, oblonga vel cylindrica nonnunquam ovoidea apicibus rotundatis. A speciebus aliis in clado africano Celoporthes (C. dispersa, woodianaque) nucleotidis 'DNA' egregie fixis in locis tribus: loco vulgo dictu 'β-tubulin-1' et 2, 243(T) et 632(C), vulgo dictu 'internal transcribed spacer rDNA' locis 111(G), 176(T), 177(G), et vulgo dictu 'translation elongation factor 1-alpha' locis 31(G), 164(T) et 217(G).

Teleomorph: Not known

Conidiomata: *Herbarium specimen:* Globose, conical to pulvinate, semi-immersed, fuscous to black, conidiomatal bases above the bark surface 290–380 µm high, conidiomatal diameter 280–590 µm. Conidiomatal locules with even to convoluted inner surfaces, multilocular. Stromatic tissue pseudoparenchymatous, cinnamon to brown. Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, (5.0–)7.5–14.0(–22.5) × (1.0–)1.5(–2.0) µm. Paraphyses occuring among conidiophores, up to 77 µm long. Conidia (3.0–)3.5(–4.0) × 1.0–1.5 µm, hyaline, non-septate, oblong to cylindrical, occasionally ovoid, with rounded apices, exuded as tendrils or droplets.

On inoculated material: Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, on *S. cordatum* stem sections (7.5–)9.0–29.0(–26.0) × 2(–3) µm, on *Eucalyptus* ZG14 stem sections (6.0–)7.5–16.0(–23.0) × (1.0–)1.5–2.0(–2.5) µm, on oats agar (10.5–)11.0–22.5(–21.5) × 2(–4) µm and on *S. cordatum* trees (7.0–)9.5–16.5(–19.0) × (1.0–)1.5(–2.0) µm. Paraphyses occurring among conidiophores, up to 88 µm long on *S. cordatum* trees. Conidia on *S. cordatum* stem sections (3.5–)4.5–5.0(–5.5) × (1.0–)1.5–1.5(–2.0) µm, on *Eucalyptus* ZG14 stem sections (4.0–)4.5–5.5(–6.0) × 1.5(–2.0) µm, on oats agar (3.5–)4.5(–5.0) × 1.5 µm and on *S. cordatum* trees

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(3.0–)3.5(–4.0) × (1.0–)(–1.5) µm, hyaline, non-septate, oblong to cylindrical, occasionally ovoid, with rounded apexes, exuded as tendrils or droplets.

Fixed base pair differences: *Celoporthe fontana* differs from other species in the African clade of *Celoporthe* (*Cel. dispersa* and *Cel. woodiana*) by uniquely fixed DNA nucleotides in three nuclear loci: β-tubulin1 and 2 positions 243 (T) and 632 (C); internal transcribed spacer rDNA (ITS1, 5.8S, ITS2) positions 111 (G), 176 (T) and 177 (G); translation elongation factor 1-alpha positions 31 (G), 164 (T) and 217 (T).

Culture characteristics: Cultures on MEA white with grey patches, fluffy with uneven margins, changing from umber to hazel to chestnut, fast-growing, covering a 90 mm diam plate in 4–5 days at the optimum temperature of 30 °C.

Specimens examined: ZAMBIA, NORTH WESTERN PROVINCE: Kaleni Hills, Ikelenge. Isolated from bark of *S. cordatum,* 2006, M. Vermeulen and J. Roux. HOLOTYPE, PREMxxxx ex-type culture CMW29376/CBSxxxx, PARATYPE, PREMxxxx (isolate CMW29375 artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), living culture CMW29375/CBSxxxx.

Celoporthe woodiana M. Verm., Gryzenh. & Jol. Roux, sp. nov. prov. nom. (FIG 3g–i)

MycoBank MB563046

Etymology: Named after Dr. Medley Wood, one of the first mycologists in South Africa, who worked in the Durban Botanic Gardens. This location represents the first and only locality where this species has been found.

Latin: Teleomorpha ignota. Conidiomata in speciminis herbarii non ad manum; in speciminis vivis conidiophorae hyalinae, basi vel supra irregulariter in cellulas cylindricas ramosae, cum vel sine septis. Conidia hyalina non septata, oblonga vel cylindrica nonnunquam allantoidea apicibus rotundatis. A speciebus aliis in clado africano Celoporthes (C. dispersa, fontanaque)

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nucleotidis 'DNA' egregie fixis in locis duobus: loco vulgo dictu 'β-tubulin-1' et 2, 133 (T), 189 (T)and 645 (A); ITS1, 5.8S, ITS2, loco 412(C).

Teleomorph: Not known

Conidiomata: *On herbarium specimens*: not available. *On inoculated material:* Conidiophores hyaline, irregularly branched at the base or above into cylindrical cells, with or without separating septa, on *S. cordatum* stem sections (6.0–)8.5–16.5(–21.5) × (1.0–)1.5–2.0(–2.5) µm, on *Eucalyptus* ZG14 stem sections (5.0–)6.0–13.0(–17.0) × (1.0–)1.5–2.0(–2.5) µm and on oats agar (8.0–)8.5–17.5(–25.0) × (1.5–)2.0(–3.0) µm. Paraphyses occuring among conidiophores, up to 55 µm long on *S. cordatum* trees. Conidia on *S. cordatum* stem sections (3.0–)3.5–4.0(–4.5) × (1.0–)1.5(–2.0) µm, on *Eucalyptus* ZG14 stem sections 4.0–4.5 × (1.0–1.5) µm and on oats agar (3.5–)–4.0(–4.5) × 1.0–1.5 µm, hyaline, non-septate, oblong to cylindrical, occasionally allantoid, with rounded apex, exuded as luteous tendrils or droplets.

Fixed base pair differences: *Celoporthe woodiana* differs from other species in the African clade of *Celoporthe* (*Cel. dispersa* and *Cel. fontana*) by uniquely fixed DNA nucleotides in two nuclear loci: β-tubulin1 and 2 positions 133 (T) 189 (T) and 645 (A); internal transcribed spacer rDNA (ITS1, 5.8S, ITS2) position 412 (C).

Culture characters: Cultures on MEA white with grey patches, fluffy with uneven margins, changing to umber, hazel to chestnut, fast-growing, covering a 90 mm diameter plates in 4–5 days at the optimum temperature of 25 °C.

Specimens examined: SOUTH AFRICA, KWAZULU-NATAL: Durban Botanical garden. Isolated from bark of *T. granulosa,* 2006, M. Gryzenhout. HOLOTYPE, PREMxxxx (isolate CMW13936 artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), ex-type culture CMW13936/CBS118785, PARATYPE, PREMxxxx (isolate CMW13937

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artificially inoculated on *S. cordatum* stem section, 2007, M. Vermeulen), living culture CMW13937/CBSxxxx.

DICHOTOMOUS KEY TO *CELOPORTHE* SPECIES (BASED ON ANAMORPH STRUCTURES ONLY)

1a. Optimal growth at 25 °C, conidiomata unilocular* ……………………………………2

1b. Optimal growth at 30 °C, conidiomata multilocular…………………………...................3

2a. Conidial length shorter than 4.5 µm……………………………...……***Cel. woodiana***

2b. Conidial length can be longer than 4.5 µm……………………………….…..***Cel. dispersa***

3a. Conidia, cylindrical, occasionally oblong...***Cel. indonesiensis***

3b. Conidia oblong to cylindrical...4

4a. Conidia occasionally ovoid..***Cel. fontana***

4b. Conidia occasionally allantoid...5

5a. Conidia shorter than 4 µm..***Cel. syzygii***

5b. Conidia longer than 4 µm...6

6a. Paraphyses shorter than 70 µm...***Cel. eucalypti***

6b. Paraphyses longer than 90 µm..***Cel. guangdongensis***

*Not known for *Cel. woodiana.*

3.3 PATHOGENICITY TESTS

Six weeks after inoculation, lesions were visible on all trees except those inoculated with sterile MEA plugs. Statistical analyses showed a significant difference in lesion lengths on trees inoculated with the test fungi and the controls for both *Eucalyptus* clone ZG14 and *S.*

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cordatum ($p<0.05$). Data for the two trails could not be combined, as there were statistical differences between repeats ($p>0.05$) (FIG 4).

No statistical differences were observed between the lengths of lesions formed on the *Eucalyptus* clone ZG14 by *Cel. dispersa* (Avg =29.8 mm), *Cel. fontana* (Avg =30.1 mm) and *Cel. woodiana* (Avg =30.5) ($p>0.05$), for both repeats one and two. Statistical differences were, however, observed for lesion lengths on *S. cordatum* ($p<0.05$), and the results for the first and second repeat differed. For the first repeat there was a statistical difference ($p<0.05$) in lesion length on *S. cordatum* between *Cel. woodiana* (Avg = 28.7 mm) and *Cel. dispersa* (Avg = 19.9 mm) as well as between *Cel. woodiana* (Avg = 28.7 mm) and *Cel. fontana* (Avg = 16.6 mm). For the second repeat there was a statistical difference ($p<0.05$) in lesion length on *S. cordatum* between *Cel. dispersa* (Avg = 29.8 mm) and *Cel. fontana* (Avg = 19.6 mm) and *Cel. dispersa* (Avg = 29.8 mm) and *Cel. woodiana* (Avg = 22.1 mm). There was also a statistical difference in lesion length formed on *Eucalyptus* clone ZG14 and *S. cordatum* seedlings for isolates CMW 9976, CMW 29375, CMW 29376 and CMW 29378 in repeat one, and CMW 29376 in repeat two ($p<0.05$). In all of these variable isolates, the lesions formed on *Eucalyptus* clone ZG14 were longer than those on *S. cordatum*. There was no statistical difference in lesion length formed on the *Eucalyptus* clone ZG14 and *S. cordatum* seedlings for the rest of the isolates ($p>0.05$). Fruiting structures formed on inoculated stems of both tree species and *Cel. dispersa, Cel. fontana* and *Cel. woodiana* was re-isolated from wounds.

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4 DISCUSSION

In this study two previously undescribed species of *Celoporthe* were identified from Africa and these occurred on native *S. guineense* and non-native *T. granulosa* trees. *Celoporthe woodiana* was originally isolated from *T. granulosa* (KwaZulu-Natal Province, South Africa), but was treated as *Cel. dispersa* because no morphological differences could be identified at that time (Nakabonge *et al*. 2006a). *Celoporthe fontana* was recently isolated from *S. cordatum* in Zambia, but this species was also not described at the time of collection due to lack of available herbarium specimens for morphological comparisons (Vermeulen *et al*. 2011). A combination of morphology from naturally infected material and inoculated specimens as well as multi-gene phylogenies generated in this study made it possible to describe these *Celoporthe* species. The known distribution of *Cel. dispersa* was expanded and it currently includes isolates from Limpopo Province and Mpumalanga Province, South Africa on *S. cordatum* and *S. legatti* (Nakabonge *et al*. 2006a, Vermeulen *et al*. 2011).

Using morphological comparisons as a taxonomic characteristic in a genus such as *Celoporthe* is difficult. This is due to the limited availability of specimens to examine, which often results in incomplete morphological descriptions, especially where one of the morphic states is absent (Nakabonge *et al*. 2006a, Chen *et al*. 2011, Vermeulen *et al*. 2011). When studying the morphology of the Cryphonectriaceae, it is best to use originally collected specimens of natural infections (Gryzenhout *et al.* 2009) because fruiting structures produced in culture or in artificial inoculations usually exhibit variation in structure, size and shape (Myburg *et al.* 2002, 2003, Hodges *et al.* 1986, Chen *et al.* 2011). The shape and position of conidiomata of *Celoporthe* species produced on inoculated stems in this study also differed from those collected in the field. It is, therefore, important to use these structures with caution

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and, where possible, to compare fruiting structures formed during artificial inoculations with those from natural infections when using them for taxonomic descriptions (Kobayashi 1970).

This study relied strongly on the phylogenetic species concept (PSC) (Taylor *et al*. 2000) to recognize the new species *Cel*. *fontana* and *Cel*. *woodiana.* This is similar to the approach recently taken to recognize *Chr. deuterocubensis* as distinct from the well-known *Eucalyptus* pathogen *Chrysoporthe cubensis* (Van der Merwe *et al.* 2010). Chen *et al.* (2011), similarly described *Cel*. *eucalypti*, *Cel. guangdongensis, Cel. indonesiensis* and *Cel*. *syzygii* based on single base pair differences between species and supporting but overlapping morphological differences.

Results of this study add substance to the view that *Celoporthe* species have a widespread, yet possibly structured occurrence in Africa and Asia. Isolates from Asia and Africa group separately, indicating a possible geographical distinction. In Asia, species currently are only known from China (3 species) and Indonesia (1 species). Given that there have been few studies of this group (Chen *et al.* 2011) the diversity of these fungi in Asia has most likely only been partially sampled and nothing is known of their origin. None of the recorded hosts in the regions are native to the areas where the *Celoporthe* species were found. In Africa, however, surveys for Cryphonectriaceae have been undertaken widely in South Africa, and also in other African countries such as Kenya, Mozambique and Namibia (Nakabonge *et al*. 2006a, 2006b, Vermeulen *et al.* 2011). Only three *Celoporthe* species were found in these surveys, of which two occur on native trees. *Celoporthe fontana* was found only in the North West Province of Zambia and *Cel. woodiana* only in the KwaZulu-Natal Province of South Africa. It seems likely that these species are native to southern Africa.

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Pathogenicity tests in this study showed that *Cel. dispersa, Cel. fontana* and *Cel. woodiana* are pathogenic to *Eucalyptus* clone ZG 14 and *S. cordatum* seedlings under greenhouse conditions. The variation observed in lesion lengths for *Cel. dispersa, Cel. fontana* and *Cel. woodiana* on *S. cordatum* between the two inoculation trials could be due to differences in host resistance as the plants were propagated from seed and individuals would, therefore, differ in susceptibility. However, the results provide clear evidence of pathogenicity in these fungi and they could emerge as important pathogens of *Eucalyptus* in the future, as has been shown with *Chr. austroafricana* (Wingfield 2003, Gryzenhout *et al.* 2009).

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TABLE I. Isolates used in DNA sequence comparison, inoculations and pathogenicity trails

Identity	Isolate number CMW [A]	CBS [B]	PREM [C]	Host	Country and province	Collector	GenBank accession numbers [D]
Cel. dispersa	13645	119119	58899	*H. canescens*	South Africa, Mpumalanga	G Nakabonge, J Roux and M Gryzenhout	DQ267134, DQ267140, DQ267146
	9978	118781	58896	*S. cordatum*	South Africa, Limpopo	M Gryzenhout	AY214316, DQ267135, DQ267141
	9976	118782	58897	*S. cordatum*	South Africa, Limpopo	M Gryzenhout	DQ267130, DQ267136, DQ267142
	9977			*S. cordatum*	South Africa, Limpopo	M Gryzenhout	
	32952			*S. cordatum*	South Africa, Mpumalanga	M Vermeulen, M Gryzenhout	
	32951			*S. cordatum*	South Africa, Mpumalanga	M Vermeulen, M Gryzenhout	
	29378			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726942, GU726954
	29898			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726943, GU726955
	29900			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	GU726944, GU726956
	29901			*S. legatti*	South Africa, Limpopo	M Vermeulen, J Roux	
	29903			*S. legatti*	South Africa, Limpopo	M Vermeulen, J	

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Identity	Isolate number CMW [A]	CBS [B]	PREM [C]	Host	Country and province	Collector	GenBank accession numbers [D]
						Roux	
	29905			*S. cordatum*	South Africa, Limpopo	M Vermeulen, J Roux	GU726945, GU726957
Celoporthe eucalypti	26900	127191		*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730816, HQ730826, HQ730836, HQ730849
	26908	127190		*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730817, HQ730827, HQ730837, HQ730850
	26911	127192		*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730818, HQ730828, HQ730838, HQ730851
	26913			*Eucalyptus* EC48 clone	China, GuangDong	XD Zhou, SF Chen	HQ730819, HQ730829, HQ730839, HQ730852
Cel. fontana	29375			*S. guineense*	Zambia, North Western	M Vermeulen, J Roux	GU726940, GU726952
	29376			*S. guineense*	Zambia, North Western	M Vermeulen, J Roux	GU726941, GU726953
Cel. guangdongensis	12750			*Eucalyptus* sp.	China, GuangDong	TI Burgess	
Cel. indonesiensis	10781	115844		*S. aromaticum*	Indonesia, North Sumatra	MJ Wingfield	AY084021, AY084033, AY08400
	10779			*S. aromaticum*	Indonesia, Somosir	MJ Wingfield	AY084019, AY084031, AY084007
	10780			*S. aromaticum*	Indonesia, Somosir	MJ Wingfield	AY084020, AY084032, AY084008
Cel. syzygii	34023	127218		*S. cumini*	China GuangDong	SF Chen	HQ730811, HQ730821, HQ730831, HQ730844

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Identity	Isolate number			Host	Country and province	Collector	GenBank accession numbers [D]
	CMW [A]	CBS [B]	PREM [C]				
	34024			*S. cumini*	China GuangDong	SF Chen	HQ730812, HQ730822, HQ730832, HQ730845
	24912	127188		*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730813, HQ730823, HQ730833, HQ730846
	24914	127189		*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730814, HQ730824, HQ730834, HQ730847
	24917			*S. cumini*	China GuangDong	MJ Wingfield, XD Zhou	HQ730815, HQ730825, HQ730835, HQ730848
Cel. woodiana	13936	118785	*58901*	*T. granulosa*	South Africa, Kwazulu-Natal	M Gryzenhout	DQ267131, DQ267137, DQ267143
	13937			*T. granulosa*	South Africa, Kwazulu-Natal	M Gryzenhout	DQ267132, DQ267138, DQ267144

A Culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

B CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

C PREM, Agricultural Research Council, Pretoria, South Africa.

D GeneBank accession numbers for sequence data of the ITS (primers ITS1/2), BT 1 and 2 (primers BT1a/1b and BT21/2b) and Translation elongation factor 1-alpha positions (primers EF1-728 and EF986R).

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TABLE II. Statistics resulting from Maximum Parsimony analyses

Statistic	ITS	BT	TEF1α	Combined gene regions
Number of taxa	31	31	27	27
Aligned characters	497	812	268	1577
Constant characters	425	696	248	
Parsimony-uninformative characters	14	7	3	
Parsimony-informative characters	58	109	17	
Tree length	94	140	22	113
Consistency index (CI)	0.989	0.921	0.955	0.920
Retention Index (RI)	0.998	0.979	0.992	0.988
Rescaled consistency index (RC)		0.902	0.947	0.910

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TABLE III. Unique fixed DNA nucleotides in ITS, BT and TEF1α sequences for African *Celoporthe* clade

Species	Isolate number	ITS			
		111	176	177	412
Cel. dispersa	9978	-	A	A	T
Cel. dispersa	9976	-	A	A	T
Cel. woodiana	13936	-	A	A	C
Cel. woodiana	13937	-	A	A	C
Cel. fontana	29375	G	T	G	T
Cel. fontana	29376	G	T	G	T

Species	Isolate number	BT											
		133	142	177	179	180	182	189	194	243	632	633	645
Cel. dispersa	9978	C	T	A	C	C	C	C	T	C	T	T	C
Cel. dispersa	9976	C	T	A	C	C	C	C	T	C	T	T	C
Cel. woodiana	13936	T	C	C	T	T	T	T	A	C	T	C	A
Cel. woodiana	13937	T	C	C	T	T	T	T	A	C	T	C	A
Cel. fontana	29375	C	C	C	T	T	T	C	A	T	C	C	C
Cel. fontana	29376	C	C	C	T	T	T	C	A	T	C	C	C

Species	Isolate number	TEF1α			
		31	164	207	217
Cel. dispersa	9978	A	A	G	-
Cel. dispersa	9976	A	A	G	-
Cel. woodiana	13936	A	A	A	-
Cel. woodiana	13937	A	A	A	-
Cel. fontana	29375	G	T	A	T
Cel. fontana	29376	G	T	A	T

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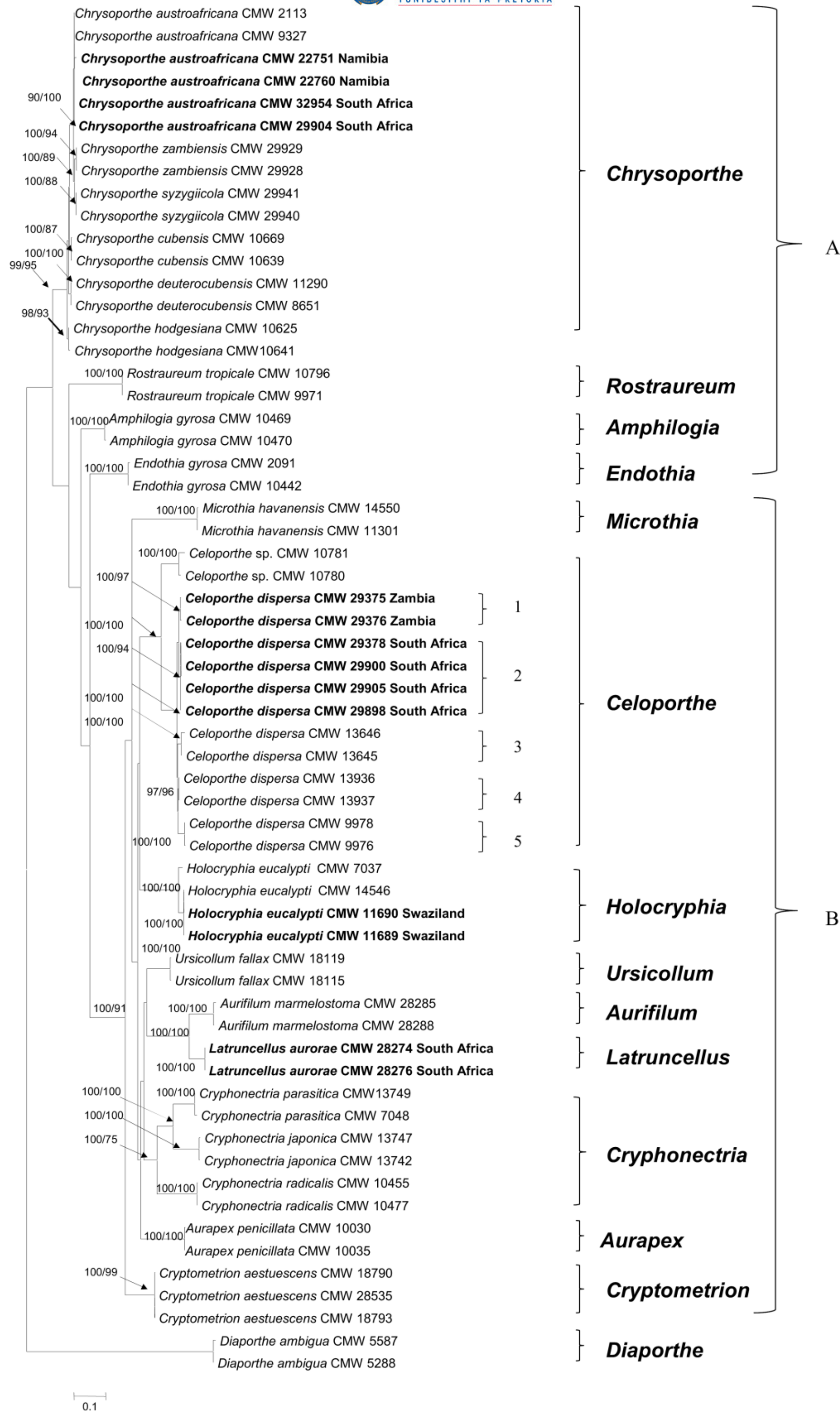

FIGURE 1. Phylogram obtained from the combined data sets of the ITS and BT gene sequences (Vermeulen *et al.* 2010). The phylogram was obtained with Maximum Likelihood analyses using the TrN+I+G parameter model. Confidence levels >70% of the tree branch nodes, determined by posterior probabilities (BPP) and ML, 1000 replicate bootstrap analysis (BS) and are indicated on tree branches (BPP/BS). Isolates sequenced in this study are in bold. Five clades of *Celoporthe dispersa* are marked 1 to 5. *Diaporthe ambigua* was defined as the outgroup taxon.

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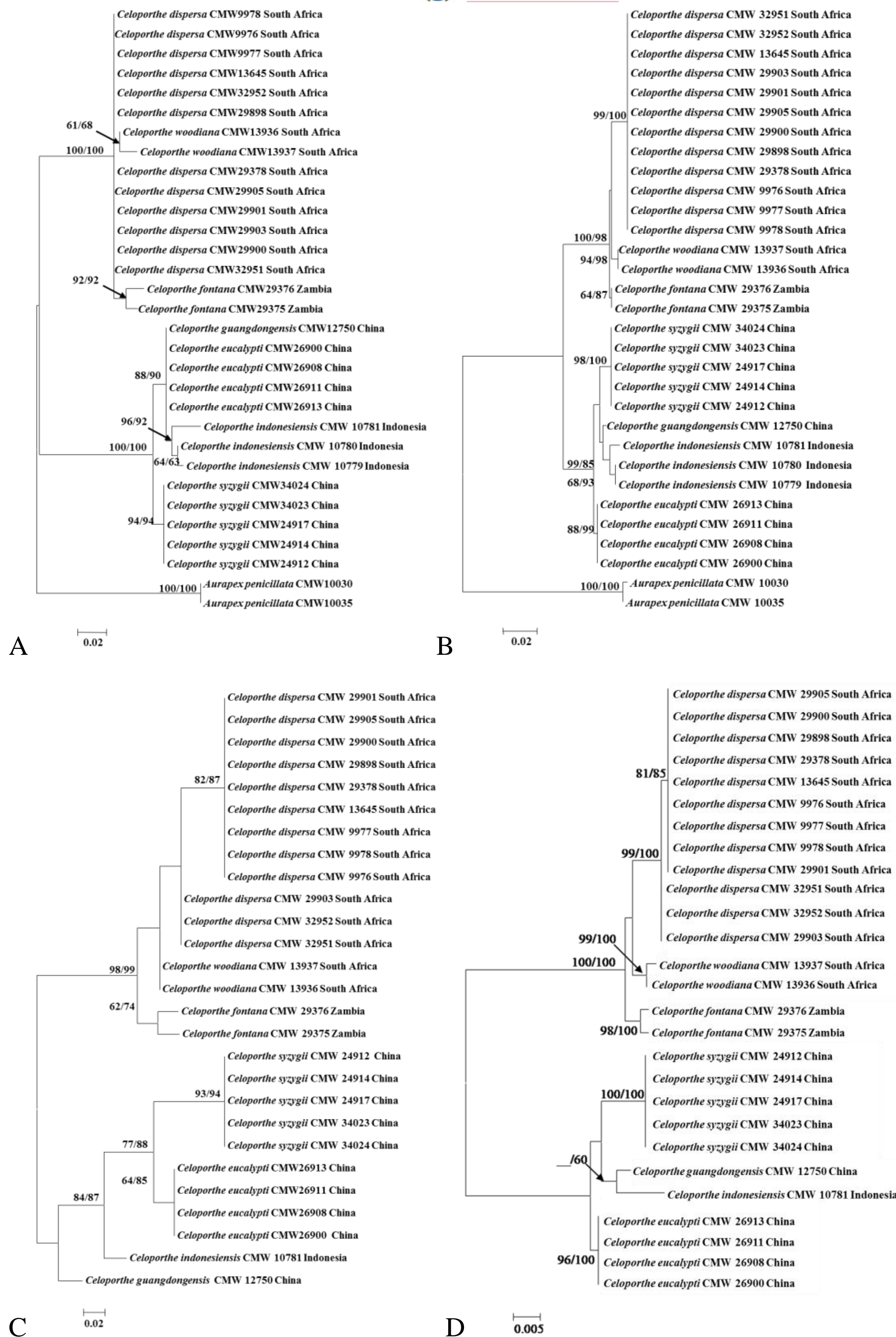

FIGURE 2. Phylograms obtained from Maximum Likelihood analyses (A) Phylogram for ITS gene sequences, obtained using the TrNef+I parameter model. (B) Phylogram for BT gene sequences, obtained using the TIM3+G parameter model. (C) Phylogram for TEF1α gene sequences, obtained using the HKY+I parameter model. (D) Phylogram for combined ITS, BT and TEF1α gene sequences, obtained using the HKY+I parameter model. For all trees confidence levels >60% (1000 replicate bootstrap analysis) of the tree branch nodes determined by MP and ML, are indicated on tree branches (MP/ML). (A–B) *Aurapex penicillata* defined as the outgroup taxon. (C–D) Trees were midpoint rooted.

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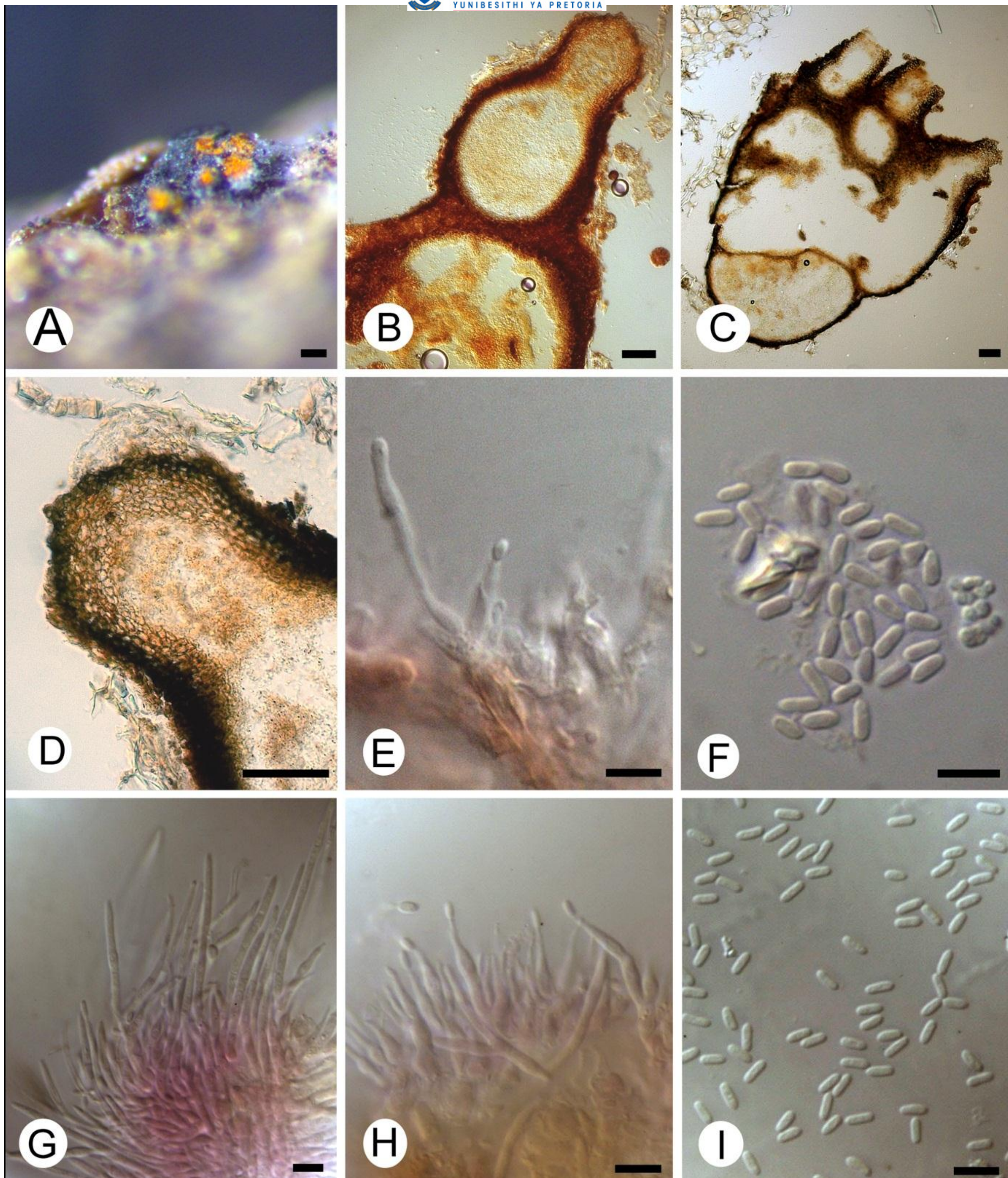

FIGURE 3. Fruiting structure of *Celoporthe fontana* and *Cel. woodiana* (A–F) Fruiting structures of *Cel. fontana* on *Syzygium guineense* trees infected in the field. (A) Conidioma on the bark. (B–C) Longitudinal sections through conidiomata. (D) Stromatic tissue of conidioma. (E) Paraphyses and conidiogenous cells. (F) Conidia. (G–I) Fruiting structures of *Cel. woodiana* on greenhouse inoculated stem tissue of *S. cordatum*. (G) Paraphyses. (H) Conidiogenous cells. (I) Conidia. (scale bar: A = 100 µm; B–D = 50 µm ; E–I = 5 µm.

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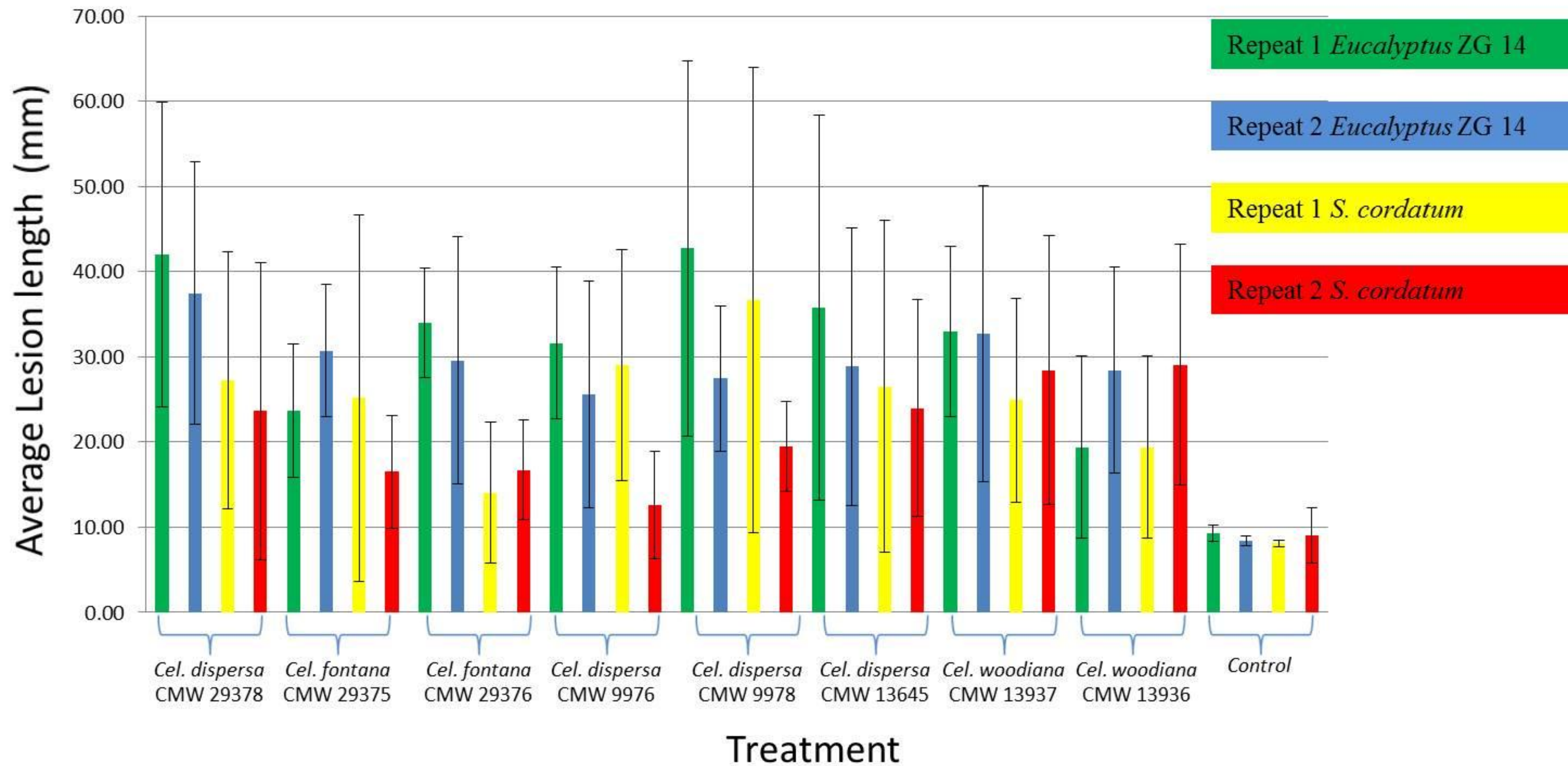

FIGURE 4. Column chart indicating the average lesion length (in millimetres) resulting from inoculation trials for repeat one and two on a *Eucalyptus* ZG 14 clone and *Syzygium cordatum* seedlings under glasshouse conditions. Error bars represent standard deviation for each treatment.

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SUMMARY

The Cryphonectriaceae is a recently established family in the Diaporthales and includes important tree pathogens. Four genera occur in Africa, namely *Aurifilum*, *Celoporthe, Chrysoporthe* and *Holocryphia* and are known tree pathogens able to infect native trees, non-native ornamentals and forestry trees in the Myrtales. In Chapter One of this thesis, the taxonomic history, pathology, possible origins, host range, distribution, symptoms, impact and possible control of Cryphonectriaceae in Africa was considered. This review aimed to provide a background for the study of specimens resembling Cryphonectriaceae collected from southern Africa during surveys throughout 2005-2009. Furthermore, it provided a background for the study of the population diversity of *Chr. austroafricana* in southern Africa and its possible movement within the country and between known hosts. Although great progress has been made in studying the Cryphonectriaceae it is clear that our knowledge of these pathogens in Africa remain limited, despite evidence that the centers of origin of some of them are most likely in Africa. Chapter Two reported, for the first time, *Chr. austroafricana* from Namibia as well as areas in South Africa, outside of its previously known range. *Holocryphia eucalypti* was reported from Swaziland and the host and distribution range of *Celoporthe* species are now known to include *S. legatti* in the Soutpansberg area in South Africa and *S. cordatum* in Zambia. *Latruncellus aurora* gen. sp. nov. was discovered on *Galpinia transvaalica* (Lythraceae, Myrtales) in Swaziland, representing the first report of a member of the Cryphonectriaceae infecting Lythraceae in Africa. In Chapter Three of this thesis the diversity of *Chr. austroafricana* was assessed based on Vegetative Compatibility Groupings (VCGs). A high diversity was observed for *Chr. austroafricana* in southern Africa, with very limited movement of VCGs between different locations and hosts. This high VCG diversity is indicative of a native pathogen,

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confirming previous hypotheses in this regard. This study provides a VCG tester database that will allow assignment of newly discovered isolates to a specific VCG grouping and allowing more rapid identification of new introductions. This technique is especially valuable in that it is cheap and easy to apply, allowing for all laboratories in Africa to be able to use it. Two previously unknown *Celoporthe* species were described in Chapter Four. These are *Cel. fontana,* described from *S. cordatum* in Zambia, and *Cel. woodiana* from *T. granulosa* in South Africa. This chapter relied strongly on the phylogenetic species concept (PSC) to recognize these new species. The PSC is especially helpful in a genus such as *Celoporthe* where limited specimens are available for morphological comparisons and where morphological differences are difficult to identify. This thesis greatly expanded our knowledge on the diversity, distribution and host range of the Cryphonectriaceae in southern Africa and described a novel genus and three novel species. Based on pathogenicity tests all three new species have the potential to cause disease on *Eucalyptus*, a tree of importance in forestry in various southern African countries, indicating that in the future these fungi could emerge as important pathogens of *Eucalyptus* species in Africa and other continents.

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