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A Study of *Chrysoporthe* and *Cryphonectria* species on Myrtales in Southern and Eastern Africa

by

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DECLARATION

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

Grace Nakabonge

June 2006

Dedicated to my family and friends

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PREFACE

Fungi and bacteria cause diseases that pose serious threats to *Eucalyptus* plantations worldwide. Plantations of exotic *Eucalyptus* spp. have tended to grow remarkably well, owing at least in part, to their removal from natural enemies found in their native habitat. However, this advantage has rapidly declined as the movement of people and products have increased the transport of pests and pathogens around the globe. Effective management of these pests and diseases relies on accurate taxonomy and detailed knowledge of the biology, origin and movement of the pathogens involved.

Cryphonectria canker is one of the important fungal diseases that reduce the productivity of *Eucalyptus* plantations in tropical and sub-tropical areas, worldwide. In a number of countries it has necessitated the development of extensive breeding programmes to develop disease-tolerant planting material. However, it still remains a threat to *Eucalyptus* plantations globally, including those in Australia where these trees are native. In recent years, the taxonomy, host range and distribution of *Cryphonectria* spp. have undergone numerous changes. However, information on these pathogens on the African continent has remained largely restricted to what has been reported from South Africa. The aims of studies making up this thesis have been to expand on the knowledge regarding this important group of pathogens in southern and eastern Africa.

In Chapter one of this thesis I present an overview of the most recent findings regarding the taxonomy, host range and distribution of *Cryphonectria cubensis sensu lato* and *Cryphonectria eucalypti* associated with canker of *Eucalyptus* trees. This includes background to the description of a new genus, *Chrysoporthe* Gryzenhout & M.J. Wingf. and three new species previously considered to represent *C. cubensis*. Emphasis is placed on these *Eucalyptus* pathogens in Africa.

The studies presented in chapter two, consider the distribution of *Chrysoporthe* spp. on non-native *Eucalyptus* spp. and native *Myrtales* in southern and eastern Africa. Previous studies have suggested that *Chr. austroafricana* occurs only in South Africa,

while *Chr. cubensis* occurs in Australia, west Africa, Zanzibar, south east Asia and the Americas. In South Africa, *Chr. austroafricana* is a pathogen on non-native *Eucalyptus* (Myrtaceae) and *Tibouchina* spp. (Melastomataceae), both residing in the order *Myrtales* and on native *Syzygium cordatum* trees in the country, leading to the hypothesis that it is native to Africa. In contrast, *Chr. cubensis* is thought to have been introduced into Africa and is known only on non-native *Eucalyptus* and *Syzygium aromaticum* (clove) in four countries. The distribution of *Chrysoporthe* spp. on non-native *Eucalyptus* spp. and native *Myrtales* in southern and eastern Africa is not fully known. The results of a survey of *Chrysoporthe* spp. in this region are discussed, specifically focusing on their identification, using both morphological and DNA sequence data.

Chrysoporthe cubensis is an important fungal pathogen of *Eucalyptus* spp. and many other trees, all-residing in the order *Myrtales*. Previous studies have suggested that *Chr. cubensis* might be native to South America and south east Asia and that it was probably introduced into Africa. Recently, surveys have been conducted in eastern and southern Africa to assess the distribution of *Chrysoporthe* spp. in this region. *Chr. cubensis* was found on *Eucalyptus* spp. in Kenya, Malawi and Mozambique. Chapter three of this thesis compares the genetic diversity of *Chr. cubensis* populations from these countries. Use was made of five pairs of microsatellite markers previously developed for *Chr. cubensis*.

Chapter four treats the development of polymorphic microsatellite markers for the fungal tree pathogen *Cryphonectria eucalypti. Cryphonectria eucalypti,* previously known as *Endothia gyrosa,* is a fungal pathogen of *Eucalyptus* spp. in South Africa and Australia. Nothing is, however, known regarding the population biology of *C. eucalypti,* although it is assumed that the pathogen is native to eastern Australia. Co-dominant markers are especially useful in studies aimed at answering questions relating to genetic diversity, origin and reproduction of fungi due to their high levels of polymorphism and high reproducibility. The technique used to develop the markers and results obtained are discussed, especially the level of polymorphism and reproducibility.

Cryphonectria eucalypti is associated with stem cankers on *Eucalyptus* species in Australia and South Africa. In South Africa it is considered opportunistic and in Australia it has been associated with occasional but serious disease problems. Chapter five of this thesis considers the structure of a South African population of *C. eucalypti* and compares it with three Australian populations of the fungus. Isolates from several *Eucalyptus* spp. and clones in South Africa, are compared with those from *E. globulus* in south western Australia, *Corymbia calophylla* in south western Australia and isolates from *E. dunnii* in eastern Australia. DNA from these isolates was amplified using eight pairs of microsatellite markers previously developed for *C. eucalypti*.

During surveys for *Cryphonectria* and *Chrysoporthe* species on *Myrtales* in South Africa, a fungus resembling *Chr. austroafricana* was collected from native *S. cordatum* near Tzaneen (Limpopo Province), *Heteropyxis canescens* near Lydenburg (Mpumalanga Province) and exotic *Tibouchina granulosa* in Durban (KwaZulu/Natal Province). The fungus was associated with dying branches and stems on *H. canescens* and *T. granulosa*. However, morphological differences were detected between the unknown fungus from these three hosts and known species of *Chrysoporthe*. Chapter six of this thesis presents a study aimed at characterising the unknown fungus using DNA sequence comparisons and morphological features. Pathogenicity tests were also conducted to assess its virulence on *Eucalyptus*, *H. natalensis* and *T. granulosa*.

This thesis is comprised of studies aimed at expanding our knowledge of the taxonomy, distribution and population diversity of *Cryphonectria sensu lato* species in Africa. It represents the first detailed survey of these pathogens in countries such as Kenya, Malawi, Mozambique and Zambia. During these studies a new taxa have emerged and several first reports of *Chrysoporthe* spp. have been made from eastern and southern African countries. Furthermore, the population diversities of *C. eucalypti* and *Chr. cubensis* are considered on different trees, and in different geographical regions, providing what I believe is valuable information regarding their possible origins and movement on the continent.

CHAPTER 1

LITERATURE REVIEW

Taxonomy, host range and geographic distribution of three Eucalyptus canker pathogens previously classified in the genus *Cryphonectria*

1.0 INTRODUCTION

Plantation forestry, particularly plantations of *Eucalyptus* spp., contributes significantly to the economy of many countries. It is currently estimated that there are approximately 14 million hectares of commercial *Eucalyptus* plantations in the world (Turnbull 2000). Furthermore, it is estimated that by the year 2010, approximately 20 million hectares of these trees will have been established globally, if the current planting trends are maintained (Evans 1982, Turnbull 2000). The success of *Eucalyptus* spp., particularly as non-native plantation species, has been due to their adaptation to a wide variety of environments, rapid growth, easy management as well as their valuable wood and pulp properties (Evans 1982, Sutton 1995, 1999, Turnbull 2000).

Diseases caused by fungi and bacteria pose a serious threat to *Eucalyptus* plantations, worldwide. One of the major reasons for this is thought to be the rather narrow genetic base of these plantations, compared to the diversity of the trees in their natural habitats (Wingfield 1990, Turnbull 2000, Potts & Pederick 2000). Another factor that contribute to disease development include offsite planting that subjects trees to stress, resulting in attack by opportunistic pathogens (Florence 2000, Ashton 2000).

Although plantations of exotic *Eucalyptus* spp. initially grew very well due to their separation from natural enemies, this advantage is rapidly disappearing as humans move plants and their natural pests and pathogens around the globe (Wingfield 1999, Keane *et al*. 2000, Wingfield *et al*. 2001a). Extended periods in new habitats, have also led to the threat of pathogens in the new habitat, adapting to *Eucalyptus* as a new host. For example, Eucalyptus rust caused by *Puccinia psidii* Wint., a native pathogen on *Myrtaceae* in South and Central America, has appeared on a variety of exotic *Eucalyptus* spp. causing disease on these exotics (Ferreira 1989, Coutinho *et al*. 1998). It is also a threat to *Eucalyptus* spp. in their native range in Australia and to plantations in other tropical and sub-tropical areas where the disease has not yet been found (Ferreira 1989, Coutinho *et al*. 1998).

The establishment of non-native tree species in new environments can potentially lead to the introduction of the pests and pathogens of those trees, into their new environments. This is not only a threat to the future of the plantations but it poses a threat to plant species native to these countries (Wingfield 1999, Keane *et al*. 2000, Wingfield *et al*. 2001a). A classic example of an exotic pathogen introduced into a native forest environment is the introduction of the chestnut blight pathogen, *Cryphonectria parasitica* (Murrill) M. E. Barr, into the eastern United States in the early 1900's. This disease has devastated *Castanea dentata* (Marsh.) Borkh. (American chestnut) and led to its reduction from a dominant forest species to scattered populations of sprout stems (Anagnostakis 1987).

Numerous diseases have been reported to reduce the productivity of *Eucalyptus* plantations (Keane *et al*. 2000, Old *et al*. 2003). Some of the more important diseases include Cryphonectria canker (Hodges *et al*. 1979, Florence *et al.* 1986, Wingfield *et al.* 1989, Wingfield 2003), Mycosphearella leaf and shoot blight (Crous 1998), Coniothyrium canker (Wingfield *et al*. 1997), Phytophthora root and collar rot (Shear & Smith 2000, Linde *et al*. 1994), Ceratocystis wilt (Roux *et al*. 2000b) Cylindrocladium leaf and shoot blight (Sharma & Mohanan 1982, 1991, Crous *et al.* 1993), bacterial wilt (Coutinho *et al*. 2000) and bacterial blight (Wardlaw *et al.* 2000, Coutinho *et al*. 2002). The occurrence of these diseases has had serious implications for commercial plantation forestry in some countries. In some instances, they have led to the abandonment of the planting of certain *Eucalyptus* spp. This was the case with *Eucalyptus globulus* Labill. in South Africa, which was seriously affected by *Mycosphaerella* spp. (Purnell & Lundquist 1986, Lundquist & Purnell 1987). In other cases, extensive breeding programmes, incorporating clonal forestry, have been initiated to produce disease tolerant material, as was the case with Cryphonectria canker in Brazil and South Africa (Ferreira 1979, Alfenas *et al.* 1983, Wingfield *et al.* 1991, Wingfield & Kemp 1993).

The genus *Cryphonectria*, as it has previously been circumscribed, includes some of the most important canker pathogens of *Eucalyptus* trees. These *Eucalyptus* pathogens are *Cryphonectria eucalypti* M. Venter and M. J. Wingf. [*Endothia gyrosa* (Schwein.) Fr.] and *Cryphonectria cubensis* (Bruner) Hodges. *C. eucalypti* is considered a stress

related yet occasionally important pathogen in Australia (Old *et al*. 1986, Old *et al*. 1990, Wardlaw 1999), but in South Africa, it is a pathogen of minor importance, infecting only stressed trees (Van der Westhuizen *et al*. 1993, Gryzenhout *et al*. 2003). Canker of *Eucalyptus* caused by *C. cubensis* is economically important in many tropical and sub-tropical countries worldwide (Boerboom & Maas 1970, Hodges *et al*. 1976, Gibson 1981, Sharma *et al.* 1985, Wingfield *et al.* 1989, Davison & Coates 1991, Roux *et al.* 2003). Although losses associated with *C. cubensis* infection have been reduced in many situations through breeding and selection of resistant clones/hybrids, the disease is still a serious problem in plantation forestry.

Numerous new hosts and new areas of occurrence have been reported for species of *Cryphonectria sensu lato*, previously only known on *Eucalyptus* spp. The aim of this review is to provide an overview of the most recent findings regarding the taxonomy, host range and distribution of *C. cubensis sensu lato* and *C. eucalypti.* This includes background to the description of a new genus, *Chrysoporthe* Gryzenhout & M.J. Wingf. and three new species previously considered to represent *C. cubensis*. Particular emphasis is placed on these *Eucalyptus* pathogens in Africa.

2.0 *CHRYSOPORTHE* SPP.

2.1 Taxonomy

Chrysoporthe cubensis (Bruner) Gryzenhout & M. J. Wingf., sp. Studies in Mycology 50: 130. 2004

Basionym: *Diaporthe cubensis* Bruner, Estac. Exp. Agron. Cuba Bull. 37: 15-16. 1917.
≡ *Cryphonectria cubensis* (Bruner) Hodges, Mycologia 72: 547. 1980
= *Cryptosporella eugeniae* Nutman & Roberts, Ann. Appl. Biol. 39: 607. 1952.
≡ *Endothia eugeniae* (Nutman & Roberts) J. Reid & C. Booth, Mycologia 78: 347. 1986

Chrysoporthe austroafricana Gryzenhout & M. J. Wingf., sp. Studies in Mycology 50: 133. 2004

The genus *Chrysoporthe* was established in 2004 for the *Eucalyptus* canker pathogen, *Cryphonectria cubensis* (Gryzenhout *et al*. 2004). *C. cubensis* was first described in 1917 by Bruner as *Diaporthe cubensis* Bruner (Bruner 1916, Hodges 1980). Later, *D. cubensis* was transferred to the genus *Cryphonectria* as *C. cubensis* (Hodges 1980). Further studies showed that *Endothia eugeniae* (Nutman & Roberts) Reid & Booth, well-known as the cause of dieback of clove [*Syzygium aromaticum* (L.) Merr. & Perry] and *C. cubensis* were conspecific (Hodges *et al*. 1986, Micales *et al.* 1987).

The first clues to possible differences between the fungus known as *C. cubensis* in different parts of the world emerged when differences in symptom expression of the disease in South America, Southeast Asia and South Africa were noted (Wingfield *et al.* 1989, Gryzenhout *et al*. 2004). Cankers on *Eucalyptus* trees in South Africa are generally limited to the bases of trees. These cankers are characterized by cracking and swelling of the bark resulting in the development of "skirts" on older trees (Wingfield *et al*. 1989, Gryzenhout *et al*. 2004). Younger trees are infected at the root collar, resulting in stem girdling, rapid wilting and death of trees (Wingfield *et al.* 1989, Conradie *et al*. 1990). In contrast, cankers caused by *C. cubensis* in South America and Asia are reported both at the bases as well as higher up on the stems of mature *Eucalyptus* trees (Sharma *et al*. 1985, Ferreira 1989, Wingfield *et al*. 1989, Wingfield 2003). These stem cankers are often formed around branch stubs and have a target shaped appearance (Hodges *et al*. 1979, Sharma *et al*. 1985, Florence *et al*. 1986, Wingfield *et al*. 1989). These differences in symptomology led to the view that the pathogen in South Africa might be different from that in the rest of the world (Myburg *et al*. 2002a). Furthermore, it was noticed that perithecia were rarely or never found on cankers in South Africa, but both perithecia and pycnidia were always abundant on the surface of cankers caused by *C. cubensis* in other parts of the world (Wingfield *et al*. 1989, Van Heerden *et al*. 1997, Van Heerden & Wingfield 2001).

Phylogenetic and morphological studies of *C. cubensis* specimens and isolates from different geographic origins have supported the suggestion that this pathogen might represent more than one species. DNA based studies using β- tubulin and histone H3 gene sequences resulted in phylogenetic trees distinguishing *C. cubensis* isolates from South America, Asia and South Africa (Myburg *et al*. 2002a). Myburg *et al*. (2002a)

showed that *C. cubensis* consists of three well resolved phylogenetic groups, which suggests that the South African fungus represents a species distinct from *C. cubensis,* occurring elsewhere in the world (Myburg *et al*. 2002a). Myburg's (2002a) data also suggested that three possible species were encompassed by the name *C. cubensis*.

Gryzenhout *et al*. (2004), not only supported the split of *C. cubensis* into two species, but they also suggested that *C. cubensis* was not appropriatly placed in the genus *Cryphonectria*. These authors thus, described the new genus, *Chrysoporthe* Gryzenh. & M. J. Wingf., including two species (Gryzenhout *et al*. 2004). *Chrysoporthe austroafricana* Gryzehn. & M. J. Wingf., was established to accommodate isolates residing in the South African phylogenetic group. *Chrysoporthe cubensis* Gryzenh. & M. J. Wingf., accommodates isolates from both South America and southeast Asia, as these two phylogenetic groups could not be separated based on morphology. The anamorph genus *Chrysoporthella* Gryzenh. & M. J. Wingf., was also described to accommodate anamorphs of *Chrysoporthe* (Fig 1) (Gryzenhout *et al*. 2004). In this genus, *Chrysop. hodgesiana* Gryzenh. & M. J. Wingf., was described from Colombia, where it causes severe cankers and dieback of *Tibouchina urvilleana* (DC). Logn. and *T. lepidota* Baill. (Wingfield *et al*. 2001b, Gryzenhout *et al*. 2004) (Fig 1).

2.2 Morphology

Sexual structures of *Chrysoporthe* spp. are characterized by black, valsoid perithecia embedded in bark tissue with perithecial necks covered with amber tissue. (Hodges *et al*. 1979, Sharma *et al*. 1985, Conradie *et al*. 1990, Gryzenhout *et al*. 2004). Each ascus contains 8 spores that are fusiod to ellipsoid (Hodges *et al*. 1979, Gryzenhout *et al*. 2004). Ascospores are hyaline, two celled, fusoid to oval with rounded apices for *Chr. austroafricana* (Fig. 2) and tapered apices for *Chr. cubensis* (Hodges *et al*. 1979, Sharma *et al*. 1985, Gryzenhout *et al*. 2004).

The anamorph of *Chrysoporthe, Chrysoporthella* is characterized by conidiomata that occur individually or at the apices of the ascostromata. They are superficial, fuscous to black in colour, generally pyriform to pulvinate with one to four attenuated necks (Gryzenhout *et al*. 2004). Stromatic tissue at the bases is composed of *textura globulosa* and at the necks of *textura porrecta*. Conidiophores are hyaline with rectangular basal cells. Conidia are hyaline, non septate and oblong, and they are

expelled as bright luteous tendrils (Hodges *et al*. 1979, Wingfield *et al*. 1989, Gryzenhout *et al*. 2004).

In culture (on malt extract agar), *Chrysoporthe* spp. are characterised by white mycelium with cinnamon to hazel patches that are fluffy and colonies have smooth margins. They are fast growing, covering a 90 mm plate in 7 days at an optimum temperature of 30°C. Fruiting structures (anamorphs) are produced in the primary culture but rarely after sub-culturing (Hodges *et al*. 1979, Wingfield *et al*. 1989, Gryzenhout *et al*. 2004).

Chrysoporthe spp. can be distinguished from *Cryphonectria* spp. by the superficial conidiomata that are fuscous to black, pyriform to globose with attenuated necks in the former genus. In contrast, those of *Cryphonectria* spp. are semi-immersed, orange and globose without necks (Myburg *et al*. 2004). Perithecial necks of *Chrysoporthe* spp. are covered with umber tissue whereas the necks of *Cryphonectria* spp. are covered with orange tissue (Myburg *et al*. 2004). The perithecial necks in *Chrysoporthe* spp. usually extend beyond the stromatal surfaces while those of *Cryphonectria* spp. do not develop beyond the stromatal tops (Gryzenhout *et al*. 2004). The ascospores of *Chrysoporthe* spp. are septate and the septa occur at the centre of the cells, whereas those of *Cryphonectria* spp. occur near the apex (Gryzenhout *et al*. 2004). The conidia of *Chrysoporthe* spp. are oblong while those of *Cryphonectria* spp. are more cylindrical (Myburg *et al*. 2004).

2.3. Distribution and host range

Chrysoporthe cubensis (*C. cubensis*) has been known in Africa since the 1950s. The fungus in Africa first entered the record books as *Endothia eugeniae* causing dieback of cloves (*S. aromaticum*) on Unguja Island (Zanzibar) (Nutman & Roberts 1952). On *Eucalyptus* spp., the fungus was first found in the Democratic Republic of Congo (Zaire) and thought to be *Cryphonectria havanensis* (Bruner) M.E. Barr (Gibson 1981) but was later identified as *Chr. cubensis* (Micales *et al.* 1987). *Chr. cubensis* has also been reported to cause disease in young *E. urophylla* S.T. Blake stands in Cameroon (Gibson 1981) and the Republic of Congo (Congo Brazzaville), causing disease on *E. grandis* and *E. urophylla* (Roux *et al*. 2000a, 2003) (Table 1, Fig. 3).

Reports of occurance of *Chrysoporthe* spp. on hosts other than *Eucalyptus* spp. and *S. aromaticum* are increasing in number. Both *Eucalyptus* spp. and *S. aromaticum*, the first known hosts of *Chr. cubensis*, belong to the family *Myrtaceae*, order *Myrtales* (Heywood 1993). The order *Myrtales* includes a relatively large number of families, namely the *Alzateaceae*, *Combretaceae*, *Crypteroniaceae*, *Heteropyxidaceae*, *Lythraceae*, *Melastomataceae*, *Memecylaceae*, *Myrtaceae*, *Oliniaceae*, *Onagraceae*, *Penaeaceae*, *Psiloxylaceae*, *Rhynchocalycaceae* and *Vochysiaceae* (Heywood 1993, Gadek *et al.* 1996, Conti *et al*. 1997).

Chrysoporthe spp. have been confirmed from two families of the order *Myrtales*. These are the *Myrtaceae* and *Melastomataceae* (Hodges 1980, Hodges *et al*. 1986, Gibson 1981, Wingfield *et al*. 2001b, Myburg *et al*. 2002b, Heath *et al*. 2006). In the *Melastomataceae, Chr. cubensis* has been reported in Colombia on indigenous *Miconia theaezans* (Bonpl.) Cogn. and *M. rubiginosa* (Bonpl.) DC. (Rodas *et al*. 2005). The recently described *Chrysop. hodgesiana,* has also been reported from *Tibouchina urvilleana, T. semidecandra* (Schrank & Mart. Ex. DC.) Cogn. and *T. lepidota* on which it causes cankers and dieback of stems and branches in Colombia (Wingfield *et al*. 2001b, Gryzenhout *et al*. 2004) (Table 1, Fig. 3).

Seixas *et al*. (2004) reported a *Chrysoporthe* sp. as a potential pathogen of *Terminalia cattapa* L. and *Laguncularia racemosa* (L.) C. F. Gaertn, which reside in the family *Combretaceae*. They further reported two potential hosts of *Chrysporthe* spp., *Persea americana* Mill. and *Pouteria caimito* (R&P) Radlk. which reside in the orders *Ranales* and *Ebenales* respectively (Seixas *et al*. 2004). The latter orders are very distantly related to the *Myrtales*, suggesting that the fungi in question are probably not related to *Chr. cubensis* and its relatives.

Seixas *et al*. (2004) identified a *Chrysoporthe* sp. on *T. granulosa* in Brazil and treated it as *C. cubensis* (Table 1). From their inoculation trials, it was further reported that *Miconia calvescens* DC. and *Clidemia hirta* (L.) D. Don., which also reside in the *Melastomataceae*, are potential hosts of this fungus. Further studies might, therefore, reveal additional hosts of this important pathogen and may aid in understanding its possible origin and spread.

Chr. cubensis has been reported from two genera in the *Myrtaceae*. The fungus has been known on *Eucalyptus* spp. for many years in countries and regions such as Brazil (Hodges *et al*. 1976, Hodges 1980), Cuba (Bruner 1916), Surinam (Boerboom & Maas 1970, Hodges 1980), Florida (Hodges *et al*. 1979, Hodges 1980), Puerto Rico (Hodges *et al*. 1979, Hodges 1980), India (Sharma *et al*. 1985, Florence *et al*. 1986), Western Samoa (Hodges 1980), Trinidad (Hodges 1980) and Australia (Davison & Tay 1983, Old *et al*. 1986, Davison & Coates 1991) (Table 1, Fig 3). In Australia the fungus has been found associated with root cankers of *Eucalyptus marginata* Donn ex Sm., (Davison & Coates 1991), however, no recent reports of new outbreaks have been documented. *Chrysoporthe cubensis* has also been known on *S. aromaticum*, for many years in countries such as Brazil, Indonesia and Tanzania (Zanzibar) (Nutman & Roberts 1952, Hodges *et al*. 1986) (Table 1, Fig 3).

Chr. austroafricana is known only from Africa, specifically South Africa. The fungus was first reported as *C. cubensis* on the African continent in the 1980's, from South Africa (Wingfield *et al*. 1989). It was found to cause serious losses to clonal *Eucalyptus* plantations in the Zululand area of the country. These outbreaks were specifically significant in as much as clonal *Eucalyptus* forestry had recently been established in the area (Wingfield *et al*. 1989).

More recently *Chr. austroafricana* has been reported from two new hosts in South Africa. They include species in the *Myrtaceae* and *Melastomataceae* (Myburg *et al*. 2002b, Heath *et al*. 2006). In South Africa, *Tibouchina* spp. (*Melastomataceae*) are widely planted in gardens and along streets, for ornamental purposes. The discovery of *Chr. cubensis* on *Tibouchina* spp. in Colombia (Wingfield *et al*. 2001b) initiated a survey of *Tibouchina* trees in South Africa and other countries where *Tibouchina* spp. are found. These surveys led to the discovery of *Chr. austroafricana* causing dieback and death of branches of these trees (Myburg *et al*. 2002b) (Table 1). Recent surveys in South Africa also revealed the presence of *Chr. austroafricana* on native South African *Syzygium cordatum* Hachst. and *S. guineense* (CD.) (Heath *et al*. 2006) (Table 1). This was the first report of *Chr. austroafricana* on native hosts in Africa (Heath *et*

al. 2006) and its discovery has supported the contention that *Chr. austroafricana* might be native to the African continent (Heath *et al*. 2006).

2.4 Origin and population diversity

Population studies provide valuable information regarding the possible origin, movement and recombination within a pathogen population. These issues are important to establish suitable quarantine and management strategies for pathogens (McDonald & McDermott 1993, McDonald 1997). Early studies on the population biology of *Chr. cubensis* and *Chr. austroafricana*, made use of vegetative compatibility group (VCG) studies (Van der Merwe 2000, Van Heerden & Wingfield 2001). More recently, this knowledge has been expanded through the application of polymorphic molecular markers in the form of microsatellite/simple sequence repeat markers (Van der Merwe *et al*. 2003, Heath 2004).

2.4.1 *Chr. austroafricana*

Population diversity studies using VCGs on a South African population of *Chr. austroafricana* consisting of 100 revealed a very low genotypic diversity (0.095%) within the population (Van Heerden & Wingfield 2001). At that stage, the pathogen was considered to be identical to *Chr. cubensis* and had been known in South/Central America and Asia for many years, including on native *S. aromaticum* in Asia. Thus, the emerging hypothesis from VCG studies was that the fungus had been introduced into South Africa, either from Latin America or Asia (Van Heerden & Wingfield 2001). Furthermore, recombination within the South African population was not observed, confirming previous findings that sexual fruiting structures (perithecia) were absent on the cankers (Van Heerden & Wingfield 2001).

Heath (2004), considered the population diversity of South African *Chr. austroafricana* isolates from *Eucalyptus* spp., native *S. cordatum* and exotic *Tibouchina* spp. using microsatellite DNA markers and VCG tests. His results using the polymorphic markers showed that there is a greater number of unique genotypes amongst isolates from *Eucalyptus* and *Tibouchina* than from native *Syzygium* species. A high genotypic diversity within all *Chr. austroafricana* populations i.e. from *Eucalyptus* (95%), *Tibouchina* (96%) and *Syzygium* (75%) spp. was also revealed. This was in contrast to the VCG data that revealed low genotypic diversity within the

native *Syzygium* (0.26%) population, exotic *Tibouchina* (0.22%) and *Eucalyptus* (0.096%) populations respectively (Heath 2004).

The study by Heath (2004) indicated that *Chr. austroafricana* has been present on native *Syzygium* spp. in South Africa for a much longer time than it is on exotic *Tibouchina* and *Eucalyptus* spp. It was thus proposed that *Chr. austroafricana* is native to South Africa and that it could have originated from native *Myrtaceae* in the country. However, it was hypothesized that *S. cordatum* might not be the native host of the founder population in the country (Heath 2004).

Heath (2004), considered a relatively small population of *Chr. austroafricana* from *S. cordatum*. Detailed studies are needed in this regard including a larger number of isolates from different hosts than those previously considered. These should ideally also include comparisons with *Chr. austroafricana* isolates that could occur in other African countries. Dealing with a wider geographic area would help to establish a deeper understanding of the possible origin of this pathogen in South Africa. Such studies should also expand our knowledge of the relationship between isolates of the pathogen occurring on native trees and those that have moved to exotic plantation trees.

2.4.2 *Chr. cubensis*

Population diversity of *Chr. cubensis* has been studied with isolates from Brazil (Van Zyl *et al*. 1998) as well as Venezuela and Indonesia (Van Heerden *et al*. 1997) using VCGs. In all countries, a large number of VCG groups were reported (Van Heerden *et al*. 1997, Van Zyl *et al*. 1998). The two studies showed that *Chr. cubensis* is well established in both South America and southeast Asia, making it difficult to determine its centre of origin (Van Heerden *et al*. 1997, Van Zyl *et al*. 1998). Previously it had been hypothesized that *Chr. cubensis* originated from Indonesia on *S. aromaticum* (Hodges *et al*. 1986, Wingfield *et al*. 2001a, Wingfield 2003), but more recently the same fungus was reported from native South American *Miconia* spp. (Rodas *et al*. 2005). This implies that the fungus has been established both in Asia and South America for a very long time and supports phylogenetic data that show that the fungi in these two areas probably represent discrete taxa (Rodas *et al*. 2005).

In Africa, *Chr. cubensis sensu stricto* has been reported only from non-native trees (Nutman & Roberts 1952, Gibson 1981, Micales *et al*. 1987, Roux *et al*. 2003). Isolates from the western part of the continent are most closely related to those from South America. This is in contrast to those from East Africa that are most closely related to isolates of the fungus from Asia (Myburg *et al*. 2002a, Gryzenhout *et al*. 2004). It is probable that the fungus was spread from South America and southeast Asia to Africa via trade.

2.5 Management

2.5.1 Breeding and selection

Canker of *Eucalyptus* spp. caused by *Chrysoporthe* spp. has been effectively managed by planting disease tolerant hybrids and clones of *Eucalyptus* spp. In South Africa and South America, tolerant clones have been identified using natural screening of trees and artificial inoculation trials (Wingfield *et al*. 1991, Wingfield & Kemp 1993, Van Heerden & Wingfield 2002). Today, in South Africa, the disease can be found only in seedling stands, or in trial plots (Wingfield & Roux 2000).

Several strategies have been employed to select disease resistant clones and hybrids. Studies conducted by Van Zyl and Wingfield (1999) evaluated wound response of *Eucalyptus* clones after inoculation with *Chr. austroafricana.* It was observed that clones tolerant to *Chr. austroafricana* had the greatest level of callus formation after inoculation. Thus clones whose wounds heal faster are more resistant to *Chr. austroafricana* and vice versa.

Van Heerden and Wingfield (2002), conducted studies to determine the influence of different environments on the response of *Eucalyptus* clones to infection with *Chr. austroafricana*. Their findings showed an association between disease severity and geographical location of the host or test trees. Recommendations were thus made to screen for disease tolerance in the specific areas where clones/hybrids are to be grown.

Brondani *et al*. (1998) and Van der Nest *et al*. (2000), developed simple sequence repeats for *Eucalyptus* spp. The markers are being used for identification of *Eucalyptus* parent trees and clones for use for vegetative propagation (Van der Nest *et*

al. 2000). These markers are also important in large-scale population diversity studies of *Eucalyptus* spp. as well as in monitoring diversity in *Eucalyptus* clone banks for genetic conservation (Brondani *et al*. 1998, Van der Nest *et al*. 2000).

Recently, studies have been initiated targeting the use of DNA microarray fingerprinting in *E. grandis* (Lezar *et al*. 2004, Lezar 2005). The studies have indicated that microarrays can be used efficiently for genome-wide fingerprinting of closely related *Eucalyptus* trees. Lezar (2005), revealed that diversity array technology together with bulk segregant analysis provide a powerful approach for the discovery of DNA based molecular markers associated with *Chrysoporthe* tolerance in *E. grandis.* Thus, in future the technology will probably be employed in tree breeding programmes and genome analysis of *Eucalyptus.*

2.5.2 Biological Control

Hypovirulence, which is the reduction of the virulence of fungal pathogens using viruses, has been documented to be a potentially effective control measure of fungal diseases (Anagnostakis 1977, Nuss & Koltin 1990, Nuss 1992, 1996). Studies have been undertaken on many fungi including *Chrysoporthte* spp., to explore viruses as possible biological control agents (Anagnostakis 1977, Nuss & Koltin 1990, Nuss 1992, 1996, Van Heerden *et al*. 2001).

Some of the most extensive studies of hypovirulence in fungi have been conducted on the chestnut blight pathogen, *Cryphonectria parasitica.* The best known of the viruses that infect this fungus and interfere with its pathogenicity are the hypoviruses, in the form of double stranded RNA and belonging to the family *Hypoviridae* (Anagnostakis 1977, 1990). This group of fungal viruses have been shown to infect and result in hypovirulence in *C. parasitica* (Grente 1965, Grente & Sauret 1969, Day *et al*. 1977, Hillman *et al*. 1995). Today, biological control of chestnut blight and spread of hypovirulent stains of *C. parasitica* has been reported in the USA and Europe (Grente & Sauret 1969, Fulbright *et al*. 1983, Garrod *et al.* 1985, Anagnostakis 1990, 2001).

Based on the success achieved with *C. parasitica*, Van Heerden *et al*. (2001), investigated the presence and possible use of hypoviruses in *Chr. austraofricana*. He isolated mitoviruses from *Chr. austroafricana*, then thought to represent *C. cubensis*, in South Africa. However, these mitoviruses did not confer hypovirulence in *Chr. austroafricana* and could not be used as biological control agents (Van Heerden *et al.* 2001). An alternative was to transfect a *C. parasitica* hypovirus, CHVI-EP713 that was shown to impart hypovirulence in the chestnut blight fungus into *Chr. austroafricana* (Van Heerden *et al*. 2001). Van Heerden *et al*. (2001), succeeded in transfecting an isolate of *Chr. austroafricanana* from South Africa with the virus. *In vitro* studies showed that the transfectants were able to show hypovirulence. However, it was noted that the virus couldn't be transmitted to conidia and spread in the field was unlikely to occur.

Several characteristics have been associated with presence of hypoviruses in fungi (Nuss 1992, 1996, Preisig *et al*. 2000). For instance, the occurrence of viruses in plant pathogenic fungi has been associated with reduced virulence (Hammer *et al.* 1989), toxin production reduction (Sutherland & Brasier 1995) and inhibition of sporulation in culture (Bottacin *et al.* 1994). Although this mode of control has not yet proven successful for *Chr. cubensis* and *Chr. austroafricana* it is still considered a possible management strategy for the future.

3.0 *CRYPHONECTRIA EUCALYPTI*

3.1 Taxonomy

Cryphonectria eucalypti M. Venter & M. J. Wingfield., Sydowia 54(1): 113. 2002.

Cryphonectria eucalypti, a fungus previously known as *Endothia gyrosa* (Schw.: Fr.) Fr. is a canker pathogen of *Eucalyptus* spp. in South Africa and Australia (Venter *et al.* 2002). In contrast to *C. eucalypti*, *E. gyrosa* is a well-known blight pathogen of Pin oak (*Quercus palustris* Muenchh.) (Stipes & Phipps 1971, Appel & Stipes 1986) and other tree species in North America (Snow *et al.* 1974, Roane *et al*. 1974, Hunter & Stipes 1978, Appel & Stipes 1986, Roane 1986, Farr *et al.* 1989). It has been documented that the fungus has a wide distribution in North America, having been reported in Kansas, Michigan, Maryland, New Jersey, Connecticut, New York,

California and Ohio (Shear *et al.* 1917, Stevens 1917, Snow *et al.* 1974, Hunter & Stipes 1978, Appel & Stipes 1986). *E. gyrosa* has also been reported in China and Europe on *Quercus* and *Fagus* spp. (Spaulding 1961, Teng 1974).

Cryphonectria eucalypti was first reported as an *Endothiella* anamorph of *C. havanensis* (Davison 1982). In 1985, Walker, Old & Murray reported the occurrence of *E. gyrosa* associated with cankers, dieback of branches and stems as well as death of *E. saligna* Sm. in New South Wales, eastern Australia (Walker *et al*. 1985). Both sexual and asexual structures were examined and it was clear that the fungus had an *Endothiella* anamorph. Using isozyme analysis Davison & Coates (1991) compared isolates of *E. gyrosa* reported in eastern Australia and *C. havanensis* that had been reported in Western Australia. Their findings showed that the fungus reported as *C. havanensis* was the teleomorph of *E. gyrosa*, the same fungus as in eastern Australia.

Morphological differences were noted between *E. gyrosa* from North America and Australia (Walker *et al*. 1985). The Australian specimens have less developed stromata and perithecial bases seated in the bark as compared to North American isolates where the perithecial bases occur in the fungal tissue. This was, however, attributed to environmental factors and the fact that the specimens from different areas occur on different hosts (Walker *et al*. 1985).

In 1990, a fungus similar to that known as *E. gyrosa* in Australia was reported in South Africa on *Eucalyptus* spp. (Van der Westhuizen *et al*. 1993). The fungus was found during surveys to establish the spread of *Chr. austroafricana* (then known as *C. cubensis*) in the country (Van der Westhuizen *et al*. 1993). The fungus was associated with a canker disease where symptoms included shallow cracks and faintly swollen patches on the bark, less severe than symptoms associated with *Chr. austroafricana* (Van der Westhuizen *et al*. 1993). The cankers were more concentrated around the bases of trees, although they were also found in other areas of the stems (Van der Westhuizen *et al.* 1993). The causal agent was morphologically different from *Chr. austroafricana* with distinct orange brown stromata and non-septate ascospores and identified as *E. gyrosa*.

Venter *et al*. (2001), conducted DNA sequence comparisons to characterize *E. gyrosa* isolates from South Africa and Australia. These studies were prompted by the distinct morphological differences revealed between the fungus occurring in these regions and that in North America (Walker *et al*. 1985, Venter *et al*. 2001). Phylogenetic analyses of DNA sequences for the Internally Transcribed Spacer Regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon, and PCR-based restriction fragment length polymorphisms (PCR-RFLP) were thus conducted (Venter *et al*. 2001). Results indicated that *E. gyrosa* from South Africa and Australia is different from the fungus occurring in North America. Although few species of *Endothia* and *Cryphonectria* were included, it also appeared that the fungus grouped closely with *C. parasitica* and not *Endothia* spp.

Venter *et al*. (2002), sequenced a portion of the β–tubulin gene region in *E. gyrosa* isolates and combined the results with those obtained from ITS1 and 1TS2 regions to confirm the differences previously revealed between *E. gyrosa* occurring in different regions. This was accompanied by morphological studies of *E. gyrosa* from Australia, South Africa and North America and comparisons with other *Cryphonectria* spp. Results confirmed the differences noted by Venter *et al*. (2001) showing that *E. gyrosa* from South Africa and Australia was different from the fungus from North America and closely related to species of *Cryphonectria*. The South African and Australian fungus was, therefore, transferred to *Cryphonectria* and described as the new species, *C. eucalypti* (Venter *et al*. 2002).

3.2 Morphology

The teleomorph of *C. eucalypti* is characterized by semi-immersed orange stromata, composed of perithecial bases seated within the host tissue. Perithecia are embedded beneath the surface of the bark at the base of stromata. Perithecia are dark brown with dark and slender necks. Asci are numerous, persistent and float freely in the perithecial cavities and they are cylindrical to fusiform in shape. Ascospores are non-septate, hyaline and cylindrical to fusiform (Walker *et al*. 1985, Venter *et al*. 2002).

The anamorph of *C. eucalypti*, is characterized by multilocular stromata, with less than 10 pycnidial locules per stroma. Conidiogenous cells are cylindrical, slightly tapered towards the apex, hyaline, septate and branched with paraphyses amongst cells.

Conidia are oblong cylindrical, hyaline and aseptate (Walker *et al*. 1985, Venter *et al*. 2002).

In culture, *C. eucalypti* appears white and fluffy with smooth margins. In some cases straw yellow patches appear as the cultures become older. Cultures grow fast, covering a 90 mm plate in nine days at an optimum temperature of 25-30°C (Walker *et al*. 1985, Venter *et al*. 2002).

Although *C. eucalypti* groups close to other *Cryphonectria* species, it has aseptate ascospores, atypical of *Cryphonectria* (Venter *et al*. 2002, Myburg *et al*. 2004). It has thus been suggested that *C. eucalypti* may reside in a discrete genus (Myburg *et al*. 2004). More recent phylogenetic and morphological studies including a great number of taxa have added weight to the view that that *C. eucalypti* represents a distinct genus in the order *Diaporthales* and it will soon be described as a new genus and species, *Holocryphia eucalypti* (Gryzenhout *et al*. 2006; Accepted for publication).

3.3 Distribution and host range

Cryphonectria eucalypti is a fungal pathogen of *Eucalyptus* spp. in South Africa and Australia (Davison 1982, Walker *et al*. 1985, Davison & Coates 1991, Venter *et al*. 2002, Gryzenhout *et al*. 2003). It has to date not been found in any other countries. The host range of *C. eucalypti* is restricted to the two genera *Eucalyptus* and *Corymbia*. Both genera are well known members of the *Myrtaceae* and were previously considered a single genus (Hill & Johnson 1995).

In Australia, the anamorph state of the fungus has been reported on *Corymbia calophylla* (Lindl.) K.D. Hill & L.A.S. Johnson (*Eucalyptus calophylla var. maideniana* Hochr.), *E. marginata* and *E. saligna* in western Australia (Davison 1982, Davison & Tay 1983, Davison & Coates 1991). *C. eucalypti* also has been reported on several other *Eucalyptus* spp. such as *E. globulus* Labill. and *E. nitens* (H. Deane & Maiden), in Tasmania and New South Wales (Davison 1982, Walker *et al.* 1985, Yuan & Mohammed 1997, 1999, Wardlaw 1999).

Africa is the only continent other than Australia from which *C. eucalypti* is known and it has been recorded only from South Africa (Van der Westhuizen *et al*. 1993). Recent surveys have shown a wide distribution of *C. eucalypti* in South Africa. It has been reported in several provinces namely the Mpumalanga, KwaZulu/Natal, and Limpopo Provinces (Van der Westhuizen *et al*. 1993, Gryzenhout *et al*. 2003). It has also been found in Swaziland, a country adjacent to South Africa (Roux, personal communication). All reports from South Africa and Swaziland are from *Eucalyptus* spp., hybrids and clones.

3.4 Origin and population diversity

Various studies have been conducted on the taxonomy, pathogenicity and phylogeny of *C. eucalypti* (Davison 1982, Walker *et al*. 1985, Van der Westhuizen *et al*. 1993, Yuan & Mohammed 1999, 2000, Venter *et al*. 2001, 2002, Gryzenhout *et al*. 2003, Myburg *et al.* 2004). Nothing is, however, known regarding the population structure or origin of this pathogen. It would be interesting to learn more regarding the origin and movement of *C. eucalypti* within and between the countries from which it is known.

3.5 Management

In Australia, *C. eucalypti* has been associated with severe cankers and in some cases tree death has been reported (Walker *et al*. 1985, Old *et al*. 1986, Yuan & Mohammed 1999). However, studies conducted by Yuan & Mohammed (2000), Davison & Tay (1983) and Old *et al*. (1990) indicated that *C. eucalypti*, results in severe damage only on stressed trees. Green house inoculations further showed less damage caused by *C. eucalypti* as compared to what had been observed in the field (Yuan & Mohammed 2000). For proper management of this disease in Australia it would be necessary to employ strategies that reduce stress on the trees and planting of disease tolerant material.

Studies done on the pathogenicity of *C. eucalypti* in South Africa have confirmed that it is a stress-related pathogen (Gryzenhout *et al*. 2003). Furthermore it was shown that, similar to other pathogens, some *Eucalyptus* clones are more tolerant to infection by *C. eucalypti*, allowing for the selection of disease free planting material (Gryzenhout *et*

al. 2003). In South Africa, avoidance of off-site planting of *Eucalyptus* spp. will reduce stress and thus the appearance of *C. eucalypti* on trees (Gryzenhout *et al*. 2003).

4.0 CONCLUSIONS

A great deal of change has occurred in recent years, regarding our understanding of the taxonomy and ecology of *Eucalyptus* fungal pathogens previously treated in the genera *Cryphonectria* and *Endothia*. *Cryphonectria cubensis* now resides in *Chrysoporthe* as two species, which are very distinct from *Cryphonectria*. The stem pathogen previously known as *E. gyrosa* is now treated as *C. eucalypti* and awaits formal transfer to a discrete genus (to be known as *Holocryphia*). It is very likely that *C. eucalypti* and *Chr. cubensis* were introduced onto the African continent, but this hypothesis remains to be tested. In contrast, *Chr. austroafricana* appears to be native to the African continent.

Changes to the taxonomy of *Chrysoporthe* and *Cryphonectria* spp. occurring on members of the *Myrtales* have international implications. Currently, *Chr. austroafricana*, which is more virulent than *Chr. cubensis*, only occurs in southern Africa. Accidental introduction of *Chr. austroafricana* into other continents with susceptible native *Myrtaceae* could thus have grave implications. Accidental introductions could also impart serious negative effects on *Eucalyptus* plantations in countries where the fungus does not currently occur. Similarly, the movement of *Chr. cubensis* into South Africa could be serious, since the clones currently planted have not been tested for tolerance to this pathogen.

Molecular genetic tools have greatly strengthened research on fungal pathogens. Apart from being used in fungal taxonomy, molecular methods have been applied in phylogenetic studies, diagnostic applications as well as epidemiology and population genetics of fungal pathogens. Such studies have already had a substantial impact on research concerning *Cryphonectria* and *Chrysoporthe* spp. and the tree diseases associated with them. With suitable populations and more detailed surveys into the host range and geographic distribution of these fungi, many important questions could be answered. These include questions pertaining to the origin and population biology of these pathogens on the African continent, the host range of these pathogens beyond

exotic *Eucalyptus* spp. and their distribution beyond South Africa, and the Republic of Congo.

The aim of studies contained in this thesis is to consider some of the above questions. This is achieved through surveys in southern and eastern Africa, of both *Eucalyptus* spp. and native tree species belonging to the *Myrtales*. Extensive use of various DNA-based techniques will be made to gain added knowledge concerning the isolates collected for this study. The intention has been that results of the studies in this thesis will contribute to a better understanding of the taxonomy, origin, distribution, host range, as well as pathogenicity of various *Cryphonectria* and *Chrysoporthe* species in eastern and southern Africa.

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Table 1. Host range and geographical distribution of *Chrysoporthe* spp.

Species identity	Host	Origin	Reference
Chrysoporthe austroafricana	*Eucalyptus grandis*	South Africa	Wingfield *et al.*1989.
"	*Syzygium cordatum*	South Africa	Heath *et al.* 2006
"	*S. guineense*	South Africa	Heath *et al.* 2006
"	*Tibouchina granulosa*	South Africa	Myburg *et al.* 2002b
Chr. cubensis	*E. marginata* Donn ex Sm	Australia	Davison & Coates 1991
"	*E. saligna* Sm., *E. maculata* Hook., *E. angulosa* Schau., *E. botryoides* Sm. E. *camaldulensis* Dehnh., *E. urophylla* S. T. Blake, *E. trabutoo* Vilmorin, *E. tereticornis*, *E. robusta*, *E. propinqua* Deane & Maid., *E. pilularis* Sm., *E. paniculata* Sm., *E. microcorys* F. Muell., *E. longifolia* Link & Otto, *E. grandis, E. citriodora*	Brazil	Hodges *et al.* 1976, Hodges 1980
"	*E. urophylla*	Cameroon	Gibson 1981
"	*E. grandis*	Colombia	Hodges 1980
"	*E. rostrata* Schlecht., *E. microphylla* Willd., *E. robusta* Sm., *E. occidentalis* Endl., E	Cuba	Bruner 1916
"	*E. saligna*	Demographic Rep. of Congo	Micales *et al.* 1987
"	*E. grandis*	Florida	Hodges 1980, Hodges *et al.* 1979
"	*E. marginata*	Indonesia	Hodges *et al.* 1986
"	*E. grandis, E. tereticornis* Sm., *E. citriodora* Hook. *E. terelliana* F. Muell, *E. deglupta, E. saligna, E. brassiana* S. T. Blake, *E. camaldulensis*, *E. pellita* F. Muell, *E. cloeziana* F. Muell.	India	Florence *et al.* 1986, Sharma *et al.* 1985
"	*Eucalyptus deglupta* Bl., *E. grandis*, *E. saligna*	Kauai, Hawaii	Hodges 1980, Hodges *et al.* 1979
"	*E. urophylla, E. deglupta*	Puerto Rico	Hodges 1980, Hodges *et al.* 1979
"	*E. urophylla*, *E. grandis*	Republic of Congo	Roux *et al.* 2003
"	*E. rostrata* Schlecht., *E. microphylla* Willd., *E. robusta* Sm., *E. occidentalis* Endl., E	Surinam	Boerboom & Maas 1970, Hodges 1980
"	*E. saligna*	Trinidad	Hodges 1980
"	*E. grandis*	Venezuela	Hodges *et al.* 1986
"	*E. saligna*	Western Samoa	Hodges 1980, Hodges *et al.* 1979
"	*Miconia rubiginosa*, *M. theaezans*	Colombia	Rodas *et al.* 2005
"	*S. aromaticum*	Brazil	Hodges *et al.* 1986
"	*S. aromaticum*	Kalimantan	Hodges *et al.* 1986
"	*S. aromaticum*	Indonesia	Hodges *et al.* 1986
"	*S. aromaticum.*	Tanzania, Zanzibar	Nutnam & Roberts 1952
"	*Clidemia sericea* D Don, *Rhynchanthera mexicana* DC	Mexico	Gryzenhout *et al.* 2005
"	*Tibouchina urvilleana*	Singapore, Thailand	Gryzenhout *et al.* 2005
"	*Melastoma malabathricum* L.	Indonesia	Gryzenhout *et al.* 2005
"	*Lagerstroemia indica* L.	Cuba	Gryzenhout *et al.* 2005
"	*Psidium cattleianum* Sabibe	S. America	Hodges 1988
Chrysoporthe sp.	*Tibouchina granulosa, Miconia calvescens, Clidemia hirta*	Brazil	Seixa *et al.* 2004
Chrysoporthella hodgesiana	*T. lepidota*	Colombia	Gryzenhout *et al.* 2004, Rodas *et al.* 2005
"	*T. semidecandra*	Colombia	Gryzenhout *et al.* 2004, Rodas *et al.* 2005
"	*T. urvilleana*	Colombia	Gryzenhout *et al.* 2004, Rodas *et al.* 2005

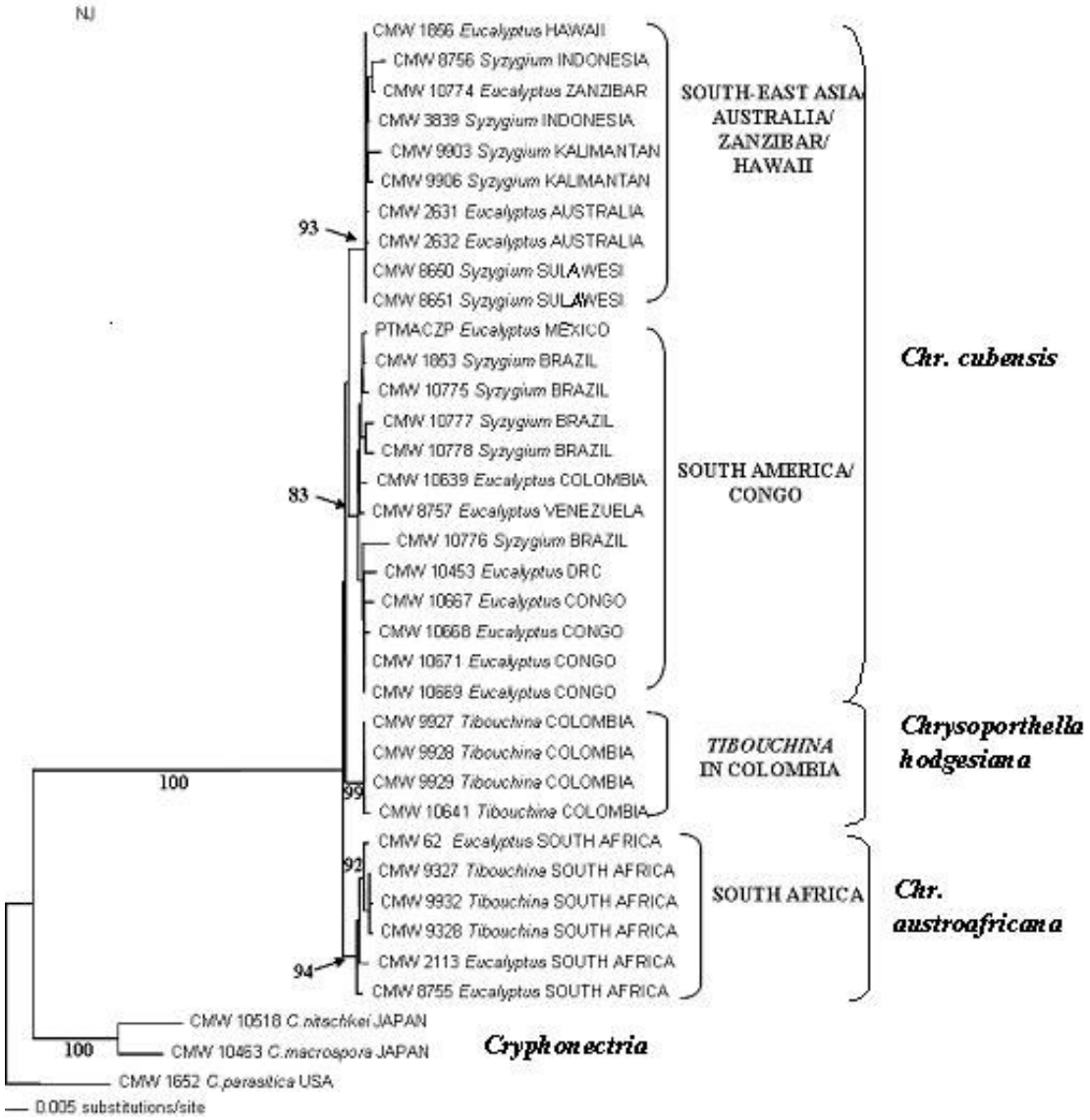

Fig 1. Distance phylogram obtained from the combined data set of the ribosomal DNA and β-tubulin gene sequences. *Cryphonectria*, *C. nitschkei* and *C. macrospora* were defined as outgroups to root the trees (phylogenetic analysis from Gryzenhout *et al*. 2004).

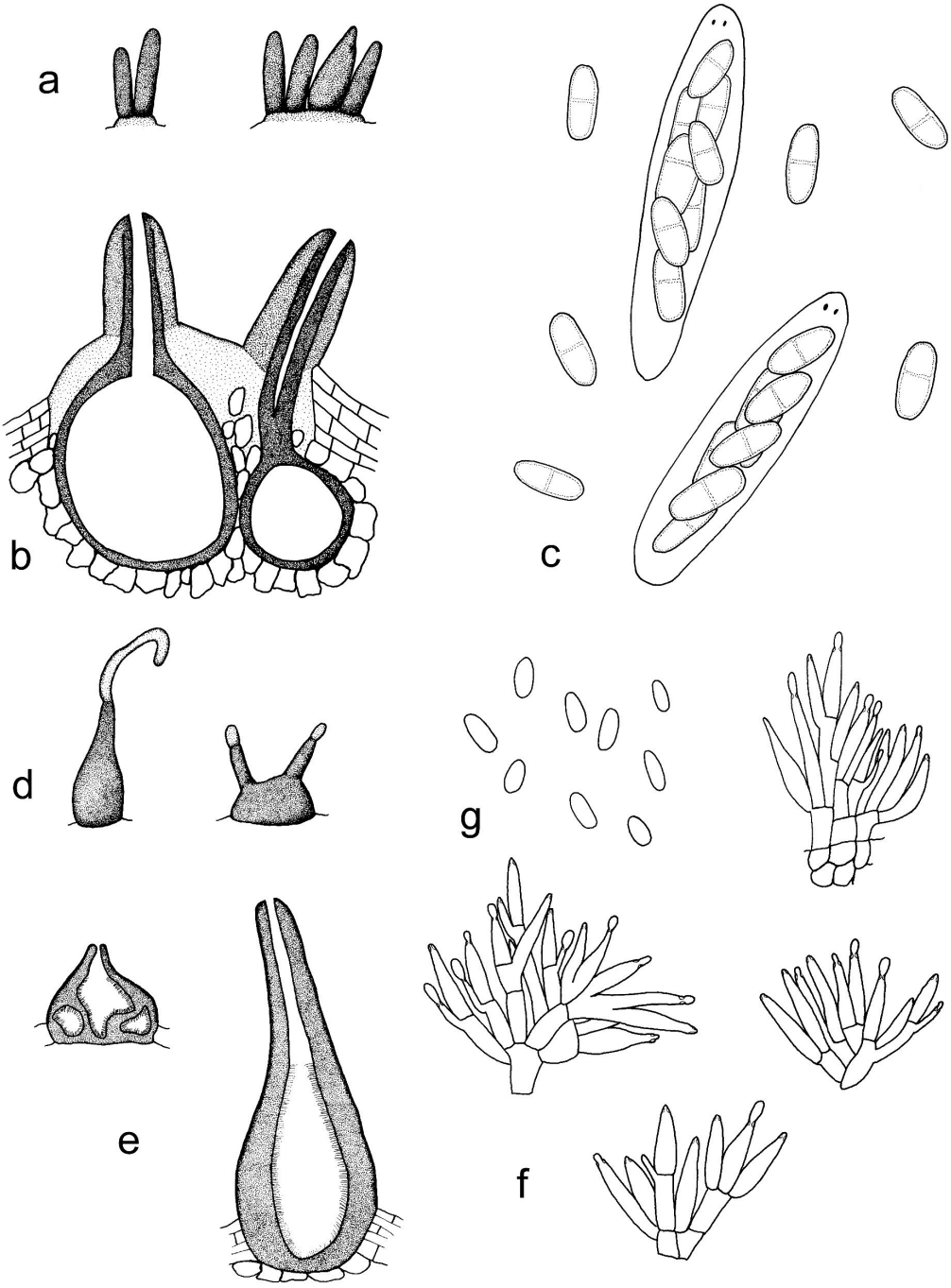

Fig 2. Line drawings of *Chrysoporthe austroafricana*. A. Shapes of ascomata and conidiomata. B. Section through ascoma. C. Asci and ascospores. D. Shapes of conidiomata. E. Section through conidiomata. F. Conidiophores and conidiogenous cells. G. Conidia. Scale Bars A-B, D-E = 100µm; C, F-G = 10µm (Drawings from Gryzenhout *et al*. 2004).

Represents areas where *Chrysoporthe cubensis* has been reported

Represents areas where *Chrysoporthe austroafricana* has been reported

Fig 3. Map showing the global distribution of *Chrysoporthe* spp.

CHAPTER 2

Distribution of Chrysoporthe canker pathogens on *Eucalyptus* and *Syzygium* species in eastern and southern Africa

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ABSTRACT

Chrysoporthe cubensis and *Chr. austroafricana*, collectively known as *Cryphonectria cubensis* in the past, are important canker pathogens of *Eucalyptus* spp. worldwide. Previous studies have suggested that *Chr. austroafricana* occurs only in South Africa, while *Chr. cubensis* occurs in Australia, Cameroon, Tanzania, Democratic Republic of Congo, Republic of Congo, South East Asia and South, Central and North America. In South Africa, *Chr. austroafricana* is a pathogen on non-native *Eucalyptus* (Myrtaceae) and *Tibouchina* spp. (Melastomataceae) both residing in the order Myrtales. Recently the fungus has also been found on native *Syzygium cordatum* trees in the country, leading to the hypothesis that it is native to Africa. In contrast, *Chr. cubensis* is thought to be introduced into Africa and is known only on non-native *Eucalyptus* and *Syzygium aromaticum* (clove) in four countries. The aim of this study was to consider the distribution of *Chrysoporthe* spp. on non-native *Eucalyptus* spp. as well as on native Myrtales in southern and eastern Africa. Isolates were collected from as many trees as possible and characterised based on their morphology and DNA sequence data for two gene regions. Results show, for the first time, that *Chr. cubensis* occurs in Kenya, Malawi and Mozambique on non-native *Eucalyptus* spp. *Chr. austroafricana* was found for the first time in Mozambique, Malawi and Zambia on non-native *Eucalyptus* spp. and native *S. cordatum*. The known distribution range of *Chr. austroafricana* within South Africa was also extended during these surveys.

INTRODUCTION

Species of *Chrysoporthe* previously treated in the genus *Cryphonectria* (Gryzenhout *et al*. 2004) are important canker pathogens of *Eucalyptus* spp. grown in plantations in both tropical and subtropical areas worldwide. They have been reported in South and Central America (Boerboom & Maas 1970, Hodges *et al*. 1976, Hodges *et al*. 1979, van der Merwe *et al*. 2001), southeast Asia (Sharma *et al*. 1985, Florence *et al*. 1986, Hodges *et al*. 1986, Myburg *et al*. 2003), Australia (Davison & Coates 1991, Myburg *et al*. 1999), North America (Hodges *et al*. 1979, Myburg *et al*. 2003), and Africa (Gibson 1981, Hodges *et al*. 1986, Wingfield *et al*. 1989, Roux *et al*. 2003, Wingfield 2003, Roux *et al*. 2005). The disease with which *Chrysoporthe* spp. is associated has been known as Cryphonectria canker in the past, and leads to the girdling of stems, wilting and death of infected trees (Hodges *et al*. 1979, Hodges 1980, Sharma *et al*. 1985, Conradie *et al*. 1990). The cankers can occur at the bases of the stems or they are found higher up on the trunks (Hodges *et al*. 1976, 1979, Sharma *et al*. 1985, Conradie *et al*. 1990). The disease caused by *Chrysoporthe* spp. has been successfully managed by breeding for disease tolerant *Eucalyptus* hybrids in some countries such as Brazil and South Africa (Alfenas *et al*. 1983, Wingfield 1990, Wingfield *et al*. 1991, Gadgil *et al*. 2000, van Heerden *et al*. 2005). It is, however, still considered a major constraint to the successful establishment of *Eucalyptus* plantations and is regarded as a high priority disease.

Chrysoporthe includes two economically important species, *Chr. cubensis* (Bruner) Gryzenhout & M. J. Wingf. and *Chr. austroafricana* Gryzenhout & M. J. Wingf., which are pathogenic to *Eucalyptus* spp. These species were previously treated in *Cryphonectria* and were collectively known as *Cryphonectria cubensis* (Bruner) Hodges (Bruner 1917, Gryzenhout *et al*. 2004). Recognition of *Chrysoporthe* spp. as distinct from those of *Cryphonectria* emerged from comparisons of DNA sequence data and clear morphological differences. The most notable morphological differences distinguishing *Chrysoporthe* from *Cryphonectria* are the limited stromatic development in the ascostromata, long and black perithecial necks, and black, pyriform and superficial conidiomata in species of the former genus (Gryzenhout *et al*. 2004).

Within *Chrysoporthe*, morphological and phylogenetic differences have been observed between isolates from South Africa and those occurring in other parts of the world (Myburg *et al*. 2002a, Gryzenhout *et al*. 2004). Comparisons of DNA sequence data, based on multiple gene regions have shown that *Chr. cubensis* isolates reside in three well-supported phylogenetic groups (Myburg *et al*. 2002a). One of these encompasses South African isolates, another includes isolates from southeast Asia, East Africa and Australia and a third group represents isolates from South America, North America, Central Africa and West Africa (Myburg *et al*. 2002a). Slightly larger asci and rounded ascospores are found in the South African isolates as compared to smaller asci and tapered ascospores in isolates occurring in other parts of the world (Gryzenhout *et al*. 2004). These differences support the treatment of the South African fungus as a distinct species known as *Chr. austroafricana* (Gryzenhout *et al*. 2004). Although isolates from southeast Asia, East Africa and Australia (southeast Asian group) group separately from the South American, North American, Central African and West African (South American group) isolates in phylogenetic comparisons, morphological differences have not been observed between the two groups (Myburg *et al*. 2002a, Gryzenhout *et al*. 2004). For the present, they are treated collectively as representing *Chr. cubensis* (Myburg *et al*. 2002a, Gryzenhout *et al*. 2004).

Chrysoporthe austroafricana has been reported only from South Africa, where it is considered to be one of the most important pathogens in non-native plantation-grown *Eucalyptus* spp. (Wingfield *et al*. 1989, Conradie *et al*. 1992). Before its recognition as a distinct species, this fungus was thought to have been introduced into South Africa (Van Heerden & Wingfield 2001). Discovery of *Chr. austroafricana* on the non-native ornamental tree *Tibouchina granulosa* Cogn. (Myburg *et al*. 2002b) provided further support for the view that the fungus had been introduced into South Africa. However, the fungus has recently been discovered causing stem and branch cankers on native *Syzygium cordatum* Hachst. and *S. guineense* (Willd.) D.C (Heath *et al*. 2006). This has given rise to the alternative view that *Chr. austroafricana* is native to Africa and has undergone a host jump to non-native *Eucalyptus* and *Tibouchina* spp. (Heath *et al*. 2006, Slippers *et al*. 2005). This might then also imply that the fungus would occur in countries neighboring South Africa. Native Myrtaceae

similar to those in South Africa occur in these countries as do non-native *Eucalyptus* spp., which have been used to establish plantations and woodlots for many years.

Chrysoporthe cubensis is known from Africa and several other regions of the world (Gryzenhout *et al*. 2004). In Africa, the fungus has been reported only from non-native hosts, namely from *Eucalyptus* spp. in the Republic of Congo (Roux *et al*. 2000, Roux *et al*. 2003), Democratic Republic of Congo (DRC) (Micales *et al*. 1987) and Cameroon (Gibson 1981) as well as from *Syzygium aromaticum* (L.) Merr. & Perry. (Clove) on Unguja Island, Zanzibar (Tanzania) (Nutman & Roberts 1952, Hodges *et al*. 1986, Myburg *et al*. 2003). It has been hypothesized that this pathogen was introduced to the African continent (Gryzenhout *et al*. 2004). Its occurrence in West and Central Africa, as well as on the eastern seaboard of Africa would suggest that it occurs on non-native plantation-grown *Eucalyptus* trees in other parts of Africa.

Recently, Roux *et al*. (2005) reported the occurrence of *Chrysoporthe* spp. from several eastern and southern African countries, where *Chrysoporthe* spp. were not previously known. Their study greatly expanded the known geographic distribution of this genus of canker pathogens in Africa. However, the study by Roux *et al.* (2005) focused only on non-native plantation-grown *Eucalyptus* spp. and isolates were not identified to species level. The aim of this study was firstly to determine the identity of the isolates collected by Roux *et al*. (2005). A second aim was to conduct additional surveys of native hosts, especially *Syzygium* spp. in South Africa and other southern and eastern African countries.

MATERIALS AND METHODS

Collection of isolates

Surveys of indigenous species residing in the Myrtales growing in the wild as well as non-native *Eucalyptus* spp. grown in plantations were conducted in Kenya, Malawi, Mozambique, South Africa, Tanzania and Zambia (Fig 1). Sampling involved selecting the trees and a subsequent search for disease symptoms. On *Syzygium* spp., dying branches and stem cankers were the symptoms of interest. On *Eucalyptus* spp., cracks, cankers on the stems and swollen bases provided the best indications that *Chrysoporthe* spp. might be present (Fig 2a-b). After the detection of fruiting

structures (Fig 2c-d), pieces of wood and bark were scraped or cut off from symptomatic trees, placed in brown paper bags and labeled for subsequent laboratory study and isolation.

Pieces of wood bearing fungal fruiting bodies were placed in moist chambers to induce spore production. Fungal fruiting bodies were identified using standard microscope techniques (Gryzenhout *et al*. 2004). Isolations were made by lifting spore drops from fruiting structures. Single spore cultures were made by suspending spores (ascospores or conidia) in sterile distilled water. Spore suspensions were spread onto the surface of 2 % malt extract agar (MEA) (20 g/l malt extract and 15 g/l agar, Biolab, Midrand, South Africa) containing 100 mg Streptomycin sulfate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in Petri dishes. These were incubated overnight and germinating single spores were selected and transferred to fresh plates. Resulting cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table 1) where cultures were preserved on Agar slants and sterile distilled water. One isolate from Cameroon that was obtained from the Centraalbureau voor Schimmelcultures (CMW 14852, CBS101281), was also included in this study.

DNA sequence comparisons

Representative isolates were selected from each host and geographic area and used for DNA sequence comparisons (Table 1). For each isolate, actively growing mycelium from one MEA plate per isolate was scraped from the surface of the agar using a sterile scalpel and transferred to a 1.5 µl Eppendorf tube. Excess liquid was removed from the tubes by centrifugation at 12000 rpm for 1 minute. DNA was extracted using a modification of the protocol described by Gryzenhout *et al*. (2004). DNA concentrations were estimated visually on a 1 % agarose gel using known concentrations of lambda DNA under UV illumination.

The polymerase chain reaction (PCR) was used to amplify the β-tubulin 1 and β-tubulin 2 and rDNA (ITS 1, 5.8S and ITS 2) regions (Glass & Donaldson 1995,White *et al.* 1990). The reactions were done in a volume of 25 µl comprising of 2 ng DNA template, 800 µM dNTPs, 0.15 µM of each primer, 5 U/µl Taq polymerase (Roche

Diagnostics, Mannheim, Germany) and sterile distilled water (17.4 µl). The PCR reactions were carried out on a thermal cycler (Master cycle® Perkin Elmer Corporation, Massachusetts, United States) consisting of an initial denaturation step at 94 °C for 2 min, followed by 30 amplification cycles consisting of 1 min at 92 °C and 30 sec of annealing at 56 °C – 60 °C, depending on the primer pair used. The PCR products were visualised under UV light on a 2 % agarose gel containing ethidium bromide to determine the presence or absence of bands. The PCR products were purified using the High Pure PCR product purification kit according to the manufacturers' protocol (Roche Diagnostics, Mannheim, Germany).

The sequencing reactions (10 µl) consisted of 5X dilution buffer, 4.5 µl H_2O, DNA (50 ng PCR product), 10X reaction mix, and ~ 2 pmol /µl of one of either reverse or forward primers that were used in the PCR reactions. The PCR sequencing product was cleaned using Sephadex G-50 beads following the manufacturers' protocol (SIGMA-ALDRICH, Amersham Biosciences Limited, Sweden). 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, Foster City, CA).

The gene sequences were analyzed and edited using Sequence Navigator Version 1.0.1™ (Perkin-Elmer Applied BioSystems, Foster City, CA). Sequences were aligned with those in the TreeBASE dataset (S 1211, M 2095) obtained from Gryzenhout *et al*. (2004). Phylogenetic analysis was performed using the software package Phylogenetic Analysis Using Parsimony (PAUP) Version 4.01b (Swofford 1998). Phylogenetic analyses were first done for each gene region separately and then for a combined data set of the ITS and β-tubulin 1 and 2 gene regions. This was preceded by a partition homogeneity test to test if the data sets of the two regions were not significantly different in their evolutionaty relationships to each other which would prevent pooling of the sequence data from the three gene regions (Heulsenbeck *et al*. 1996).

The most parsimonious trees were obtained with heuristic searches using stepwise addition and tree bisection and reconstruction (TBR) as the branch swapping

algorithms. All equally parsimonious trees were saved and all branches equal to zero were collapsed. Gaps were treated as fifth character. Bootstrap replicates (1000) were done on consensus parsimonious trees (Felsenstein 1985). Three *Cryphonectria* species, namely *C. parasitica* (Murrill) M. E. Barr, *C. nitschkei* (G. H. Otth) M. E. Barr and *C. macrospora* (Tak. Kobay. & Kaz. Itô) M. E. Barr were used as the outgroup taxa to root the trees (Gryzenhout *et al*. 2004).

RESULTS

Collection of Isolates

Chrysoporthe samples were collected from Kenya, Malawi, Mozambique, South Africa and Zambia both from non-native *Eucalyptus* spp. and native *Syzygium* spp. *Eucalyptus* spp. had typical symptoms of canker caused by *Chrysoporthe* spp. The majority of symptoms on *Eucalyptus* spp. were characterized by swollen basal cankers (Fig. 2a). However, in one plantation in South Africa and one compartment in Malawi near Mt. Mulanje, cankers higher on the tree stems, similar to those observed in South American and Asian countries were found (Fig. 2b). Symptoms on *Syzygium* spp. consisted mostly of cankers on dying branches and stems. Both sexual and asexual structures were encountered in all the areas surveyed and on both host genera considered.

In Zambia, most samples collected were from *Eucalyptus* trees (20 trees) near Kitwe and a few *Syzygium* trees from Kitwe and Chati. In Mozambique, more than 100 *S. cordatum* trees were sampled over a wide area (Maputo, Gaza, Inhambane and Sofala Provinces), and more than 100 *Eucalyptus* trees were sampled in the Chimoio and Manica areas. In Kenya, more than 50 *Eucalyptus* trees were sampled. Although surveys included *Eucalyptus* spp. in several areas of Kenya, the disease was found only near the coastal town of Malindi. Both *Eucalyptus* and *Syzygium* spp. were surveyed in Tanzania (Njombe area), but no *Chrysoporthe* spp. were obtained from trees in this area. In Malawi, surveys were conducted in several areas, but the disease was found only in the Mt. Mulanje area, both on *E. grandis* and *S. cordatum*. The distribution of *Chrysoporthe* spp. was also extended in South Africa, with isolates collected from *S. cordatum* in the Port Edward and Umzinto areas. Although other *Syzygium* spp., *Heteropyxis* spp., and a limited number of *Eugenia* spp. were also

surveyed and sampled in South Africa, no *Chrysoporthe* spp. were found on these trees.

DNA sequence comparisons

Sequences were obtained for both the ITS rDNA and β-tubulin 1 and β-tubulin 2 gene regions. The β-tubulin regions were approximately 500 bp whereas the ITS rDNA amplified was approximately 558 bp in size. Results of the partition homogeneity test showed that all sequences could be aligned for both regions (p-value = 0.13). The aligned sequences of the combined regions generated 1439 characters of equal weight, with 1194 constant characters of which 101 were parsimony uninformative and 144 were parsimony informative. One hundred most parsimonious trees were retained. A consensus tree (70 % majority rule) with a length of 318, a consistency index (CI) of 0.945 and retention index (RI) of 0.959, was computed (Fig 3).

Isolates from Kenya, Malawi and Mozambique that were collected from *Eucalyptus* spp. grouped with *Chr. cubensis* isolates from southeast Asia and formed a distinct clade (96% bootstrap). Isolates from *Eucalyptus* and *Syzygium* spp. from Malawi (CMW17098, CMW17101, CMW17110, CMW17115), Mozambique (CMW1902, CMW13929, CMW13926) and Zambia (CMW13877, CMW13976) grouped with *Chr. austroafricana* isolates from South Africa, collected from *Eucalyptus, Tibouchina* and *Syzygium* spp. Isolates from the newly sampled areas in South Africa including those collected from stem cankers from KwaMbonambi (CMW13878, CMW13879) also grouped in this clade (94 % bootstrap). An isolate from Cameroon (CMW14852) that was obtained from the CBS and isolates from DRC and Congo that were included in this analysis grouped together with the South American isolates of *Chr. cubensis* (94 % bootstrap).

DISCUSSION

This study has greatly increased our knowledge of the distribution of two of the most important *Eucalyptus* pathogens currently known. The geographic range of *Chrysoporthe* spp. on native *Syzygium* spp. in eastern and southern Africa has also been expanded considerably. We have shown that *Chr. austroafricana* causes cankers

at the base and higher up on stems of *Eucalyptus* trees in South Africa and Malawi, which is contrary to prior knowledge. Likewise the sexual state of this fungus has been shown to be equally abundant as the asexual state in countries north of South Africa, contrary to the situation in southern Africa where the asexual state predominates (Van Heerden & Wingfield 2001).

Chrysoporthe austroafricana was previously known only from South Africa on non-native *Eucalyptus* spp. (Wingfield *et al*. 1989), *T. granulosa* (Myburg *et al*. 2002b) and native *S. cordatum* and *S. guineense* (Heath *et al*. 2006). Results of this study have shown that the fungus is also present in Malawi, Mozambique and Zambia, both on non-native *Eucalyptus* spp. and native *S. cordatum*. The fungus is widespread in Mozambique and was collected from the southern (Maputo) and central (Chimoio) parts of the country, stretching over a distance of about 1200 km. Surveys in Zambia were limited to one area and *Chr. austroafricana* was common on *Eucalyptus* trees in plantations near the town of Kitwe. On *Syzygium* sp. the fungus was found in the same area but only on one tree. In Malawi, *Chr. austroafricana* was collected from one area (Mt. Mulanje), both from *Eucalyptus* and native *S. cordatum*. The occurrence of *Chr. austroafricana* in Malawi, Mozambique and Zambia suggests that the fungus might also be present in other East African countries such as Tanzania and Zimbabwe.

Chr. austroafricana has recently been suggested to be native to Africa (Heath *et al*. 2006). Our results, showing that the fungus has a wide geographic distribution in southern and eastern Africa on both non-native and native trees, support this hypothesis. This wide distribution and the absence of *Chr. austroafricana* from other continents, despite extensive surveys, suggests that the fungus is limited to southern Africa. In this respect, it represents a potentially important threat to Myrtaceae elsewhere in the world. The fungus causes a canker disease, which results in reduced growth rates, reduced coppicing and death of infected *Eucalyptus* trees (Wingfield *et al*. 1989). On native *Syzygium* trees it is found primarily on dead or dying branches (Heath *et al*. 2006). Limited studies by Roux *et al*. (2000) and Rodas *et al*. (Rodas *et al*. 2005) have shown that *Chr. austroafricana* isolates from South Africa are more virulent than *Chr. cubensis* isolates. The introduction of *Chr. austroafricana* isolates to other continents could, therefore have serious negative impacts on commercial forestry and biodiversity.

It was initially believed that *Chr. austroafricana* causes only basal cankers on the stems of *Eucalyptus* in South Africa, whereas *Chr. cubensis* gives rise to both basal cankers and cankers higher up on the stems of trees in southeast Asia and South America (Wingfield *et al*. 1989, Conradie *et al*. 1992, Van Heerden & Wingfield 2001, Wingfield 2003). During the course of our surveys, *Chr. austroafricana* was isolated from cankers, up to 3 m above ground level on *Eucalyptus* trees in the KwaZulu/Natal Province (KwaMbonambi) in South Africa and in Malawi. This symptom is clearly less common than it is with *Chr. cubensis* elsewhere in the world. It is highly possible that environmental factors have an influence on areas of infection on the stems.

One of the early indications that *Chr. austroafricana* and *Chr. cubensis* might really be two speceis was the fact that cankers of the former fungus are typically covered with asexual structures (pycnidia) whereas those of *Chr. cubensis* more typically bear only perithecia (Wingfield *et al*. 1989, Van Heerden & Wingfield 2001, Wingfield *et al*. 2001). During the present surveys, both sexual and asexual structures of *Chr. austroafricana* were commonly found on *Eucalyptus* spp. as well as on native *Syzygium* spp. in Malawi, Mozambique and Zambia. In South Africa the sexual state of this fungus is abundant on native *Syzygium* spp. but not on *Eucalyptus* (Heath *et al*. 2006). This characteristic might thus be associated with environmental factors such as temperature and humidity, which are lower in South Africa than more northern African countries.

Chrysoporthe cubensis has been known in Africa since the early 1960s where it has been recorded on *Eucalyptus* spp. and *S. aromaticum* (Gibson 1981, Micales *et al*. 1987, Myburg *et al*. 2003). Our surveys have extended the geographic range of the fungus to include Kenya, Malawi and Mozambique, where it occurs on *Eucalyptus* spp. Phylogenetic analyses showed that *Chr. cubensis* from Kenya, Mozambique and Malawi groups in the same sub-clade as *Chr. cubensis* from Zanzibar and southeast Asia, but separate from isolates from South America, the Republic of Congo, DRC and Cameroon. This suggests that East African isolates could have been introduced from Asia. This finding should now be tested at the population biology level. It might

thus raise clues as to how the pathogen has moved around the world and provide knowledge that will reduce the risks of future introductions into new areas.

The question regarding the origin of *Chr. cubensis* remains to be resolved. A previous view has been that the fungus originated in Indonesia on native *S. aromaticum* (Hodges *et al*. 1986). An alternative hypothesis has been that the fungus has originated on native plants in South America (Wingfield *et al*. 2001, Wingfield 2003). There have been more recent reports of *Chr. cubensis* from native plants in South America (Rodas *et al*. 2005) adding support to the view that this area could represent the origin of the fungus. Population biology studies using Vegetative Compatibility Groups (VCGs) on *Chr. cubensis* isolates from South America (Venezuela, Brazil) and southeast Asia (Indonesia) have shown that a large number of VCGs occur in each country (van Heerden *et al*. 1997, van Zyl *et al*. 1998). This suggests either a high level of out-crossing within the populations or well-established native populations in both areas. The fact that the Indonesian population is also highly diverse, together with the clear phylogenetic distinction between Asian and South American isolates, supports suggestions that these two groups of *Chr. cubensis* isolates might represent distinct species (Myburg *et al*. 2002a, Gryzenhout *et al*. 2004).

The results of this study, combined with previous findings (Hodges *et al*. 1986, Micales *et al*. 1987, Wingfield *et al*. 1989, Myburg *et al*. 2003, Roux *et al*. 2003, Roux *et al*. 2005), show that *Chrysoporthe* spp. have a wide distribution in Africa. In East Africa, all isolates of *Chr. cubensis* collected reside in the southeast Asian group defined for the fungus. In contrast, all isolates from west and central Africa reside in the South American clade of *Chr. cubensis.* In Mozambique, South Africa and Zambia only *Chr. austroafricana* is present. The populations of isolates collected in the surveys presented in this study, will make it possible to consider the origin of *Chrysoporthe* spp. on the African continent and to better understand how these fungi are moving within the region.

The knowledge generated in this study is important to *Eucalyptus* plantation managers. For example, disease caused by *Chr. austroafricana* in South Africa is largely managed through the planting of disease tolerant clones (van Heerden &

Wingfield 2002, van Heerden *et al*. 2005). However, our study shows that within 2000 km, *Chr. cubensis* also occurs, and this is a pathogen against which South African *Eucalyptus* stock has not been tested. Future outbreaks of canker caused by *Chrysoporthe* spp. in South Africa should thus be carefully monitored. Countries in central and west Africa, Asia, Australia and South America, where *Chr. austroafricana* is still unknown, should also take note of the potential threat of the fungus in their areas.

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Table 1: Isolates included in this study

Isolate number[a]	Alternative isolate number[a]	Species identity	Host	Origin	Collector	GenBank accession numb
CMW 1856		*Chr. cubensis*	*Eucalyptus* sp.	Kauai, Hawaii	n.a	AY 083999, AY 084010, AY 084022
CMW 8756		*Chr. cubensis*	*S. aromaticum*	Indonesia	MJ Wingfield	AF 046896, AF 273077, AF 285165
CMW 3839		"	"	"	"	AF 046904, AY 084011, AY 084023
CMW 11288	CBS 115736	"	*Eucalyptus* sp.	"	"	AY 214302, AY 214230, AY214266
CMW 11289	CBS 115737	"	"	"	"	AY 214303, AY 214231, AY 214267
CMW 10774		"	*S. aromaticum.*	Zanzibar	CS Hodges	AF 492130, AF 492131, AF 492132
CMW 2631		"	*E. marginata*	Australia	E Davison	AF 543823, AF543824, AF523825
CMW 10671	CBS 115752	"	*Eucalyptus* sp.	Rep. of Congo	J Roux	AF 254219, AF 254221, AF 254223
CMW 10453	CBS 505.63		"	DRC	E Davison	AY063476, AY063478, AY063480
CMW 10639	CBS 115747	"	*E. grandis*	Colombia	CA Rodas	AY 263419, AY 263420, AY 263421
CMW 8757		"	*Eucalyptus* sp.	Venezuela	MJ Wingfield	AF 046897, AF 273069, AF 273464
CMW 10777		"	*S. aromaticum*	Brazil	CS Hodges	AY 084005, AY 084017, AY 084029
CMW 10778	CBS 115755	"	"	"	CS Hodges	AY 084006, AY 084018, AY 084030
CMW 9432	CBS 115724	"	*E. grandis*	Mexico	MJ Wingfield	AY 692321, AY 692324, AY 692323
CMW 13915[b]		"	*Eucalyptus* sp.	Mozambique	G Nakabonge	DQ246552, DQ246575, DQ246552
CMW 13912[b]		"	"	"	G Nakabonge	DQ246554, DQ246577, DQ246554
CMW 13883[b]		"	"	"	G Nakabonge	DQ246553, DQ246576, DQ246553
CMW 13944[b]		"	"	Kenya	J Roux	DQ246550, DQ246573, DQ246550
CMW 13949[b]		"	"	"	J Roux	DQ246551, DQ246574, DQ246551
CMW 14774[b]		"	"	Malawi	J Roux	DQ246555, DQ246578, DQ246555
CMW 14769[b]		"	"	"	J Roux	DQ246556, DQ246579, DQ246556
CMW 62		*Chr. austroafricana*	*E. grandis*	South Africa	MJ Wingfield	AF 292041, AF 273063, AF 273458
CMW 2113[d]	CBS 112916	"	"	"	MJ Wingfield	AF 046892, AF 273067, AF 273462
CMW 9327[d]	CBS 115843	"	*T. granulosa*	"	MJ Wingfield	AF 273473, AF 273060, AF 273455
CMW 9328		"	"	"	MJ Wingfield	AF 292040, AF 273064, AF 273458
CMW 13902[b]		"	*S. cordatum*	Mozambique	G Nakabonge	DQ246572, DQ246595, DQ246572
CMW 13926[b]		"	"	"	"	DQ246571, DQ246594, DQ246571
CMW 13929[b]		"	*Eucalyptus* sp.	"	G Nakabonge	DQ246570, DQ246593, DQ246570
CMW 14561[b]		"	*S. cordatum*	South Africa	"	DQ246559, DQ246582, DQ246559
CMW 14562[b]		"	"	"	"	DQ246560, DQ246583, DQ246560
CMW 13878		"	*Eucalyptus* sp.	South Africa	J Roux	DQ246566, DQ246589, DQ246566
CMW 13879		"	"	"	"	DQ246567, DQ246590, DQ246567
CMW 13977[b]		"	*Eucalyptus* sp.	Zambia	J Roux	DQ246569, DQ246592, DQ246569
CMW 13976[b]		"	*S. cordatum*	"	J Roux	DQ246568, DQ246591, DQ246568
CMW 17098[b]		"	"	Malawi	J Roux	DQ246561, DQ246584, DQ246561
CMW 17096[b]		"	"	"	J Roux	DQ246565, DQ246588, DQ246565
CMW 17101[b]		"	*Eucalyptus* sp.	"	"	DQ246562, DQ246585, DQ246562
CMW 17110[b]		"	"	"	"	DQ246563, DQ246586, DQ246563
CMW 17115[b]		"	"	"	"	DQ246564, DQ246587, DQ246564
CMW14852[b]	CBS 101281	"	*Eucalyptus* sp.	Cameroon	na	DQ246557, DQ246580, DQ246557
CMW 10790		*C. parasitica*	*Quercus serrata*	Japan	M Kusunoki	AF 140243, AF 140253, AF 140255
CMW 10518	CBS 112919	*C. nitschkei*	*Quercus* sp.	"	T Kobayashi	AF 452118, AF 525706, AF 525713
CMW 10463	CBS 112920	*C. macrospora*	*Castanopsis cupsidata*	"	T Kobayashi	AF 368331, AF 368351, AF 368350

[a]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. CBS refers to Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. [b]Isolates sequenced in this study. [c]GeneBank accession numbers are sequence data of the β-tubulin 1 & 2 (primers Bt1a/1b & Bt2a/2b) and ITS (primers ITS1/4) regions. [d]ex–type cultures.

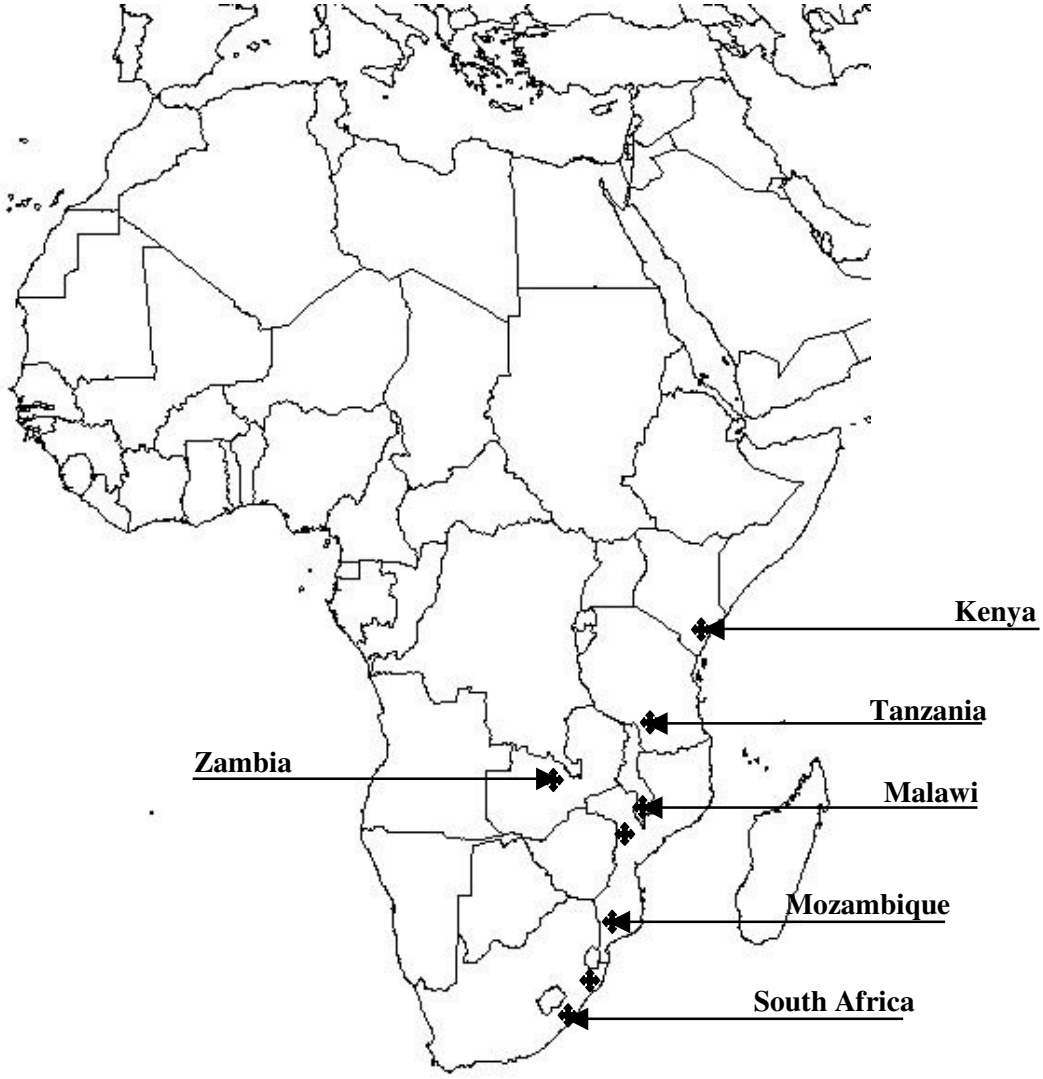

Fig. 1. Map of Africa showing different countries where surveys were conducted (http://www.africaguide.com/afmap.htm). Arrows/ crosses indicate general areas within each country where samples were collected.

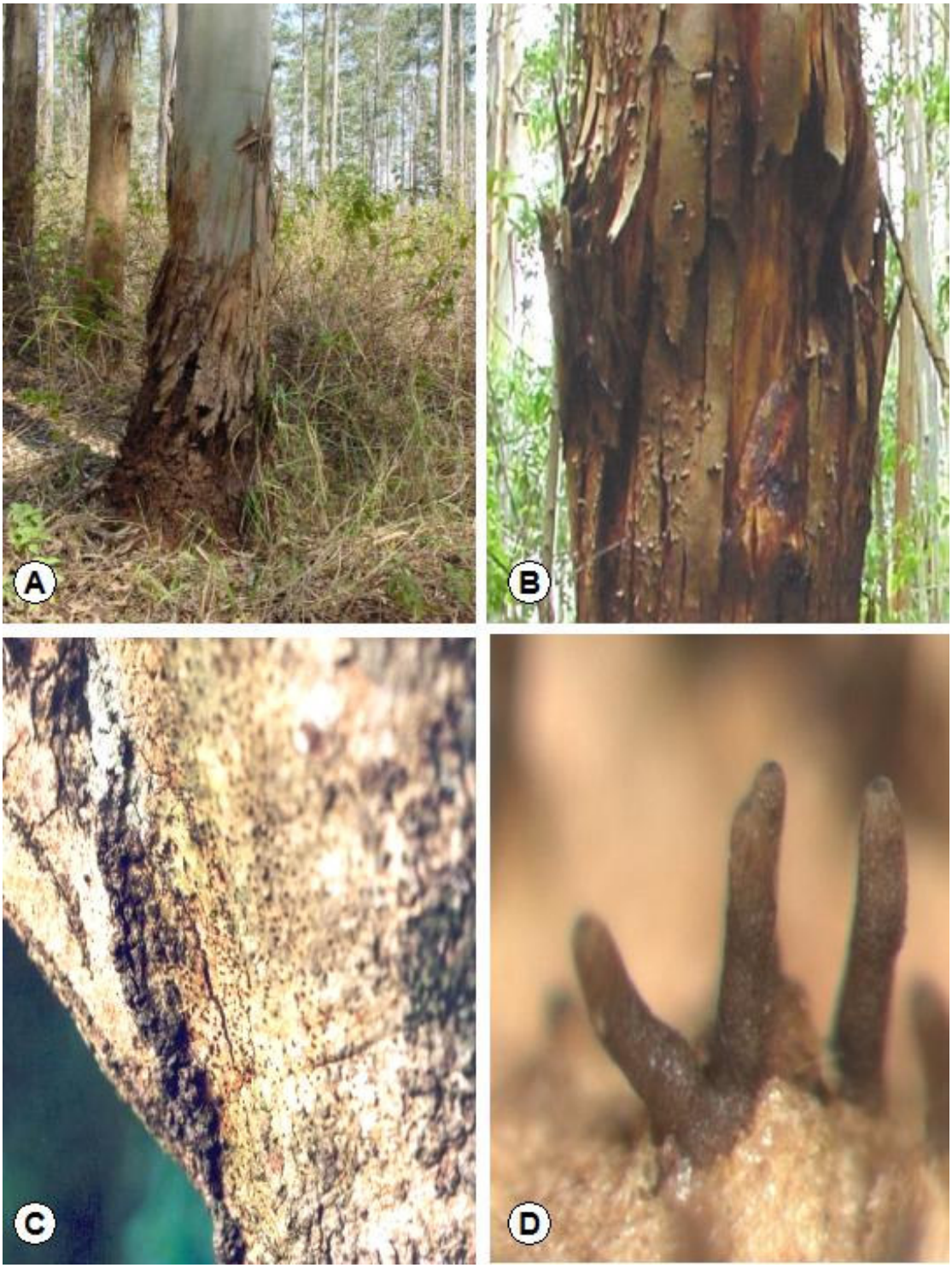

Fig. 2. Signs and symptoms of *Chrysoporthe* infection on *Eucalyptus* and *Syzygium cordatum*. **A.** Basal canker caused by *Chrysoporthe* sp. on *Eucalyptus* sp. **B.** Stem canker caused by *Chrysoporthe* sp. on *Eucalyptus* sp. **C.** Fruiting structures of *Chrysoporthe* sp. on *S. cordatum* (Figure provided by R. N. Heath, FABI, University of Pretoria). **D.** Ascomata of a *Chrysoporthe* sp.

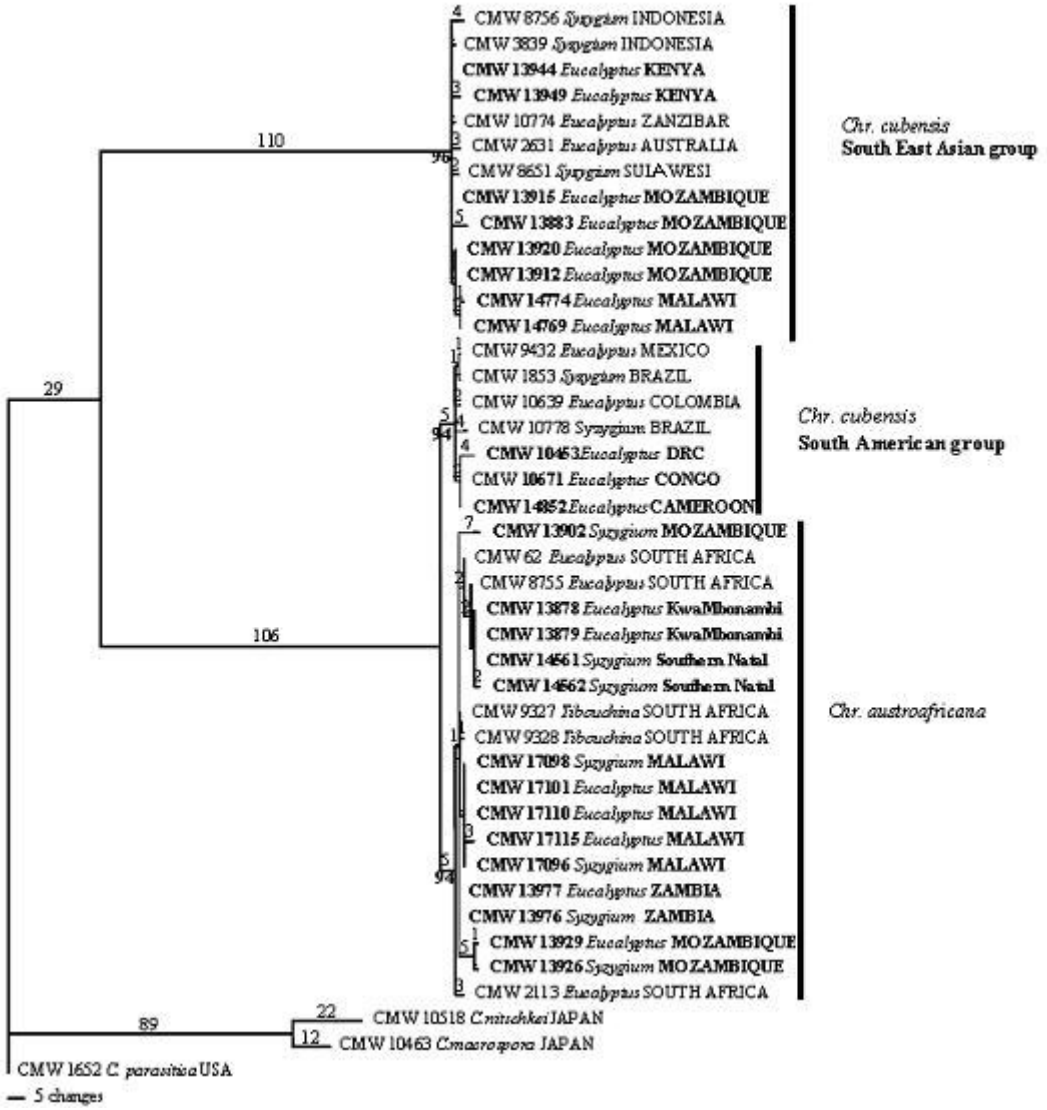

Fig. 3. Phylogenetic tree generated from ITS rDNA and β-tubulin gene sequence data (Tree length = 318, CI = 0.945, RI = 0.959) showing relatedness of the isolates of *Chrysoporthe* spp. collected from African countries. Branch lengths are indicated above the branches and bootstrap values above 50 % are indicated below the branches. *Cryphonectria parasitica, C. nitschkei* and *C. macrospora* were used as outgroups. Isolates collected in this study and isolates included in the study from Republic of Congo, DRC and Cameroon are in bold.

CHAPTER THREE

Genetic diversity of *Chrysoporthe cubensis* in eastern and southern Africa

ABSTRACT

Chrysoporthe cubensis is an important fungal pathogen of *Eucalyptus* spp. worldwide. The fungus is also known on many other hosts all residing in the order *Myrtales*. Previous studies have suggested that *Chr. cubensis* might be native to South America and southeast Asia and that it might have been introduced into Africa. Recently surveys have been conducted in eastern and southern Africa to assess the distribution of *Chrysoporthe* spp. in this region. *Chr. cubensis* was found on *Eucalyptus* spp. in Kenya, Malawi and Mozambique. The aim of this study was to determine the genetic diversity of *Chr. cubensis* populations from these countries. Population diversity studies were conducted using five pairs of microsatellite markers previously developed for *Chr. cubensis*. Results show that there is a very low genetic diversity within the populations of *Chr. cubensis* from Kenya, Malawi and Mozambique implying that the fungus is probably newly introduced in these countries. Based on phylogenetic analyses the origin of eastern African *Chr. cubensis* is most likely Asia.

INTRODUCTION

Chrysoporthe cubensis (Bruner) Gryzenh. & M. J. Wingf., previously known as *Cryphonectria cubensis* (Bruner) Hodges (Gryzenhout *et al.* 2004), is a fungal pathogen of *Eucalyptus* species in tropical and subtropical areas worldwide (Wingfield 2003). The canker disease caused by *Chr. cubensis* is characterized by the formation of stem cankers, wilting and death of trees (Hodges *et al*. 1976, Sharma *et al*. 1985). The disease is common on *Eucalyptus* spp. in areas with high temperatures and rainfall (Boerboom & Mass 1970, Hodges *et al.* 1976, Sharma *et al*. 1985) such as South America (Hodges *et al*. 1976), Central and North America (Hodges *et al*. 1979), Asia (Boerboon & Mass 1970, Sharma *et al*. 1985, Florence *et al*. 1986), Australia (Davison & Tay 1983) and Africa (Gibson 1981, Roux *et al*. 2003). Cankers are generally found at the bases of trees, but are often also found higher up on the stems (Hodges *et al*. 1976, Sharma *et al*. 1985, Roux *et al*. 2003). Management of the disease is most typically by planting resistant hybrids and clones (Alfenas *et al*. 1983, Sharma *et al*. 1985, Wingfield 2003, van Heerden *et al*. 2005).

In Africa, *Chr. cubensis* has been known since the 1950s. The fungus is known from the Democratic Republic of Congo (Zaire) where it was thought to be *Cryphonectria havanensis* (Bruner) M.E. Barr (Gibson 1981) but later identified as *C. cubensis* (Micales *et al.* 1987). *Chr. cubensis* is also known from Cameroon (Gibson 1981) and the Republic of Congo (Congo Brazzaville), on *E. grandis* and *E. urophylla* S.T. Blake (Roux *et al*. 2000, Roux *et al*. 2003).

The host range of *Chr. cubensis* is restricted to members of the order *Myrtales*. In the family *Myrtaceae*, apart from *Eucalyptus* spp., *Chr. cubensis* has been found on *Syzygium aromaticum* (L.) Merry. & Perry. (Clove) in Indonesia (Hodges *et al*. 1986), Sulawesi (Myburg *et al*. 2003), Malaysia (Reid & Booth 1969) and Zanzibar (Nutman & Roberts 1952). In the family *Melastomataceae,* the fungus has been reported from Colombia on indigenous *Miconia theaezans* (Bonpl.) Cogn. and *M. rubiginosa* (Bonpl.) DC., in Mexico on native *Clidemia sericea* D. Don and *Rhynchanthera mexicana* DC. (Gryzenhout *et al*. 2006), in Singapore and Thailand on exotic *Tibouchina urvilleana* (DC). Logn. (Gryzenhout *et al*. 2006) and in Indonesia on native *Melastoma malabathricum* L. (Gryzenhout *et al*. 2006). Recently,

Chr. cubensis has also been reported from Cuba on the non-native *Lagerstroemia indica* L. (Lythraceae). This represents the first record of the fungus on a plant family other than the *Myrtaceae* and *Melastomataceae* (Gryzenhout *et al*. 2006), but still within the order *Myrtales*.

Until recently, *Chrysoporthe* spp. were treated under the single name *C. cubensis,* responsible for a disease known collectively as Cryphonectria canker (Gryzenhout *et al*. 2004). Morphological characteristics and phylogenetic analysis of DNA sequence data of the Histone H3 gene and β- tubulin 1&2 gene regions revealed that within *C. cubensis* there are distinct phylogenetic groups (Myburg *et al*. 1999, 2002, Wingfield *et al.* 2001a, Gryzenhout *et al*. 2004, 2006). The South African group includes isolates that have been described as *Chr. austroafricana* Gryzenh. & M. J. Wingf. (Gryzenhout *et al*. 2004). Another group comprises of the American (Central North and South) and West and Central African isolates (South American group). A third group includes the southeast Asian, East African and Australian isolates (southeast Asian group) (Myburg *et al*. 2002, Nakabonge *et al*. 2005a). The latter two groups are phylogenetically clearly different but are treated as *Chr. cubensis* since no distinct morphological differences have been observed between them (Gryzenhout *et al*. 2004).

There have been two hypotheses regarding the possible origin of *Chr. cubensis.* One is that the fungus lives on clove trees (*S. aromaticum*) native to southeast Asia, where it causes little if any conspicous symptoms (Hodges *et al.* 1986). It is presumed that it spread to *Eucalyptus* growing areas worldwide, possibly via the spice trade. This is supported by the high genetic diversity revealed using Vegetative Compatibility Groups (VCGs) within the Indonesian population of *Chr. cubensis* (Van Heerden *et al*. 1997). Another hypothesis is that *Chr. cubensis* originated from South America (Wingfield *et al*. 2001b, Wingfield 2003). This is supported by the high genetic diversity revealed using VCGs within the Venezuelan population of *Chr. cubensis* (Van Heerden *et al*. 1997). Studies conducted on Brazilian isolates by Van Zyl *et al*. (1998) also revealed a high diversity in that region. Recently *Chr. cubensis* has been found on native *Miconia* species in Colombia (Rodas *et al*. 2005) and on native *C. sericea* and *R. mexicana* in Mexico (Gryzenhout *et al*. 2006). These South American

collections provide support for the view that the fungus might have originated in that part of the world (Gryzenhout *et al*. 2006).

Several disease surveys have been conducted in southern and eastern Africa to assess the distribution of *Chrysoporthe* spp. in the region (Nakabonge *et al*. 2005a, Roux *et al*. 2005). *Chr. cubensis* was subsequently reported for the first time from Kenya, Malawi and Mozambique. All the isolates from these areas were shown to group in the southeast Asian clade of *Chr. cubensis* (Nakabonge *et al*. 2005a). The aim of the present study was to assess the level of genetic variability in Kenyan, Malawian and Mozambican populations of *Chr. cubensis*. This would provide information on the possible origin of this spceis, which has been hypothesized to be introduced into Africa.

MATERIALS AND METHODS

Fungal isolates

Pure cultures of *Chr. cubensis* used in this study were those from collections made by Roux *et al*. (2005) and identified by Nakabonge *et al*. (2005a) (Chapter 2). Fifty-one isolates were obtained from *Eucalyptus* trees growing in two adjacent plantations in Malawi, ten isolates from *E. urophylla* in a single plantation from Kenya and nine isolates from the Manica area in Mozambique (Table 1). All isolates used in this study are housed in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Isolates were grown in Petri plates on 2% malt extract agar (MEA) (20 g/l malt extract and 15 g/l agar, Biolab, Midrand, South Africa) containing 100 mg Streptomycin sulfate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) at 26 ºC for seven days. Mycelium was scraped from the plates, transferred to 1.5 ml Eppendorf tubes and DNA extracted as previously described by Nakabonge *et al.* (2005b) (Chapter 6).

Simple Sequence Repeats (SSR) PCR

Eight pairs of PCR-based simple sequence repeat (SSR) primers previously developed by Van der Merwe *et al*. (2003) for *Chr. cubensis* were tested on the African isolates.

The PCR reaction mixes and conditions were the same as those described by Van der Merwe *et al*. (2003). The DNA concentrations of the PCR products were visually measured against the intensity of a 100 bp marker (Roche Molecular Biochemicals, Mannheim, Germany) on a 2 % Agarose gel stained with ethidium bromide and exposed to UV illumination.

PCR products were diluted for genescan analysis based on the approximate concentrations of the PCR products. Samples were separated on a 4.25 % PAGE gel, using an ABI Prism^{TM} 377 DNA sequencer (Applied Biosystems). Allele sizes were estimated by comparing the mobility of the SSR products to that of the TAMRA internal size standard (Applied Biosystems, Perkin Elmer Corp) as determined by Genescan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 2 (Applied Biosystems). To ensure reproducibility, a reference sample was run on every gel.

Genetic diversity and differentiation

Isolates were scored based on allele size at each locus. This information was used to generate a multilocus profile or genotype for each isolate. Identical genotypes were treated as clones and statistics were calculated for clone-corrected populations. Allele frequencies in each population were then calculated by dividing the number of times an allele occurred in the population by the population sample size. The allele frequencies were used to calculate the gene diversity (Nei 1973), $H = 1 - \sum_k x_k^2$ where x_k is the frequency of the k^{th} allele for each population using the programme POPGENE version 1.31 (Yei *et al.* 1999). Differences in allele frequencies for clone corrected populations were estimated by calculating Chi-square tests (χ^2) (Workman & Niswander 1970).

Genotypic diversity was calculated using the formula $G = 1/\Sigma [f_x (x/n)^2]$, where, n is the sample size and f_x is the number of genotypes (haplotypes) occurring x times in the population and G being the effective number of equally frequent haplotypes (Stoddart & Taylor 1988). The genotypic diversities between populations was compared by obtaining the maximum percentage of genotypic diversity using the formula $\hat{G} = G/N*100$, where N is the sample size (McDonald *et al*. 1994).

Genetic distance

The genetic distance was calculated between *Chr. cubensis* genotypes based on Nei's (1972) unbiased genetic distance. The distance matrix was generated using the program POPGENE version 1.31 and a tree constructed using UPGMA (Unweighted Pair – Group Method with Arithmetic mean) in MEGA version 2.1 (Kumar *et al.*, 2001).

RESULTS

Simple Sequence Repeats (SSR) PCR

Five of the eight pairs of PCR-based simple sequence repeat (SSR) primers (SA6, SA9, COL3, SA10, SA1) amplified the microsatellite regions for the African isolates. Allele sizes were successfully estimated for all microsatellite regions.

Genetic diversity and differentiation

A total of seven alleles were amplified across the five loci for the *Chr. cubensis* population from Kenya and 10 alleles for the population from Malawi (Table 2). Isolates from Mozambique were not included in the analysis because they all belonged to a single clone that occurred in both the Kenyan and Malawian populations. Locus SA1 was monomorphic in both populations. The remaining four loci had a total of three alleles each. Six alleles were shared between populations. Thus there were a total of seven unique alleles (alleles not shared between populations). The χ^2 tests for the five microsatellite regions showed no significant difference in allele frequencies at any loci between the Kenyan and Malawian populations of *Chr. cubensis* (Table 3).

A total of nine genotypes were obtained when the *Chr. cubensis* populations were combined. One genotype was shared between the Kenyan and Malawian populations giving a total of 8 unique genotypes. The maximum genotypic diversity for the Kenyan population was 17.2 % and 5.4 % for Malawi (Table 2).

Genetic distance

The UPGMA tree constructed from the matrix obtained using Nei's (1972) genetic distance clearly showed that there was no grouping of isolates according to the areas sampled (Fig 1). Different genotypes were equally distributed throughout the populations sampled.

DISCUSSION

The population structure of a collection of *Chr. cubensis* isolates from *Eucalyptus* spp. from Kenya, Malawi and Mozambique was considered for the first time in this study, using microsatellite markers specifically developed for this fungus. This represents the first attempt to consider the genetic structure of the fungus from eastern and southern Africa. The very low genetic diversity obtained for these populations is indicative of a newly introduced pathogen. The hypotheses that *Chr. cubensis* has a South American or southeast Asian origin can therefore not be rejected in this study. The low genetic diversity in combination with phylogenetic data (Myburg *et al*. 2002, Gryzenhout *et al*. 2004, Nakabonge *et al*. 2005a) suggest that *Chr. cubensis* from eastern and southern Africa did not originate from Africa.

Newly introduced populations are expected to possess lower diversities and very few private alleles, compared to native populations (Taylor *et al*. 1999, McDonald & Linde 2002). Very low genetic diversities were observed within the Kenyan and Malawian populations and only a single clone in Mozambique. Migration was also observed between the Kenyan and Malawian populations. There were no significant differences between chi square values at all loci in both populations. The low diversities obtained strongly suggest that *Chr. cubensis* has been introduced into these countries relatively recently. Processes such as mutation that result in increased diversity in a population occur in well-established populations (Taylor *et al*. 1999, McDonald & Linde 2002). The preliminary results obtained in this study imply that there has not been sufficient time to allow such processes to occur in the African populations. More isolates are required from Mozambique before any specific conclusions can be arrived at for this region. However, the combination of the fact that the Mozambican isolates examined are clonal (and identical to a clone that occurs

in both Malawi and Kenya) and that the fungus is found rarely (of a collection of 89 *Chrysoporthe* spp. collected from Mozambique only nine isolates proved to be *Chr. cubensis*; unpublished data) suggest that the fungus was only recently introduced.

Several population diversity studies have previously been conducted on *Chr. cubensis* from Asia and South America (Van Heerden *et al*. 1997, Van Zyl *et al*. 1998, Van der Merwe 2000). The majority of these studies were, however, based on VCG's. Studies conducted by Van Heerden *et al*. (1997) showed that populations from Venezuela and Indonesia are highly diverse. Van Zyl *et al*. (1998) also reported a large number of VCG groups from a Brazilian population of *Chr. cubensis*. These studies support the dual hypothesis that South America or southeast Asia both represent possible areas of origin for *Chr. cubensis*. Our study provides added evidence to suggest that eastern and southern African populations of *Chr. cubensis* are newly introduced. The fact that the populations considered in this study are genetically uniform could imply that management strategies to reduce the impact of the disease in eastern and southern Africa have a good chance of succeeding.

Previous studies have shown that *Chr. cubensis* forms two distinct phylogenetic groups (Myburg *et al*. 2002, Gryzenhout *et al*. 2004). Nakabonge *et al*. (2005a) showed that *Chr. cubensis* isolates from Kenya, Malawi and Mozambique group with the isolates from southeast Asia and Zanzibar, different from isolates from South, Central and North America. This would imply that the eastern and southern African isolates have a southeast Asian origin. The results of this study should be considered as preliminary due to the fact that the fungus was difficult to isolate and available cultures were relatively few in number despite the large numbers of samples collected. The disease was easy to find once an infected plantation was found. Isolation success was, however less than 50 %, in some cases as low as 10 %. In Kenya for example, we collected from ~50 trees but only 10 isolates were obtained. However, it is reasonably clear that the *Chr. cubensis* populations treated in this study have been introduced into the region.

Chr. cubensis has never been reported on a native host in Africa. This makes this continent a highly unlikely center of origin for the fungus. From the results obtained in this study we hypothesize that *Chr. cubensis* was introduced from Zanzibar, to

mainland Tanzania where it spread through East Africa into Mozambique. In South Africa, where *Eucalyptus* hybrids and clones are widely grown in plantations, *Chr. cubensis* has not been reported. However, the occurrence of the fungus in bordering Mozambique, is threatening to an important *Eucalyptus* plantation industry in this country. Every effort must thus be made to slow the advance of *Chr. cubensis* into South Africa and to start screening South African material for tolerance to this pathogen.

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Table 1: Isolates of *Chrysoporthe cubensis* used in this study.

Isolate numbers (CMW) [a]	**Origin**	**Host**	**Collector**
13941, 13942, 13945–13947,13951, 13953, 13955, 13956, 14412	Kenya	*Eucalyptus urophylla*	J. Roux
13943, 13944, 13948–13950, 13954, 14411,14757–14765, 14767–14774, 17095–17097, 17099,17101, 17103, 17104, 17106, 17111, 17113, 17114, 17116, 17117, 17119, 17120–17130, 17134, 17135	Malawi	*E. grandis*	J. Roux
13880, 13883, 13912, 13915, 13920, 13928, 17064, 17071, 17073	Mozambique	*E. grandis*	J. Roux

[a]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Table 2: Allele size (bp) and frequency at 5 loci for clone corrected populations of *Chr. cubensis* from *Eucalyptus* spp. from Kenya and Malawi.

Locus	**Allele Length**	**Allele frequencies**	
		Kenya	**Malawi**
SA6	206	-	0.14
	210	1.0	0.71
	245	-	0.14
SA9	194	0.5	1.0
	195	0.5	-
	196	-	-
COL3	167	-	0.28
	176	1.0	0.71
	177	-	-
SA10	181	-	0.14
	182	1.0	0.42
	183	-	0.42
SA1	319	1.0	1.0
	-	-	-
	-	-	-
N(g)		**2**	**9**
N		**10**	**51**
Number of alleles		**7**	**10**
G		**1.72**	**2.75**
Ĝ (%)		**17.2**	**5.4**

N = number of isolates

N (g) = number of multilocus genotypes

G = Genotypic diversity (Stoddart & Taylor 1988)

Ĝ = percent maximum diversity

Table 3: Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 5 polymorphic SSR loci across clone corrected populations of *Chr. cubensis* collected from *Eucalyptus* spp. in Kenya and Malawi. There were no significant differences between allele frequencies at any loci (P<0.005).

Locus	Kenya	Malawi	X^2	df
SA6	0	0.44	0.73	2
SA9	0.5	0.00	3.9	1
Col3	0	0.40	0.73	1
SA10	0	0.61	2.0	2
SA1	0	0.00	0	0
N	2	7		
Mean	0.1	0.29		

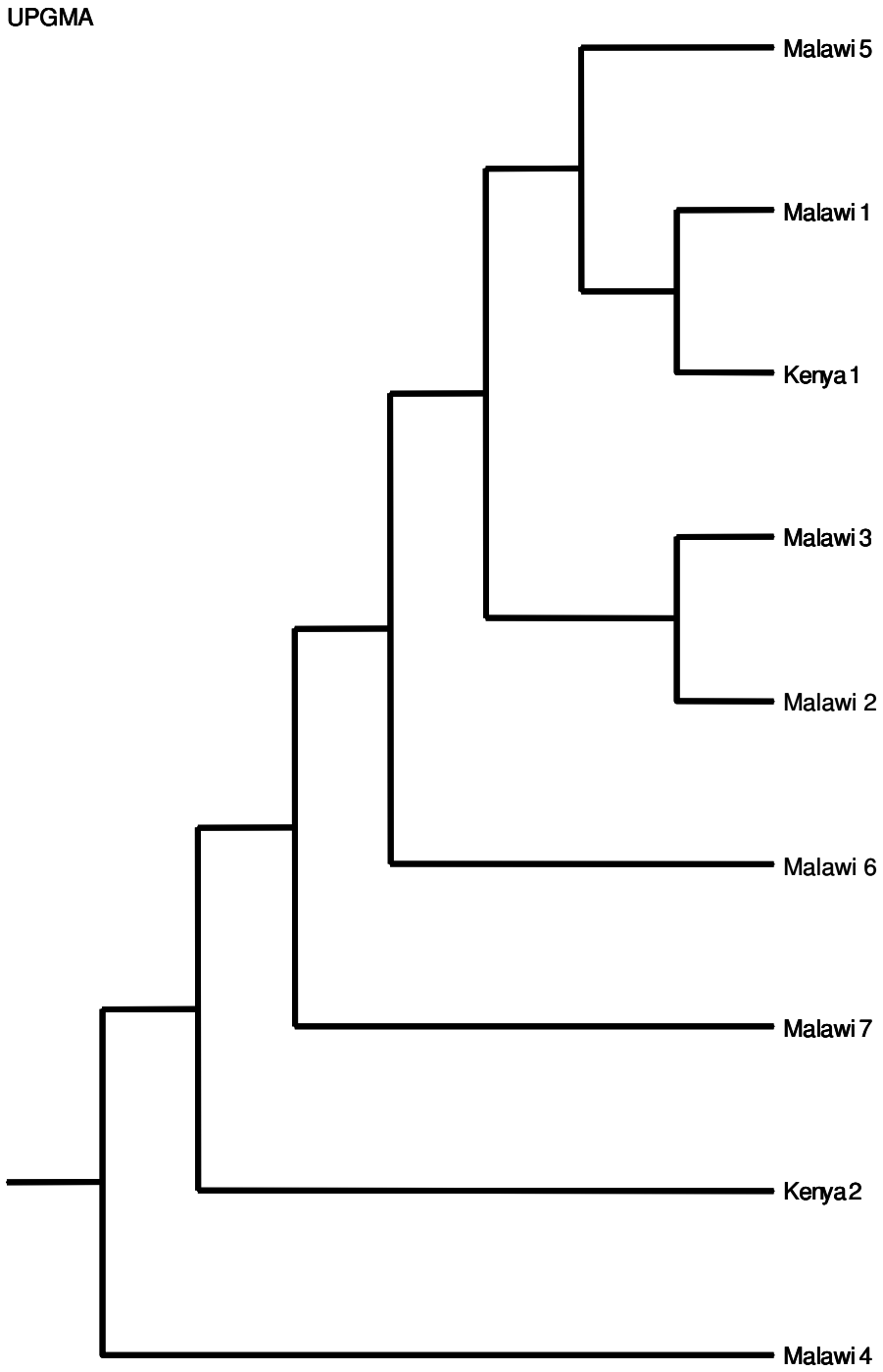

Fig. 1. UPGMA dendrogram of *Chr. cubensis* genotypes from Malawi and Kenya collected from *Eucalyptus* spp. constructed with clone corrected data obtained using 5 polymorphic microsatellite markers.

CHAPTER 4

Development of polymorphic microsatellite markers for the fungal tree pathogen *Cryphonectria eucalypti*

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ABSTRACT

Polymorphic microsatellite DNA markers were developed from a single spore isolate of *Cryphonectria eucalypti* collected from *Eucalyptus* stem canker in South Africa. Markers were obtained using the enrichment technique known as FIASCO (Fast Isolation by AFLPs of Sequences Containing Repeats). Ten polymorphic markers were isolated, of which 2 were discarded due to their high polymorphism in the flanking region. The mean number of alleles produced by the remaining eight markers from 20 isolates was 7.25 and alleles per locus ranged from 4 to 12. The markers will be used to study populations of *C. eucalypti*.

INTRODUCTION

Cryphonectria eucalypti, previously known as *Endothia gyrosa* is a fungal pathogen of *Eucalyptus* species (Venter *et al.* 2002). It is an ascomycete with a life cycle predominantly occurring in the haploid phase (Kendrick 1985). *C. eucalypti* has been reported from South Africa, Tasmania and Australia (Venter *et al.* 2002). In Australia, it has been associated with bark cracks, cankers, dieback of coppice shoots, branches and stems and in severe cases, tree death (Walker *et al*. 1985, Old *et al*. 1986). In South Africa, infection by *C. eucalypti* results in superficial cracks on the bark of trees, but under stress conditions, infection can result in large cankers and death of young trees (Gryzenhout *et al*. 2003).

Virtually nothing is known regarding the population biology of *C. eucalypti,* although it is assumed that the pathogen is native in eastern Australia. There are, however, intriguing patterns regarding the distribution of the pathogen that could be elucidated through studying its population biology. For example, in Western Australia only the asexual state of *C. eucalypti* occurs on cankers, while in eastern Australia both the sexual (teleomorph) and asexual (anamorph) states occur (Walker *et al*. 1985, Davison & Coates 1991). This might imply that the fungus has been accidentally introduced into western Australia. Both the teleomorph and anamorph of *C. eucalypti* occur in South Africa, which is a situation very much like that in eastern Australia (Venter *et al*. 2002).

Co-dominant markers are especially useful in studies aimed at answering questions relating to genetic diversity, origin and reproduction of fungi (Weising *et al*. 1995). This is due to their high levels of polymorphism and high reproducibility (Rafalski *et al*. 1996). This study was, therefore, undertaken to develop and characterise polymorphic microsatellite markers for the fungal pathogen *C. eucalypti,* to be used in population genetics studies. Modification of the enrichment technique FIASCO (Fast Isolation by AFLPs of Sequences Containing Repeats), described by Zane *et al*. (2002), was used to isolate the microsatellite regions.

Genomic DNA of approximately 200 ng/µl was extracted from mycelia (haploid) produced from a single ascospore isolate (CMW 2151) collected from a canker on the stem of a *Eucalyptus* sp. in South Africa. This was simultaneously digested with *Mse*I restriction enzyme and ligated to a *Mse*I AFLP adaptor (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG-3'). The reaction mix included 200 ng of genomic DNA, 100X BSA (Bovine Serum Albumin) (50 µg/ml), 10 µM Adaptor, 1 Mm ATP, 1X Enzyme buffer 2 (New England Biolabs), 2000 U/µl of DNA ligase (New England Biolabs) and 2.5 U *Mse*I (New England Biolabs) in a total volume of 120 µl. We used two enzymes from the same manufacturer. This enabled a single step digestion with a compatible buffer (Buffer 2). A higher concentration of ligase enzyme resulted in enhanced PCR patterns after digestion-ligation.

The reaction mixture was incubated overnight at 37 ^{o}C after which it was inactivated at 65 ^{o}C for 20 min. The digestion-ligation mixture was purified using phenol and chloroform and the DNA was precipitated overnight using 0.1 volumes of 3 M Sodium acetate (NaOAc – Ph 4.6) and 1 volume absolute ethanol.

The mixture was diluted (1:10) and amplified in a total of 25 µl consisting of 2 ng DNA, 10X PCR reaction buffer, 200 Mm dNTPs, 5 U/µl expand high fidelity enzyme (Roche Diagnostics) and 8.1 µl H_20. PCR products showing a visible smear were selected for further use and hybridized with the biotinylated probes CA_{10} and CT_{10} as described by Zane *et al*. (2002). DNA denaturation was carried out at 96 ^{o}C for 10 min and annealing at 62 ^{o}C for 1 hr. Trna (1 µg) was mixed with streptavidin coated beads (Dynal Biotech ASA) to minimize nonspecific binding of genomic DNA.

DNA was separated from the beads-probe complex by denaturation. To do so, TLE (150 µl) (10 Mm Tris-HCl, 0.1 Mm EDTA) was added to the beads and incubated at 95 ^{o}C for 10 min. After magnetizing, the supernatant containing DNA was rapidly removed and stored for further use. DNA was precipitated by adding 1 volume isopropanol (150 µl) and 3 M NaOAc Ph 4.6 (7.5 µl) which was incubated overnight at -20 ^{o}C, washed with 70% ethanol and re-suspended in 30 µl sterile distilled water. The product was amplified using the same conditions described above but rather using *Taq* DNA polymerase (Roche Diagnostics). The product was run on a 1% agarose gel

after which PCR products showing a visible smear were cleaned using 0.06 g/ml of Sephadex G-50 (SIGMA-ALDRICH, Inc.).

Cloning was executed with a TOPO-TA-cloning Kit (Invitrogen, Clareinch) following the manufacturer's protocol. Bacterial colonies containing plasmids were selected by performing colony PCR in a total volume of 50 µl, using 2 ng DNA obtained from a 2:5 dilution of an overnight grown colony, 10X PCR reaction buffer, 1.5 Mm $MgCl_2$, 300 Mm of each TOPO (M13) modified primer (5'-GTAAAACGACGGCCAG-3'/ 5'-CAGGAAACAGCTATGAC-3'), 200 Mm dNTPs, 5 U/µl *Taq* polymerase enzyme (Roche Diagnostics) and 8.1 µl H_20.

A sequencing PCR was performed in 10 µl volume containing 10X concentration of ready reaction mix BD (ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems), ~2.0 pmol/µl forward or reverse primer for each area sequenced (using the same primers used for PCR amplification), 5X dilution buffer, purified DNA (PCR product ~50 ng DNA) and 4.5 µl sterile distilled water. The reaction was performed using the following parameters: 96 ^oC for 10 s, 56 ^oC for 30 s and 60 ^oC for 4 min for a total of 25 cycles. Automated sequencing was performed on an ABI Prism 3100 auto sequencer (Perkin-Elmer Applied Bio Systems). Fragments containing repeats were selected and primer pairs were designed flanking the microsatellite repeats (Table 1).

PCR using the designed primers was conducted on DNA from 5 isolates of *C. eucalypti* including three isolates (CMW 2186, CMW 7034 and CMW 7036) from South Africa and two isolates (CMW 7038, CMW 7037) from Australia. PCR was performed in a 25 µl volume containing 2 ng DNA template, 0.2 Mm dNTPs (Promega, Madison), 0.15 Mm of each primer, 0.1 µl *Taq* DNA polymerase (Roche Molecular Biochemicals), 1X buffer with $MgCl_2$ (10 Mm Tris-HCL, 1.5 Mm $MgCl_2$, and 50 Mm KCl) and 17.4 µl water, under the following conditions: 96 ^oC for 1 min, 94 ^oC for 30 s for 35 cycles, annealing at 60 ^oC for 1 min and extension at 72 ^oC for 1.5 min.

The PCR reactions were purified using Sephadex G-50 and sequenced using the same conditions as described in the previous paragraph. The sequences obtained for all the

microsatellite regions in all five isolates were compared with the sequences from the isolate (CMW2151) that was used to develop the primers. This comparison was made in order to verify whether polymorphisms were present in the repeats or flanking regions.

Of the markers produced, ten were polymorphic. Of these eight had polymorphisms in the microsatellite regions but two had polymorphism in the flanking region and were not considered for further use. The forward primers of the eight polymorphic loci were labelled with fluorescent dyes (NED, VIC, FAM or PET) (Applied Biosystems). DNA from 15 additional isolates from Australia and South Africa (Table 2) were amplified using the labelled primers. Allele sizes were determined using ABI PRISM® Gene Mapper Software Version 3.0 (Applied Biosystems) using the LIZ™ 500 size standard.

A total of 56 alleles were obtained across eight loci for the isolates of *C. eucalypti* tested. The most polymorphic locus had 12 alleles while the least polymorphic locus had 4 alleles (Table 2). The average allelic diversity (H*) (Nei 1973) was 0.73 (Table 1). A total of 19 genotypes were obtained from the 20 isolates used in this study with isolates from the same areas exhibiting different genotypes. These markers have shown to be useful to elucidate population genetic parameters in *C. eucalypti* populations. They will consequently provide useful tools for future investigations considering the population biology and especially the global spread of *C. eucalypti*.

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Table 1. Characteristics of polymorphic microsatellite markers designed for the fungal pathogen *Cryphonectria eucalypti*. H* and PCR products sizes were computed from 20 isolates.

Primer pair	Fluorescent Label	5' 3' oligonucleotide sequence	T_m (°C)	H*	PCR product size (bp)	Core Sequence	Gen Bank accession No.
10A FF	PET	CTC TTG CAG CCT CGG AGA CTG	65	0.80	388-403	(TA)2 (CGCA)2 (CA)18	AY770525
10A RR		GAG TGG CCA TAT TCA GCT TGG C	64				
1B FF	6-FAM	GCA TCT CAA CAG TGC ACT CCA G	64	0.62	185-191	(CA)16	AY770523
1B RR		CAC ATA CAC TCT CAT AGC TCT CGG	65				
2B FF	PET	GCC CAA AGG ATG TGT GAA TGT G	62	0.58	218-222	(TGCG)3 (GT)11 (A)3	AY770529
2B RR		CAA ACT GGC GGA TGA CAG GC	63				
7A FF	VIC	CCT GAC AGA GAA GCG ACC CT	63	0.77	196-219	(CA)18 (CT)15	AY770522
7ª RR		GCA TCA GCT CAG GGC ATA GAG	63				
8A FF	6-FAM	CCG AGG GTT AGA CAT CAC CC	63	0.69	238-276	(G)4 (GT)16 (T)3	AY770526
8A RR		ACC TGA CGC TCC ATC TGC AC	63				
9A FF	VIC	CTG CTG ACA AGG ACG AGG AC	63	0.76	256-292	(GA)2 (G)3 (GT)16	AY770528
9A RR		CGT TTC GTG GCT GGA TCT CG	63				
5A FF	NED	GGT CCA TCA GTC GTC TCA GC	63	0.87	240-336	(CT)52	AY770524
5ARR		GCA GCA ATG AGG TGC CTT GG	63				
5B FF	NED	GTG TCG TCG CTC GCG AAT AG	63	0.76	342-375	(AC)15	AY770527
5B RR		CAG GAG AGG ACA TGC GAG AC	63				

H* = Nei's (1973) gene diversity, T_m (°C) = Melting temperature.

Table 2. Allelic properties of 20 isolates of *Cryphonectria eucalypti*. Each locus comprises of an allele obtained for each isolate.

		Loci							
Isolate No.[a]	**Origin**	**10A**	**1B**	**2B**	**7[A]**	**8[a]**	**9[a]**	**5[a]**	**5B**
CMW 15172	Albany, WA , Australia	403	185	220	198	259	289	269	375
CMW 15143	Brunswick Junction, WA, Australia	389	191	222	219	238	277	265	342
CMW 15144	"	389	191	222	219	259	277	240	368
CMW 15168	"	389	191	222	219	260	277	279	358
CMW 15197	Bunbary, WA, Australia	388	198	232	215	257	278	283	358
CMW 15195	"	390	191	222	219	277	277	269	344
CMW 15181	Esperance, WA, Australia	392	191	222	198	276	277	267	344
CMW 15180	"	403	185	222	198	262	284	269	375
CMW 15178	Manjimup, WA, Australia	389	191	222	211	259	277	256	344
CMW 15176	"	390	191	222	198	259	267	267	344
CMW 15185	Walpole, Australia	389	191	222	211	260	278	243	344
CMW 15150	"	389	191	222	219	259	260	271	344
CMW 2367	Flatcrown, KZN, South Africa	399	196	218	203	260	292	320	373
CMW 2554	"	399	196	218	203	255	256	322	373
CMW 2216	Graskop, Mpumalanga, South Africa	398	196	218	203	259	292	320	373
CMW 2159	"	399	196	218	203	259	292	322	374
CMW 2151	"	399	196	218	203	259	292	320	373
CMW 2188	Nyalazi, KZN, South Africa	398	196	218	196	260	292	320	373
CMW 2379	Tzaneen , Limpopo, South Africa	398	196	218	203	259	292	336	373
CMW 2373	"	399	196	218	198	260	292	320	373
	TOTAL NUMBER OF ALLELES	**7**	**4**	**4**	**6**	**8**	**8**	**12**	**7**

[a]CMW represents the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

CHAPTER FIVE

Population structure of the fungal pathogen *Holocryphia eucalypti* in Australia and South Africa

ABSTRACT

Holocryphia eucalypti (=*Cryphonectria eucalypti*) is a fungal pathogen causing stem cankers on *Eucalyptus* species in South Africa and Australia. In South Africa it is considered opportunistic and in Australia it has been associated with occasional but serious disease problems. The aim of this study was to determine the population structure of a South African population of *H. eucalypti* and compare it with three Australian populations of the fungus. Seventy two isolates from several *Eucalyptus* spp. and clones in South Africa, were compared with thirty isolates from *E. globulus* in south western Australia, twenty four isolates from *Corymbia calophylla* in south western Australia and twenty three isolates from eastern Australia on *E. dunni.* DNA of these isolates was amplified using eight pairs of microsatellite markers previously developed for *H. eucalypti*. Nei's gene diversity (*H*) showed that the eastern Australian population is most genetically diverse and the Western Australian populations from *Corymbia* and *Eucalyptus* somewhat less diverse. The South African population displayed the lowest genetic diversity. The high genetic diversity in the Australian populations supports the view that *H. eucalypti* is native to that region. In addition to a spartial effect a temporal effect may also explain these results as the migration into South Africa may have taken place a considerable time ago. This is consistent with the fact that *Eucalyptus* spp. are also native to the Australian continent.

INTRODUCTION

Holocryphia eucalypti (M. Venter & M. J. Wingf.) Gryzenh. & M. J. Wingf. prov. nom previously known as *Cryphonectria eucalypti* M. Venter and M.J. Wingf. (Venter *et al*. 2002, Gryzenhout *et al*. 2006), is a fungal pathogen causing a stem canker disease on *Corymbia* spp. and *Eucalyptus* spp. in mainland Australia (Walker *et al*. 1985, Davison & Coates 1991), Tasmania (Yuan & Mohammed 1997, Wardlaw 1999) and *Eucalyptus* spp. in South Africa (Van der Westhuizen *et al*. 1993). In Australia, *H. eucalypti* causes bark cracks, cankers, dieback of coppice shoots and in severe cases tree death has been reported (Walker *et al*. 1985, Old *et al*. 1986, Wardlaw 1999, Jackson 2004). In South Africa, infection typically results in superficial cracks in the bark and, only occasionally, severe cankers have been reported under environmental conditions stressful to the trees (Gryzenhout *et al*. 2003). Kino exudation or damage to the cambium is rarely observed in South Africa. However, *H. eucalypti* has recently been found on *E. smithii* R.T. Baker near Pietermaritzburg (KwaZulu/Natal Province) where cankers extended into the cambium (Gryzenhout *et al*. 2003).

Eucalyptus spp. were introduced to South Africa from Australia in the early 1800's and there are now over 600 000 ha of commercial *Eucalyptus* plantations in the country (Anonymous 2004). *E. grandis* W. Hill is most commonly planted, while in sub-tropical areas, clones of hybrids between *E. grandis* and *E. urophylla* S. T. Blake. or *E. camaldulensis* Dehnh. are commonly planted. Breeding programmes are used to improve wood quality, growth rates as well as resistance to pests and diseases (Denison & Kietzka 1993, Wingfield & Roux 2000).

Management and control of plantation diseases has been widely achieved via breeding for species, hybrids and clones with different levels and sources of resistance (Wingfield *et al.* 2001, Wingfield 2003). In order to effectively manage resistant hybrids, it is important to understand the genetic diversity of the pathogen population. This knowledge provides insight into the capability of the pathogen to overcome host resistance (McDonald & McDermott 1993, McDonald & Linde 2002). Processes such as mutation, gene flow, reproduction/recombination, population size and selection, result in increased diversity in a pathogen population (Taylor *et al*. 1999,

McDonald & Linde 2002). Molecular markers can give an indication of the processes occurring in populations of pathogens such as *H. eucalypti* and they can often provide an insight as to the origin of a pathogen, which contributes to quarantine legislation (Milgroom & Fry 1997). Co-dominant markers have been effectively applied in population genetic studies due to their high level of polymorphism and reproducibility (McDonald 1997). Microsatellite markers have been widely used to examine diversity, mode of reproduction, gene flow and speciation in many fungi (McDonald 1997, Burgess *et al*. 2004a,b, Barnes *et al*. 2005).

Phylogenetic and taxonomic studies have been used to show that the fungus now known as *H. eucalypti* is very different to *Endothia gyrosa,* which is the name originally used for the *Eucalyptus* pathogen in Australia and South Africa (Venter *et al*. 2001, 2002). Because *H. eucalypti* was first found in Australia and is commonly found on native trees in that country, it has been assumed that the fungus in South Africa has an Australian origin. However, there are no experimental data to support this view and nothing is known regarding the genetic diversity of the fungus in either country. The aim of this study was thus to determine the population diversity of *H. eucalypti* using polymorphic microsatellite markers recently developed for this fungus (Nakabonge *et al*. 2005).

MATERIALS AND METHODS

Fungal isolates

H. eucalypti was isolated from trees showing typical canker symptoms associated with this fungus (Fig. 1). All isolates (Table 1) were from individual trees growing either in plantations or natural forests in Australia and South Africa. Seventy-two isolates were obtained from *Eucalyptus* trees growing in plantations in South Africa, from an area of approximately 1000 km^2. Twenty-three isolates were obtained from plantation-grown *E. globulus* Labill. in Western Australia from an area of approximately 3000 km^2. Thirty isolates were obtained from native *C. calophylla* (Lindl.) K. D. Hill & L. A. S. Johnson in Western Australia from an area of similar size. Twenty-four isolates were obtained from eucalypt plantations in three states in eastern Australia.

Isolations

Isolates from South African trees were obtained from pieces of bark bearing fungal fruiting structures, which were placed in moist chambers to induce sporulation. Droplets of spores were picked up with a sterile needle and spread onto 2 % Malt Extract Agar (MEA) (20 g/L malt extract and 20 g/L agar, Biolab, Midland, Johannesburg) with 100 mg/L streptomycin sulphate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). After overnight incubation, single germinating spores were picked up using sterile needles and grown on fresh plates. Isolates of *H. eucalypti* from Australia were collected during disease surveys (Table 1). Fruiting structures of the fungus are much less common in Australia than in South Africa, particularly in Western Australia where only the asexual state of the fungus is found. Isolates from Australia were obtained by placing pieces of wood from the edges of cankers directly onto half strength PDA (½ PDA; Becton, Dickinson and Company, Sparks, USA). Fruiting structures of *H. eucalypti* form rapidly on this agar and pure cultures were obtained by streaking spores from a single structure onto fresh plates. Pure cultures have been maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction and SSR PCR

Isolates were grown in Petri plates on MEA at 26 ºC for 7 days. The mycelium was scraped from the plates, transferred to 1.5 ml Eppendorf tubes and DNA extracted as previously described by Nakabonge *et al.* (2005). Eight pairs of PCR-based, short simple repeat (SSR) primers (Table 2), previously developed by Nakabonge *et al.* (2005) for *H. eucalypti*, were used to amplify the preferred microsatellite regions. The PCR reaction mixes and conditions were the same as those described by Nakabonge *et al*. (2005). The DNA concentrations of the PCR products were visually measured against the intensity of a 100 bp marker (Roche Molecular Biochemicals, Mannheim, Germany) on a 2 % Agarose gel stained with ethidium bromide, exposed to UV illumination.

PCR products were diluted for genescan analysis based on the approximate sizes of the PCR products and the type of fluorescent label attached to the primers. Allele sizes were estimated by comparing the mobility of the microsatellite products with

that of a LIZ™ 500 size standard (Applied Biosystem, Warrington, UK). Genescan analysis was executed using an ABI Prism™ 3100 DNA sequencer (Perkin – Elmer, Warrington, United Kingdom). The allele sizes for the DNA fragments were determined using a combination of the GeneScan® 2.1 analysis software (Applied Biosystems) and GeneMapper (Applied Biosystems).

Genetic diversity and population differentiation

Isolates were scored based on allele size at each locus. This information was used to generate a multilocus profile or haplotype for each isolate. Identical haplotypes were treated as clones and removed and statistics were calculated for clone-corrected populations. Allele frequencies in each population were then calculated by dividing the number of times an allele occurred in the population by the population sample size. The allele frequencies were used to calculate the gene diversity, $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} allele for each population (Nei 1973) using the programme POPGENE version 1.31 (Yei *et al.* 1999). Differences in allele frequencies for clone corrected populations were estimated by Chi-square tests (χ^2) (Workman & Niswander 1970). Allele frequencies of populations from the two hosts from Western Australia were compared. South African, eastern Australian and Western Australian populations were also determined to assess the level of gene diversity within these populations and the level of population differentiation between them.

Population differentiation (G_{ST}), as measured by theta (Weir 1996), was calculated between all pairs of clone corrected populations in Multilocus v. 1.3 (Agapow & Burt 2000). The statistical significance was determined by comparing the observed G_{ST} value to that of 1000 randomized datasets in which individuals were randomized among the populations being compared. The number of migrants (M) that must be exchanged between populations for each generation, to give the observed G_{ST} value, was calculated using the equation $M = (1/\theta)-1)/2$ (Cockerham & Weir 1993).

Genotypic diversity was calculated using the formula $G = 1/\Sigma [f_x (x/n)^2]$, where, n is the sample size and f_x is the number of genotypes (haplotypes) occurring x times in the population and G being the effective number of equally frequent haplotypes (Stoddart & Taylor 1988). The genotypic diversities between populations was compared by

obtaining the maximum percentage of genotypic diversity using the formula $\hat{G}$ = G/N*100, where N is the sample size (McDonald *et al*. 1994).

Genetic distance

The genetic distance between all *H. eucalypti* haplotypes from Australia and South Africa was calculated based on Nei's (1972) unbiased genetic distance. The distance matrix was generated using the program POPGENE version 1.31 and a tree constructed using UPGMA (Unweighted Pair – Group Method with Arithmetic mean) in MEGA version 2.1 (Kumar *et al*. 2001).

RESULTS

Genetic diversity

A total of 28 alleles were amplified across the eight loci for the Western Australian population from *C. callophylla* and 45 alleles for the population from *E. globulus*. Thirty alleles were amplified in the eastern Australian population and only 17 alleles from the South African population (Table 3). Locus 5A was the most polymorphic with a total of 16 alleles and locus 1B the least polymorphic with a total of four alleles. The South African population was monomorphic at three loci. There were 21 unique alleles among the Western Australian populations, the majority of which were rare (only occurring in one isolate), however, allele 222 at locus 2B was common as were allele 267 at locus 5A, allele 342 at locus 5B and allele 211 at locus 7A. There were six unique alleles in the eastern Australian population and allele 259 at locus 5A and alleles 190 and 208 at locus 7A had a frequency of greater than 25 %. Of the nine unique alleles in the South African population, three had a frequency of greater than 90 % and all except two were common (Table 3). In Western Australia, more alleles were found in the *E. globulus* population, however of the 28 alleles found in *C. callophylla* population, 25 were also present in the *E. globulus* population and the three that differed were of very low frequency. Twenty-three of the 30 alleles present in the eastern Australian population were also present in Western Australia, however, the frequencies were very different (Table 3). Only 45 % of the alleles found in the South African isolates were found elsewhere and at very different frequencies.

Monomorphic loci and unique alleles affect gene diversity, which was high for the Australian populations and low for the South African population (Table 4, 5).

A total of 69 haplotypes were obtained when the three *H. eucalypti* populations were combined. However, three were shared between the Western Australian population from *Corymbia* and the Western Australian population from *Eucalyptus*, thus there was a total of 66 unique haplotypes. No haplotypes were shared between regions. The maximum genotypic diversity was 63.2 % for the Western Australian population from *C. callopylla,* 55.7 % from *E. globulus*, 43.7 % for the eastern Australian population and 3.6 % for the South African population (Table 3).

Genetic differentiation and Gene flow

The χ^2 tests for the eight microsatellite regions showed no significant difference in allele frequency at any loci between the Western Australian population of *H. eucalypti* which originated from two different but closely related tree genera (Table 4). For analysis purposes, the lack of significant difference implies that these isolates can be combined to give a single population from Western Australia. Conversely, when the populations from the different regions were compared, χ^2 tests were highly significant at all loci (Table 5). This is reflected in the G_{ST}, a statistic used to measure population differentiation. G_{ST} values were highly significant when comparing the populations from different regions and gene flow was very low (Table 6).

Genetic distance

The UPGMA tree constructed from the matrix obtained using Nei's (1972) genetic distance clearly separated the South African population from the Australian populations (Fig. 2). There was no grouping of isolates according to the regions sampled (Fig. 2). The majority of isolates from eastern Australia formed a distinct clade. Five isolates, however, grouped with the Western Australian isolates, and two Western Australian isolates grouped within the eastern Australian clade.

DISCUSSION

Microsatellite markers specifically developed for *H. eucalypti* were effectively used in this study to compare populations of the fungus from eastern Australia, Western Australia and South Africa. Two populations collected from different hosts in Western Australia showed no significant differences, indicating a lack of host specificity. Australian populations showed high gene and genotypic diversity compared to very low gene and genotypic diversity within the South African population. As *H. eucalypti* is thought to be native to eucalypts in Australia and the only record of occurrence outside Australia is that in South Africa (Van der Westhuizen *et al*. 1993), the low diversity observed in South Africa is indicative of an introduced pathogen. However, over 50 % of the alleles in the South African population were unique, suggesting that they were introduced from a region of Australia not sampled in the current study.

The forestry landscape in Western Australia has been greatly altered in recent years following the signing of Regional Forestry Agreements. This has led to more emphasis being put on plantation forests (www.rfa.gov.au/rfa/national/nfps/). In the last 15 years, 300 000 ha of Tasmanian blue gum (*E. globulus*) has been planted as an exotic in Western Australia (National Forest Inventory 2004). These plantations are closely associated with remnant native forests or state forests. For this reason, it is perhaps not surprising that no barrier to gene flow was found for *H. eucalypti* isolated from planted *E. globulus* and the native *C. callophylla.*

The South African population of *H. eucalypti* exhibited extremely low genotypic diversity. This was predominantly because 44 of the 72 isolates had the same multilocus haplotype. This haplotype was widely distributed throughout the regions sampled. This is particularly interesting as the sexual state of the fungus is commonly encountered in South Africa (Van der Westhuizen *et al*. 1993, Venter *et al*. 2002, Gryzenhout *et al*. 2003). Such patterns are commonly observed when there have been limited introductions of a fungus into a new area (Barton & Charlesworth 1984, McDonald 1997). Whilst reproduction is sexual, it is probably homothallic as is

commonly found in other relatives of this fungus (Milgroom *et al*. 1993). Under these circumstances, it would also be expected that alleles are linked (Milgroom *et al*. 1993, McDonald 1997).

Western Australia is separated from eastern Australia by 3000 km of desert. This desert has been an effective barrier to gene flow in flora and fauna and not associated with diseases since the early Tertiary period (Beadle 1981, Boland *et al.* 1984). Thus, if *H. eucalypti* is considered endemic to both Western and eastern Australia, the populations would have been completely isolated, gene flow would be non-existent, and different alleles would have been fixed in each region and the populations structure would vary greatly. Due to big differences in allele frequencies, population differentiation between the two regions in this study was significant and gene flow very low. The observation of 23 shared alleles between the two regions is probably indicative of historical, human assisted gene flow and not representative of two completely isolated populations.

Due to the high proportion of unique alleles in the South African population, and divergent allele frequencies between Australia and South Africa, the populations from the two continents are separated by large genetic distances and form separate clades. Thus there are no Australian haplotypes with a similar multilocus profile to those found in South Africa. We suspect the source of *H. eucalypti* may be from a region in Australia not surveyed in this study. Results, however, clearly showed that the population in Western Australia has a very high diversity, despite the fact that the sexual state has never been encountered in that area. This strongly supports the view that *H. eucalypti* is native to this continent.

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Table 1. Isolates of *Holocryphia eucalypti* from South Africa and Australia used in this study.

Isolate numbers (CMW) [a]	**Origin**	**Code**	**Host**	**Collector**
18970, 18971, 18972, 18974, 18975, 18976, 19158, 19159,	Nyalazi, KZN, South Africa	N	*Eucalyptus* (GC/ GU clones)	M Gryzenhout
18985, 18977, 18986 -18995, 18998, 18999 – 19002, 19021 – 19032, 19160 – 19162, 7034, 7035, 8541	KZN, South Africa	KZN	*Eucalyptus* (GC/ GU clones)	M Gryzenhout
18983, 18984, 18996, 18997, 19164, 19165, 19033, 18973, 18978, 18979, 18980 – 18982	Mpumalanga, South Africa	MPUM	*Eucalyptus* (GC/ GU clones)	M Gryzenhout
19003 – 19006, 19163, 19007 – 19020	Tzaneen, South Africa	TZ	*E. saligna*	M Gryzenhout
15172, 15174	Albany, Western Australia	ALB	*E. globulus*	T Jackson
15187 – 15191	Augusta, Western Australia	AUG	*E. globulus*	T Jackson
15167, 15168,15173 – 15179,	Brunswick Junction, Western Australia	BJ	*E. globulus*	T Jackson
15198, 15193 – 15197	Bunbury, Western Australia	BUN	*E. globulus*	T Jackson
15182 – 15186	Denmark, Western Australia	DEN	*E. globulus*	T Jackson
15180, 15181	Esperance, Western Australia	ESP	*E. globulus*	T Jackson
7038	Denmark, Western Australia	DEN	*E. globulus*	MJ Wingfield
15142 – 15148, 15153, 15154, 15156 – 15158, 15160 – 15164,	Manjimup, Western Australia	MAN	*C. calophylla*	T Paap
15166, 15165	Perth, Western Australia	PER	*C. calophylla*	T Paap
15152	Albany, Western Australia	ALB	*C. calophylla*	T Paap
15159, 15149 – 15151, 15155,	Denmark, Western Australia	DEN	*C. calophylla*	T Paap
6240, 6241, 6242	Canberra, eastern Australia	ACT	*Eucalyptus* sp.	MJ Wingfield
6268, 6673, 6683, 6687, 6693, 6695 – 6697,	NSW, eastern Australia	NSW	*Eucalyptus* sp.	MJ Wingfield
18689 – 18700	Brisbane, eastern Australia	QLD	*E. dunii*	Gilbert Whyte

[a]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Table 2. Microsatellite DNA markers used to amplify South African and Australian populations of *H. eucalypti.*

Primer pair	Fluorescent Label	Sequence	PCR product size (bp)
5A-FF	NED	GGT CCA TCA GTC GTC TCA GC	240-336
5A-RR		GCA GCA ATG AGG TGC CTT GG	
7A-FF	VIC	CCT GAC AGA GAA GCG ACC CT	190-219
7A-RR		GCA TCA GCT CAG GGC ATA GAG	
8A-FF	6-FAM	CCG AGG GTT AGA CAT CAC CC	238-277
8A-RR		ACC TGA CGC TCC ATC TGC AC	
9A-FF	VIC	CTG CTG ACA AGG ACG AGG AC	256-292
9A-RR		CGT TTC GTG GCT GGA TCT CG	
10A-FF	PET	CTC TTG CAG CCT CGG AGA CTG	388-403
10A-RR		GAG TGG CCA TAT TCA GCT TGG C	
5B-FF	NED	GTG TCG TCG CTC GCG AAT AG	342-377
5B-RR		CAG GAG AGG ACA TGC GAG AC	
2B-FF	PET	GCC CAA AGG ATG TGT GAA TGT G	216-232
2B-RR		CAA ACT GGC GGA TGA CAG GC	
1B-FF	6-FAM	GCA TCT CAA CAG TGC ACT CCA G	185-198
1B-RR		CAC ATA CAC TCT CAT AGC TCT CGG	

Table 3. Allele size (bp) and frequency at 8 loci for clone corrected populations of *Holocryphia eucalypti* from Western Australia on *Corymbia callophylla* (WAC) and *Eucalyptus globulus* (WAE), eastern Australia (EA) and *Eucalyptus* spp. in South Africa (RSA).

		Allele Frequencies			
Locus	**Allele length**	**WAC**	**WAE**	**EA**	**RSA**
1B	185	-	0.083	0.214	-
	191	0.833	0.833	0.357	-
	196	-	0.042	0.286	-
	198	0.167	0.042	0.143	1.000
2B	216	-	-	0.071	-
	218	-	-	-	1.000
	220	-	0.042	-	-
	222	0.833	0.875	-	-
	224	-	0.042	0.857	-
	232	0.167	0.042	0.071	-
5A	240	0.056	0.042	-	-
	243	-	0.042	-	-
	250	-	-	0.071	-
	256	-	0.083	0.286	-
	259	-	-	0.286	-
	261	-	0.042	-	-
	265	0.167	0.042	-	0.077
	267	0.167	0.375	-	-
	269	0.333	0.208	0.143	-
	271	0.111	0.083	-	-
	273	0.056	-	-	-
	279	0.056	0.042	-	-
	283	0.056	0.042	0.214	-
	320	-	-	-	0.462
	322	-	-	-	0.308
	336	-	-	-	0.154
5B	342	0.222	0.167	-	-
	344	0.611	0.500	0.071	-
	358	0.056	0.083	-	-
	368	0.111	0.083	-	-
	373	-	0.042	-	-
	375	-	0.083	0.571	0.846
	377	-	0.042	0.357	0.154
7A	190	-	-	0.214	-
	196	-	-	-	0.077
	198	-	0.167	-	0.077
	203	-	-	-	0.846
	208	-	-	0.286	-
	211	0.222	0.208	-	-
	213	-	0.083	0.357	-
	215	0.111	0.042	-	-
	219	0.667	0.500	0.143	-
8A	238	0.056	-	-	-

	250	-	-	0.286	-
	255	-	-	0.071	0.077
	257	0.111	0.042	0.071	0.231
	259	0.778	0.792	-	0.692
	262	-	0.042	-	-
	264	-	0.042	0.071	-
	276	-	0.042	-	-
	277	-	0.042	0.500	-
9A	256	-	-	-	0.077
	260	0.056	-	-	-
	267	-	0.042	-	-
	277	0.772	0.667	0.286	-
	278	0.222	0.208	0.286	-
	284	-	0.042	0.214	-
	289	-	0.042	0.214	-
	292	-	-	-	0.923
10[a]	388	0.056	0.125	-	-
	390	0.944	0.750	0.429	-
	392	-	0.042	-	-
	399	-	-	-	1.000
	403	-	0.083	0.571	-
N(g)		**18**	**24**	**14**	**13**
N		**24**	**30**	**23**	**72**
No. alleles		**28**	**45**	**30**	**17**
No. unique alleles		**21**		**6**	**9**
G		**15.15**	**16.69**	**10.04**	**2.55**
Ĝ (%)		**63.15**	**55.65**	**43.67**	**3.55**

N = number of isolates

N(g) = number of multilocus haplotypes

G = Genotypic diversity (Stoddart & Taylor 1988)

Ĝ = percent maximum diversity

Table 4. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Holocryphia eucalypti* collected from *Corymbia calophylla* and *Eucalyptus globulus* in Western Australia. There was no significant difference between allele frequencies at any loci (P<0.001).

	Gene diversity (H)			
Locus	***Corymbia***	***Eucalyptus***	χ^2	**df**
1B	0.28	0.30	3.2	3
2B	0.28	0.23	3.9	3
5A	0.81	0.79	3.2	10
5B	0.56	0.70	5.7	6
7A	0.49	0.67	4.3	4
8A	0.38	0.36	3.6	6
9A	0.43	0.51	8.4	5
10A	0.10	0.41	3.6	3
N	18	24		
MEAN	0.41	0.50		

Table 5. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Holocryphia eucalypti* collected from western Australia (WA), eastern Australia (EA) and South Africa (RSA).

	Gene diversity (H)				
Locus	**WA**	**EA**	**RSA**	**χ^2**	**df**
1B	0.31	0.72	0.00	53.5[A]	6
2B	0.27	0.25	0.00	121.0[A]	10
5A	0.83	0.76	0.66	99.9[A]	30
5B	0.67	0.54	0.26	55.9[A]	12
7A	0.65	0.72	0.27	97.4[A]	16
8A	0.36	0.65	0.46	55.4[A]	16
9A	0.50	0.75	0.14	82.4[A]	14
10A	0.31	0.49	0.00	89.4[A]	8
N	39	14	13		
MEAN	0.49	0.61	0.23		

[A] indicates significant χ^2 values,P<0.001

Table 6. Pairwise comparisons of population differentiation, G_{ST}, (above the diagonal) and number of migrants, M, (below the diagonal) among clone corrected populations of *Holocryphia eucalypti* collected from Western Australia (WA), eastern Australia (EA) and South Africa (RSA).

	WA	**EA**	**RSA**
WA	-	0.328[A]	0.551[A]
EA	1.024	-	0.490[A]
RSA	0.407	0.520	-

[A] indicates significant G_{ST} values,P<0.001

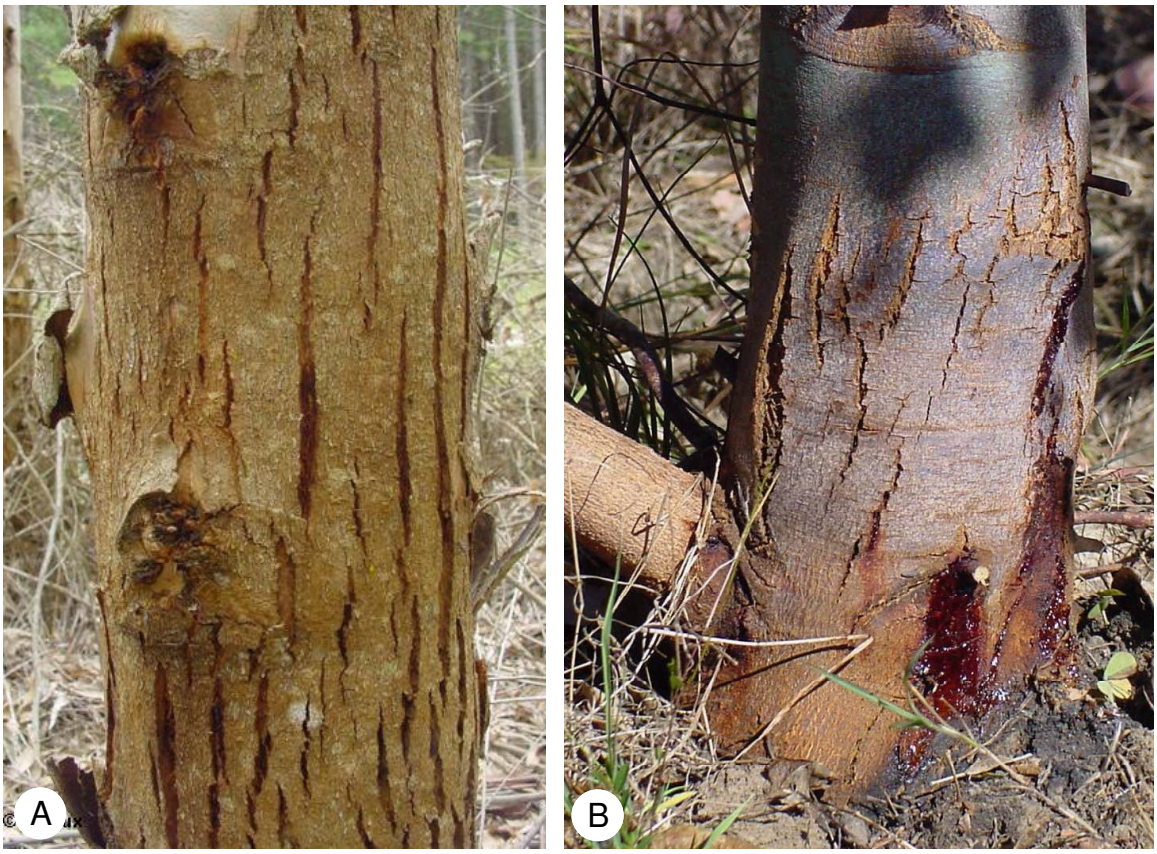

Fig. 1. Symptoms associated with *Holocryphia eucalypti* infection. A) Cracks on *Eucalyptus* bark in South Africa. B) Cracks and cankers on *E. dunnii* in eastern Australia (Photographs by Dr Treena Burgess and Prof. Jolanda Roux).

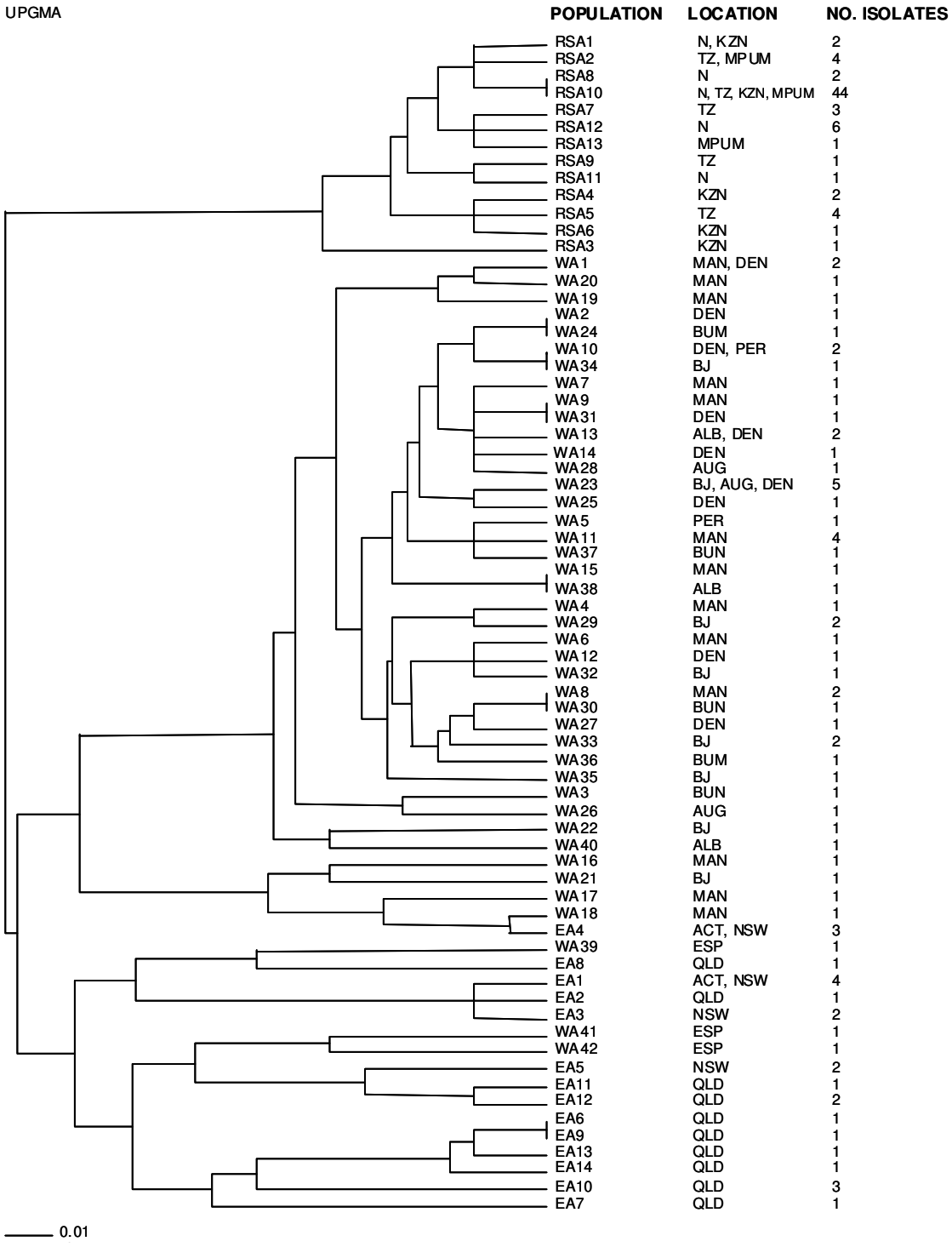

Fig. 2. UPGMA dendrogram of *Holocryphia eucalypti* haplotypes from South Africa (RSA), Western Australia (WA) and eastern Australia (EA) constructed using clone collected data obtained using 8 polymorphic microsatellite markers.

CHAPTER 6

Celoporthe dispersa gen. et sp. nov. from native *Myrtales* in South Africa

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ABSTRACT

In a survey for *Cryphonectria* and *Chrysoporthe* species on *Myrtales* in South Africa, a fungus resembling the stem canker pathogen *Chr. austroafricana* was collected from native *Syzygium cordatum* near Tzaneen (Limpopo Province), *Heteropyxis canescens* near Lydenburg (Mpumalanga Province) and exotic *Tibouchina granulosa* in Durban (KwaZulu/Natal Province). The fungus was associated with dying branches and stems on *H. canescens* and *T. granulosa*. However, morphological differences were detected between the unknown fungus from these three hosts and known species of *Chrysoporthe*. The aim of this study was to characterise the fungus using DNA sequence comparisons and morphological features. Pathogenicity tests were also conducted to assess its virulence on *Eucalyptus* (ZG 14 clones), *H. natalensis* and *T. granulosa*. Plants of *H. canescens* were not available for inoculation. Results showed distinct morphological differences between the unknown fungus and *Chrysoporthe* spp. Phylogenetic analysis showed that isolates reside in a clade separate from *Chrysoporthe* and other related genera. *Celoporthe dispersa gen. et sp. nov*. is, therefore, described to accommodate this fungus. Pathogenicity tests showed that *C. dispersa* is not pathogenic to *H. natalensis*, but that it is a potential pathogen of *Eucalyptus* and *Tibouchina* spp.

INTRODUCTION

The taxonomy of *Cryphonectria* species associated with cankers of *Eucalyptus* spp. and the worldwide distribution of these fungi has undergone numerous revisions and changes in recent years (Venter *et al*. 2002, Gryzenhout *et al*. 2004, 2005a). Studies have shown that the important *Eucalyptus* canker pathogen, *Cryphonectria cubensis* (Bruner) Hodges (Sharma *et al*. 1985, Hodges *et al.* 1986, Wingfield *et al*. 1989, Roux *et al.* 2003, Wingfield 2003), is different from other *Cryphonectria* spp. and has been placed in a new genus, *Chrysoporthe,* that includes at least two distinct species, *Chr. cubensis* (Bruner) Gryzenh. & M. J. Wingf. and *Chr. austroafricana* Gryzenh. & M. J. Wingf. (Gryzenhout *et al*. 2004). Similarly, the opportunistic *Eucalyptus* canker pathogen, *Cryphonectria eucalypti* M. Venter & M. J. Wingf., formally known as *Endothia gyrosa* (Schwein.: Fr.) Fr. (Venter *et al*. 2002), now resides in the new genus *Holocryphia* prov. nom. as the species *H. eucalypti* (M. Venter & M. J. Wingf.) Gryzenh. & M. J. Wingf. prov. nom. (Gryzenhout *et al*. 2005a).

Chrysoporthe cubensis occurs in South America on native *Psidium cattleianum* Sabine (Hodges 1988), and on exotic *Eucalyptus* spp. and *Syzygium aromaticum* (L.) Merr. & Perry (Boerboom & Maas 1970, Hodges *et al.* 1976, 1986, Van der Merwe *et al*. 2001), all of which reside in the family *Myrtaceae*; as well as on native *Miconia rubiginosa* (Bonpl.) DC. and *M. theaezans* (Bonpl.) Cogn. belonging to the family *Melastomataceae* (Rodas *et al*. 2005). In southeast Asia and Australia the pathogen has been reported from *Eucalyptus* spp. (Sharma *et al*. 1985, Hodges *et al.* 1986, Davison & Coates 1991, Myburg *et al.* 1999) and *S. aromaticum* (Hodges *et al.* 1986, Myburg *et al.* 2003). In Africa, *Chr. cubensis* has been reported from Cameroon, Republic of Congo, Democratic Republic of Congo and Unguja Island Zanzibar on *Eucalyptus* spp. and *S. aromaticum* (Nutman & Roberts 1952, Gibson 1981, Hodges *et al.* 1986, Micales *et al.* 1987, Roux *et al*. 2000, Myburg *et al*. 2003, Roux *et al.* 2003).

Chrysoporthe austroafricana has, until recently, been known only from South Africa. In this country, it has been reported from both native South African tree species and non-native ornamental and plantation forest trees (Wingfield *et al*. 1989, Myburg *et al*. 2002, Heath *et al*. 2006). The fungus was the cause of an important disease of

Eucalyptus spp. in the 1990's (Wingfield *et al*. 1989) and has recently also been reported from the non-native ornamental tree *Tibouchina granulosa* Cogn. (*Melastomataceae*) (Myburg *et al*. 2002) and native *Syzygium cordatum* Hachst. and *S. guinense* (Willd.) DC. (*Myrtaceae*) (Heath *et al.* 2006) in South Africa.

Holocryphia eucalypti is an opportunistic pathogen of *Eucalyptus* spp. in South Africa, mostly resulting in only superficial bark cankers on trees (Van der Westhuizen *et al.* 1993, Gryzenhout *et al.* 2003). The fungus is also known to occur in Australia on *Corymbia* and *Eucalyptus* spp. (Walker *et al.* 1985, Old *et al*. 1986), where it has been associated with cankers and tree death (Walker *et al*. 1985, Davison & Coates 1991, Wardlaw 1999).

Chrysoporthe spp. can be confused with *Holocryphia* because species in both genera have orange stromata in their teleomorph states (Venter *et al*. 2002, Gryzenhout *et al.* 2004, 2005a) and they share the same hosts and geographical distributions (Old *et al*. 1986, Wingfield *et al*. 1989, Davison & Coates 1991, Van der Westhuizen *et al.* 1993). However, there are distinct morphological differences between the genera. For example, the conidiomata of *Chrysoporthe* are superficial, fuscous-black, pyriform to orange with attenuated necks (Gryzenhout *et al.* 2004, Myburg *et al.* 2004), whereas those of *Holocryphia* are semi-immersed, orange and globose without necks (Venter *et al.* 2002, Myburg *et al.* 2004, Gryzenhout *et al.* 2005a). Furthermore, the ascospores of *Chrysoporthe* are septate, whereas those of *Holocryphia* are aseptate. Phylogenetic analyses have also shown that the two genera form distinct well-supported groups (Myburg *et al.* 2004, Gryzenhout *et al.* 2005a), separate from each other and from the genus *Cryphonectria,* in which both previously had been placed.

Like *Chr. cubensis*, *Chr. austroafricana* is an economically important pathogen of commercially grown *Eucalyptus* spp. (Sharma *et al*. 1985, Hodges *et al.* 1986, Wingfield *et al.* 1989, Wingfield 2003). In South Africa, *Chr. austroafricana* has caused substantial damage to clonal plantation forestry, which has been partially mitigated through the selection and planting of disease resistant clones (Wingfield *et al.* 1989, Wingfield 2003). The recent discovery of *Chr. austroafricana* on native *S. cordatum* and *S. guineense* in South Africa has led to a change of view regarding its

possible origin. Where it was once thought to be an introduced pathogen (Wingfield *et al.* 1989, Van Heerden & Wingfield 2001, Wingfield 2003), there is now substantial evidence to suggest that it is a native pathogen that could have moved from native South African *Syzygium* spp., to exotic species such as *Eucalyptus* and *Tibouchina* (Hodges *et al.* 1986, Myburg *et al*. 2002, Heath *et al*. 2006, Slippers *et al*. 2005).

Although only two species of *Syzygium* are known as hosts of *Chr. austroafricana*, it is highly likely that this fungus occurs on other *Myrtales* in South Africa. For this reason surveys were conducted in the country to establish the occurrence of *Chrysoporthe* spp. on indigenous trees belonging to this plant order. These surveys yielded a fungus similar to *Chr. austroafricana* that was collected from three hosts in three geographic areas of the country. The aims of this study were to characterize the unknown fungus based on morphology and DNA sequence comparisons and to assess its pathogenicity in greenhouse inoculations on plants of *Heteropyxis, Eucalyptus* and *Tibouchina*.

MATERIALS AND METHODS

Isolates and specimens

Isolates were obtained from bark material that was collected from *S. cordatum* from Tzaneen, *Heteropyxis canescens* Oliv. from Lydenburg and *T. granulosa* from Durban (Table 1; Fig. 1). Fungal cultures for all isolates have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and duplicates in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Bark specimens have been deposited in the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA sequence comparisons

Actively growing mycelium of each isolate was scraped from the surface of one plate each containing MEA (20 g/l malt extract and 20 g/l agar, Biolab, Midland, Johannesburg) and 100 mg/l streptomycin sulfate (Sigma – Aldrich, Chemie, Gmbh, Steinheim, Germany) using a sterile scalpel, and transferred to 1.5 µl Eppendorf tubes.

DNA was extracted as described by Myburg *et al.* (1999). Using primers ITS1 and ITS4 (White *et al*. 1990) and Bt1A/Bt1B and Bt2A/Bt2B (Glass & Donaldson 1995), the β-tubulin 1 and 2 and rDNA (ITS 1, 5.8S and ITS 2) regions respectively, were amplified. The reactions were performed in a volume of 25 µl comprising of 2 ng DNA template, 800 µM dNTPs, 0.15 µM of each primer, 5 U/µl Taq polymerase (Roche Diagnostics, Mannheim, Germany) and sterile distilled water (17.4 µl). Polymerase chain reactions (PCR) and purification of the PCR products were carried out as described by Nakabonge *et al.* (2005).

The purified PCR products were sequenced in a reaction volume of 10 µl consisting of 5X dilution buffer, 4.5 µl H_2O, DNA (50 ng PCR product), 10X reaction mix BD (ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA), and ~ 2 pmol /µl of one of either the reverse or forward primers that were used in the PCR reactions. The PCR sequencing products were cleaned by using 0.06 g/ml Sephadex G-50 (Sigma-Aldrich, Amersham Biosciences Limited, Sweden) according to the manufacturer's protocol. 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 gene sequences were analyzed and edited using Sequence Navigator Version 1.0.1™ (Perkin-Elmer Applied BioSystems, Foster City, CA). Sequences were compiled into a matrix using a modified data set (S1128, M1935) of Myburg *et al.* (2004) as template. Additional sequences that included sequences of *Chrysoporthe* (Gryzenhout *et al*. 2004), *Holocryphia* (Venter *et al*. 2002, Gryzenhout *et al.* 2005a), *Cryphonectria* (Venter *et al*. 2002, Myburg *et al.* 2004), *Endothia* (Venter *et al*. 2002, Myburg *et al.* 2004), *Rostraureum* (Gryzenhout *et al.* 2005b) and *Amphilogia* (Myburg *et al*. 2004, Gryzenhout *et al.* 2005c) were added to the data matrix. Sequences representing an un-described genus identified by Myburg *et al*. (2003) and originating from clove in Indonesia were also added. The alignment was executed using the web interface (http://timpani.genome.adjp/%7Emafft/server/) of the alignment program MAFFT ver. 5.667 (Katoh *et al*. 2002).

Phylogenetic analysis was performed using the software package Phylogenetic Analysis Using Parsimony (PAUP) Version 4.01b (Swofford 1998). A partition homogeneity test (Huelsenbeck *et al*. 1996) to determine the similarity and combinability of the data for the ITS, β-tubulin 1 and 2 regions was run. The most parsimonious trees were obtained with heuristic searches using stepwise addition and tree bisection and reconstruction (TBR) as the branch swapping algorithms. All equally parsimonious trees were saved and all branches equal to zero were collapsed. Gaps were treated as a fifth character. Bootstrap replicates (1000) were done on consensus parsimonious trees (Felsenstein 1985). Two isolates of *Diaporthe ambigua* Nitschke (CMW 5288 and CMW 5587) were used as out-group to root the tree (Myburg *et al*. 2004).

Morphology

Fruiting structures of the unknown fungus were cut from the bark under a dissection microscope, boiled for 1 min and sectioned (12 µm thick) using a Leica CM1100 cryostat (Setpoint Technologies, Johannesburg, South Africa) as described by Gryzenhout *et al.* (2004). Fruiting structures were also crushed on microscope slides in 85 % lactic acid and 3 % KOH in order to study the asci, ascospores, conidia, conidiophores and conidiogenous cells. Measurements were then taken for the above-mentioned structures. For the holotype specimen, PREM58896 (ex-type culture CMW 9976) fifty measurements were made for each character. Only twenty measurements per character were made for the remaining isolates (CMW13936, CMW13645). A HRc Axiocam digital camera with Axiovision 3.1 software (Carl Zeiss Ltd., Germany) was used to capture digital images and to compute measurements. Characteristics of specimens were compared with those published for *Chrysoporthe* and *Holocryphia* (Gryzenhout *et al*. 2004, 2005a).

Two representative isolates from *H. canescens* (CMW13645, CMW13646), *T. granulosa* (CMW13936, CMW13937) and *S. cordatum* (CMW9976, CMW9978) were used for studies of cultural characteristics. Discs (4 mm diam) taken from the margins of actively growing young cultures were placed onto the centers of 90 mm Petri dishes containing MEA. These were grown in the dark in incubators set at five temperatures ranging from 15 to 35 ^{o}C. Four plates per isolate were inoculated and two measurements perpendicular to each other were taken daily until the fastest

growing culture covered the plate. For each isolate, colony diameter was calculated as an average of eight readings. Colour notations of Rayner (1970) were used for the descriptions of cultures and fruiting bodies.

Pathogenicity tests

The pathogenicity of two isolates (one isolate from each host) of the unknown fungus from *H. canescens* (CMW13645) and from *T. granulosa* (CMW13936) was tested on 25 trees each of an *E. grandis* clone (ZG14) that is known to be highly susceptible to fungal pathogens (Van Heerden & Wingfield 2001) and *T. granulosa* seedlings respectively, in a greenhouse set at 25 ^{o}C. The *Eucalyptus* clones were approximately 2 m tall while the *Tibouchina* seedlings were approx. 1 m tall. In order to expose the cambium, wounds were made in the bark using a cork borer (4 mm diam). Discs of the same size, from the actively growing edges of four-day-old colonies, were inserted into the wounds with the mycelium facing the xylem. To prevent desiccation and contamination, wounds were covered with parafilm (Pechiney plastic packing, Chicago, USA). Twenty-five trees each of the *E. grandis* clone (ZG14) and *T. granulosa* served as negative controls and were inoculated with sterile water agar (WA: 20 g agar Merck, South Africa / 1000 mL water). Lesion development was evaluated after 8 weeks by taking measurements of the lengths of lesions in the xylem. The trial was repeated after four months. Re-isolations were made from lesions by plating small pieces of discoloured xylem onto MEA.

Regeneration of *Heteropyxis* trees such as *H. canescens* in nurseries is seldom achieved. Only three trees (~1 m tall) of a related species, *H. natalensis* Harv., could be obtained for pathogenicity tests. Two isolates (CMW13645, CMW13646) of the unknown fungus from *H. canescens* were inoculated into the stems of two *H. natalensis* trees. The third tree was inoculated with a sterile agar disc to serve as a negative control. The inoculation procedure was the same as that used when inoculating *Eucalyptus* and *Tibouchina* plants but each of the three trees had two inoculation points opposite sides of the stem at the same height. Lesion lengths were measured eight weeks after inoculation and re-isolations were made using the same procedures as with the *Eucalyptus* and *Tibouchina* inoculations.

Data were analysed using the general linear model of analysis of variance (ANOVA). Means were separated using the Least Significant Difference (LSD) method available in STATISTICA for Windows (StatSoft 1995).

RESULTS

Isolates and specimens

Specimens of the unknown fungus were collected from cracked stems of two *S. cordatum* trees near Tzaneen in the Limpopo Province (Table 1). Fruiting structures occurred between structures of *Chr. austroafricana* that were also fruiting profusely on these trees. A similar fungus was collected from six native *H. canescens* trees exhibiting severe cankers and dieback growing in the private Buffelskloof Nature Reserve near Lydenburg in Mpumalanga Province. Some of the trees were dying or dead (Fig. 1A-C). Additional collections were made from the stems of two non-native *T. granulosa* trees from the Durban Botanic Gardens in KwaZulu/Natal Province. These trees displayed symptoms of branch dieback (Fig. 1D).

DNA sequence comparisons

PCR amplicons for the two regions of the β-tubulin gene were approximately 500 bp in size. Those for the ITS rDNA region amplified were approximately 600 bp in size. Results obtained from the partition homogeneity test showed that the data for each gene region were significantly congruent (p-value = 0.02). The aligned sequences of the combined regions generated 1528 characters of equal weight, with 853 constant characters of which 208 were parsimony uninformative and 645 were parsimony informative. Sixteen most parsimonious trees were generated with similar branch lengths and topology and one was chosen for presentation. This tree had a length of 1620, a consistency index (CI) of 0.740 and retention index (RI) of 0.920 (Fig. 4).

Isolates representing species of *Amphilogia, Chrysoporthe, Cryphonectria*, *Endothia, Holocryphia* and *Rostraureum* formed distinct and well-supported clades reflecting the different genera. The isolates of the unidentified fungus from *H. canescens, S. cordatum* and *T. granulosa* in South Africa, grouped separately from these genera, specifically separately from isolates of *Chr. austroafricana* and *H. eucalypti*, which

also occur on *Myrtales* in South Africa. The isolates from *Heteropyxis, Syzygium* and *Tibouchina* in South Africa formed a clade with the isolates of the undescribed fungus from *S. aromaticum* from Indonesia (Myburg *et al.* 2003). However, within this clade, isolates formed sub-clades linked to the collections from different hosts. These were based on constant single base pair differences between isolates from the different hosts. These sub-clades include the Indonesian *Syzygium* clade (100 %), and the *Syzygium* clade (96 % bootstrap support), *Heteropyxis* clade (100 % bootstrap support) and the *Tibouchina* clade (96 % bootstrap support) from South Africa. Differences were most pronounced between the South African isolates and those from Indonesia (100 % bootstrap support), strongly suggesting that they represent different species.

Morphology

The fungus on *H. canescens, S. cordatum,* and *T. granulosa* in South Africa is characterised by fruiting structures (Figs 2A–G, 3A–F) that are morphologically very similar to those of *Chrysoporthe* species (Table 2) and the *Chrysoporthella* anamorph of *Chrysoporthe* (Gryzenhout *et al.* 2004). In the teleomorph states of both genera, the perithecial necks are covered in umber tissue as they extend beyond the bark surface (Figs 2A–B) and limited orange to cinnamon stromatic tissue can be seen at the bases of the necks (Figs 2A–B). Ascospores are one-septate, hyaline, and oblong to elliptical (Figs 2F, 3C). In the anamorph of the unknown fungus conidiomata are pulvinate to conical, fuscous black and superficial (Figs 2G, 3D), similar (Table 2) to the conidiomata of the same shape and colour in *Chrysoporthella* (Gryzenhout *et al.* 2004).

The fungus characterised in this study differs from *Chrysoporthe* in several morphological characters (Table 2). Perithecial necks of the fungus are about 50 µm long (Figs 2A–B, 3A–B), while *Chrysoporthe* spp. have long necks extending up to 240 µm long (Gryzenhout *et al.* 2004). Conidiomata are often without a neck or have necks with slightly attenuated apices (Figs 2G, 3D), differing from those of *Chrysoporthella* spp. that have long attenuated necks (Gryzenhout *et al.* 2004). The basal cells of the conidiophores in the unknown fungus (Figs 2J–K, Fig. 3F) are not as prominent as those of members of *Chrysoporthe*. Conidia are oblong to cylindrical to ovoid and occasionally allantoid (Figs 2L, 3F), differing from those of *Chrysoporthe*

spp. that are typically oblong (Gryzenhout *et al.* 2004). The stromatic tissue at the base of the conidiomata is pseudoparenchymatous (Fig. 2I), differing from that of *Chrysoporthe*, which consists of larger cells of *textura globulosa* (Gryzenhout *et al.* 2004). Lastly, cultures of the unknown fungus are white with grey patches, eventually becoming umber to hazel to chestnut. This is different from cultures of *Chrysoporthe* spp. that are white with cinnamon to hazel patches (Gryzenhout *et al.* 2004).

Phylogenetic analyses suggested that the collections from *H. canescens, S. cordatum* and *T. granulosa* might represent three related but cryptic species. However, no significant morphological differences were found among specimens from *H. canescens* (PREM58898, PREM58899), *S. cordatum* (PREM58896, PREM58897) and *T. granulosa* (PREM58900, PREM58901). There were also no clear differences in cultural morphology.

Phylogenetic analyses showed that an unnamed fungus previously treated by Myburg *et al*. (2003) from clove in Indonesia is related to the unknown fungus from South Africa, which formed the focus of the present study. It was, however, not possible to compare the South African and the Indonesian fungus based on morphology, because the latter fungus is known only from culture without any connection to morphological structures on the bark (Myburg *et al*. 2003). Some poorly formed conidiomata obtained for the Indonesian fungus by artificially inoculating it into *Eucalyptus* twigs, however, suggested that the fungus is similar to the South African collections and probably represents the same genus.

Taxonomy

Morphological characteristics combined with DNA sequence data show that the unknown fungus collected from *H. canescens*, *S. cordatum* and *T. granulosa* in South Africa can be distinguished from *Chrysoporthe*, *Cryphonectria* and other closely related genera. Based on morphology, the fungus most closely resembles *Chrysoporthe* but clearly represents an undescribed genus. The taxon also appears to include an unnamed fungus previously collected from clove in Indonesia (Myburg *et al*. 2003). Based on these differences, a new genus is thus established for the fungi from South Africa and Indonesia.

DNA sequence data showed that more than one species exists for the new genus. The sub-clade representing the Indonesian isolates was distinctly different from the South African isolates, but could not be described because there are insufficient structures on which to base a meaningful description. The isolates from the different hosts in South Africa formed another closely related group in the genus, although three possibly cryptic species, representing the isolates from three areas (Mpumalanga, Limpopo and KwaZulu/Natal Provinces) and three hosts (*H. canescens, S. cordatum*, and *T. granulosa*), respectively, could be identified based on distinct sequence differences. However, no morphological differences could be observed for these apparent cryptic species, and at present there is insuffient material or ecological information available regarding these groups to support the separation of three species. For the present, we have chosen to retain the South African collections in a single species. The isolates from Indonesia most likely do not belong to this species, but must remain un-described until fresh host material bearing fungal structures can be collected.

The specimens from *S. cordatum* in Tzaneen include both the anamorph and teleomorph, while specimens from *Heteropyxis* and *Tibouchina* have only the anamorph present. For the purpose of this study, a single species is described in a new genus, and this is based on specimens from *S. cordatum* as the holotype. Descriptions of the new genus and species follow:

Celoporthe Nakab., Gryzenh., J. Roux & M. J. Wingf., **gen. nov.**
Etymology: Greek, *celo*, referring to the fact that the fungus is difficult to find deliberately, and *porthe*, destroyer, referring to its pathogenic nature.

Ascostromata e peritheciis nigris facta, collis cum textura umbrina tectis, textura stromatica limitata cinnamomea vel aurantiaca presente. *Ascosporae* uniseptatae, oblongo-ellioticae. *Conidiomata* superficialia, juventute aurantiaca, cum maturitate fusco-nigra, pulvinata vel conica, cum vel sine collis. *Textura stromatica* pseudoparenchymatosa. *Conidiophorae* cylindricae, ramosae. *Conidia* non septata, oblonga, cylindrica vel ovoidea, interdum allantoidea.

Ascostromata consisting of black, valsoid perithecia embedded in bark tissue, with the cylindrical perithecial necks covered with umber tissue as they protrude through the

bark surface. Limited cinnamon to orange prosenchymatous to pseudoparenchymatous stromatic tissue present around the upper parts of the perithecial bases, usually beneath the bark or erumpent through the bark surface. *Asci* 8-spored, fusoid to ellipsoid. *Ascospores* hyaline, with one median septum, oblong-elliptical.

Conidiomata superficial, orange to scarlet when young, fuscous-black when mature, pulvinate to conical with or without short attenuated necks, unilocular with even inner surface. *Stromatic tissue* pseudoparenchymatous. *Conidiophores* hyaline, branched irregularly at the base or above into cylindrical cells, separated by septa or not. *Conidiogenous cells* phialidic, apical or lateral on branches beneath the septa. *Conidia* hyaline, non-septate, oblong to cylindrical to ovoid, occasionally allantoid, exuded as bright luteous spore tendrils or droplets.

Type species: *Celoporthe dispersa* Nakab., Gryzenh., J. Roux & M. J. Wingf., sp. nov. 2005.

Celoporthe dispersa Nakab., Gryzenh., J. Roux & M. J. Wingf., **sp. nov**. Figs 2–3.
Etymology: Greek, ***dispersa***, referring to the conidiomata scattered on the bark surface.

Ascostromata perithecia nigra continentia, collis perithecialibus brevibus extensis cum textura umbrina tectis, et textura stromatica limitata aurantiaca vel umbrina. *Ascosporae* uniseptatae, oblongo-ellioticae. *Conidiomata* superficialia, pulvinata vel conica cum vel sine collis, fusco-nigra. *Textura stromatica* pseudoparenchymatosa. *Conidiophorae* cylindricae, ramosae, cellulae conidiogenae apicibus attenuatis. *Conidia* non septata, oblonga, cylindrica vel ovoidea, interdum allantoidea.

Ascostromata semi-immersed in bark, recognizable by short, extending, umber, cylindrical perithecial necks, occasionally erumpent, limited, orange to umber ascostromatic tissue that cover the tops of the perithecial bases; ascostromata extending 100–400 µm high above the bark, 320–505 µm diam (Figs 2A, 3A–B). Stromatic tissue cinnamon and pseudoparenchymatous at edges, prosenchymatous in centre (Fig. 2D). *Perithecia* valsoid, 1–6 per stroma, bases immersed in the bark, black, globose to sub-globose, 100–300 µm diam, perithecial wall 30–50 µm thick (Figs 2B–C, 3B). *Perithecial necks* black, periphysate, 80–100 µm wide (Figs 2B,

3B), emerging through the stromatal surface, covered in umber stromatic tissue of *textura porrecta* thus appearing umber (Fig. 2A), extended necks up to 50 µm long, 100–150 µm wide. *Asci* 8-spored, biseriate, unitunicate, free when mature, non-stipitate with a non-amyloid refractive ring, fusoid to elliptical, (19.5–) 23.5–29.5(–33.5) × (4.5–) 5.5–7(–7.5) µm (Figs 2E, 3C). *Ascospores* hyaline, one median septum, oblong-elliptical, with rounded apices, (4.5–)6–7(–8) × (2–)2.5–3(–3.5) µm (Figs 2F, 3C).

Conidiomata eustromatic, superficial to slightly immersed, pulvinate to conical without necks, occasionally with neck which is slightly attenuated (Figs 2G, 3D), fuscous-black, conidiomatal bases above the bark surface 300–500 µm high, 200–1000 µm diam. *Conidiomatal locules* with even to convoluted inner surfaces, occasionally multilocular, locules 100–550 µm diam (Figs 2H, 3E). *Stromatic tissue* pseudoparenchymatous (Fig. 2I). *Conidiophores* hyaline, branched irregularly at the base or above into cylindrical cells, with or without separating septa, (9.5–)12–17(–19.5) × 1.5–2.5 µm (Figs 2J, 3F). *Conidiogenous* cells phialidic, determinate, apical or lateral on branches beneath a septum, cylindrical with attenuated apices, (1.5–)2–3 µm wide, collarette and periclinal thickening inconspicuous (Figs 2K, 3F). *Conidia* hyaline, non-septate, oblong to cylindrical to ovoid, occasionally allantoid, (2.5–)3–4(–5.5) × (1–)1.5(–2.5) µm (Figs 2L, 3F), exuded as bright luteous tendrils or droplets.

Cultural characteristics: On MEA, *C. dispersa* appears white with grey patches, eventually becoming umber to hazel to chestnut, fluffy with an uneven margin, fast-growing, covering a 90 mm diam plate in a minimum of five days at the optimum temperature of 25 ºC. Minimal growth was observed at 15 ºC. Cultures rarely sporulate after sub-culturing and teleomorph structures are not produced in culture.
Substratum: Bark of *Heteropyxis canescens, Syzygium cordatum* and *Tibouchina granulosa*.

Distribution: South Africa
Specimens examined: **South Africa**, Limpopo province, Tzaneen, *Syzygium cordatum*, 2003, M. Gryzenhout, **holotype** PREM 58896, culture ex-type CMW9976; PREM58897; living culture CMW9978. KwaZulu/Natal, Durban, Durban Botanic

Gardens, *Tibouchina granulosa*, M. Gryzenhout, May 2004, PREM58900; living culture CMW13936, PREM 58901; living culture CMW13937. Mpumalanga, Lydenburg, Buffelskloof private nature reserve, *Heteropyxis canescens*, G. Nakabonge, J. Roux & M. Gryzenhout, October 2003, PREM58899; living culture CMW 13645, PREM 58898; living culture CMW13646.

Pathogenicity tests

Eight weeks after inoculation with *C. dispersa*, lesions were observed on the stems of the *Eucalyptus* clone (ZG 14) and on those of *T. granulosa* (Figs 5A–D). These lesions were light to dark brown, and stretched up and down the stems from the inoculation points. Similar results were obtained in both repeats of the inoculation study. Mean lesion lengths were 106 mm for *Eucalyptus* and 29 mm for *Tibouchina* in the first experiment and 104 mm and 25 mm, respectively, in the second experiment. The differences observed between hosts were significant ($P < 0.001$) and were similar in both trials. *C. dispersa* was re-isolated from the lesions. No lesions developed on the controls, and the margins of the points of inoculation callused over (Figs 5–6).

Inoculation of *C. dispersa* on stems of *H. natalensis* showed no obvious lesion development after eight weeks. Similarly, no lesions developed on the controls.

DISCUSSION

In this study, we have shown that the fungus isolated from *H. canescens, S. cordatum* and *T. granulosa* in South Africa represents a new genus and species related to, but distinctly different from, *Chrysoporthe*. Description of this new taxon, *C. dispersa*, is supported by both morphological characteristics and DNA sequence data. These have clearly shown that isolates of *C. dispersa* form a clade distinct from *Chrysoporthe*, *Holocryphia* and other taxa, which it resembles morphologically.

Celoporthe dispersa most closely resembles species of *Chrysoporthe* and may appear indistinguishable from *Chrysoporthe* spp. when it is observed in the absence of light microscopy. Species of both genera have black conidiomata of similar shape. The

ascostromata are in both cases semi-immersed, with limited orange to cinnamon stromatic tissue and perithecial necks covered in umber tissue as they extend beyond the bark surface. Both genera have conidia and ascospores that are expelled as bright luteous spore tendrils. The ascospores of both *Celoporthe* and *Chrysoporthe* are one-septate, hyaline and oblong to elliptical. Furthermore, *C. dispersa* occurs on the same hosts as *Chrysoporthe*. The fungus was isolated from *T. granulosa* and *S. cordatum,* two hosts on which the morphologically similar *Chr. austroafricana* also occurs (Myburg *et al.* 2002, Heath *et al*. 2006). However, to the best of our knowledge this is the first fungus belonging to the group that has been collected from a species of *Heteropyxis*.

Although *Celoporthe* resembles *Chrysoporthe*, distinct morphological differences separate these two fungi. The presence of short perithecial necks, pulvinate to conical conidiomata without necks, conidia that are oblong to cylindrical to ovoid, and pseudoparenchymatous stromatic tissue in the conidiomatal base, distinguish *Celoporthe* from *Chrysoporthe* spp. *Chrysoporthe* spp. have long cylindrical perithecial necks, the conidiomata are pyriform to pulvinate with attenuated necks, conidia are oblong and uniform in shape, and stromatic tissue of the conidiomatal base is of *textura globulosa* and that of the neck of *textura porrecta* (Gryzenhout *et al.* 2004). *C. dispersa* produces cultures that are white with grey to chestnut-colored patches, in contrast to *Chrysoporthe* spp. that have white to cinnamon-colored cultures with hazel patches. Careful morphological and cultural comparisons thus make it relatively easy to distinguish *C. dispersa* from *Chrysoporthe* spp.

Three distinct but closely related and morphologically similar pathogenic fungi occur on exotic and native *Myrtales* in South Africa. These are *Chr. austroafricana*, which is a highly pathogenic fungus on *Eucalyptus* spp. grown in South Africa (Wingfield *et al.* 1989, Conradie *et al*. 1990) and which also occurs on *T. granulosa* (Myburg *et al.* 2002) and native *S. cordatum* (Heath *et al*. 2006). *C. dispersa* has been described in this study and occurs on native *S. cordatum*, *H. canescens* and exotic *T. granulosa* in South Africa. The third fungus, *H. eucalypti*, has been recorded only from *Eucalyptus* spp. in South Africa (Van der Westhuizen *et al.* 1993, Gryzenhout *et al.* 2003), but is common in and probably originates from Australia (Old *et al*. 1986). *Holocryphia eucalypti* can easily be distinguished from *C. dispersa* and *Chr. austroafricana* based

on differences in the colour and shape of conidiomata as well as cultural morphology (Venter *et al.* 2002, Gryzenhout *et al*. 2004).

DNA based comparisons in this study have shown that there are different phylogenetic groups represented by the isolates now treated as the single species *C. dispersa.* Thus, *C. dispersa* is represented by isolates from *Heteropyxis*, *Tibouchina* and *Syzygium* spp. in South Africa, and these isolates form three closely related sub-clades. A fourth sub-clade represents isolates from clove in Indonesia and was previously studied by Myburg *et al.* (2003). Based on DNA sequence data, this fungus clearly represents a distinct species, but could not be described because there are insufficient fungal structures typically produced on bark in nature to be able to characterize it. The fact that the unknown Indonesian fungus is now known to reside in *Celoporthe* should facilitate the collection of additional samples from clove in Indonesia.

The three closely related sub-clades consisting of isolates of *C. dispersa* from South Africa, were consistent with their three different host genera (*Heteropyxis*, *Syzygium* and *Tibouchina*) and areas of collection (Lydenburg, Tzaneen and Durban). These sub-clades are, however, represented by a limited number of isolates and a larger collection of isolates will be required to better understand the relationship among them. We were unable to detect clear morphological differences between the fungi in these three sub-clades and this was also hindered by the absence of teleomorph structures on the specimens from *H. canescens* and *T. granulosa*. Description of different species for the fungi represented by the three phylogenetic groupings contained in *C. dispersa* must await the acquisition of additional material and isolates. The ecological data and distribution of these fungi in South Africa is also largely unknown, and such information would be useful in studying the taxonomic status of the fungi in the three sub-clades of *C. dispersa.*

Heteropyxis canescens is a rare and endangered tree species in South Africa. Currently it is found only in Mpumalanga Province (John Burrows, *pers. comm.*, Lawes *et al.* 2004). Fruiting structures of *C. dispersa* were collected from dying trees in the Buffelskloof Nature Reserve near Lydenburg and it was thought that the fungus might be responsible for the death of the trees. However, pathogenicity tests

conducted using a limited number of trees of a closely related species, *H. natalensis*, showed that *C. dispersa* is not pathogenic to that species. Although it is possible that *H. canescens* is more susceptible to *C. dispersa* than is *H. natalensis*, the fungus might not be the cause of tree death at Buffelskloof. However, in order to understand the pathogencity of *C. dispersa* more clearly, the fungus will need to be inoculated on *H. canescens* and on a larger number of trees than was possible in this study. This will be difficult to achieve because *H. canescens* is endangered and extremely difficult to propagate artificially. The cause of tree mortality in the Buffelskloof Nature Reserve thus remains unclear. The possibility that another organism is responsible for the death of trees must also be investigated.

Pathogenity trials conducted on *E. grandis* and *T. granulosa* showed that *C. dispersa* is pathogenic on both these hosts. In these trials, the *Eucalyptus* clone was more susceptible than *T. granulosa*. *Celoporthe dispersa* is thus a newly discovered pathogen of these trees and it could become important on commercially grown *Eucalyptus* trees in South Africa.

Celoporthe dispersa and *Chr. austroafricana* are present on both native and non-native *Myrtales* in South Africa. This raises many important issues pertaining to the origin and distribution of these fungi. Both fungi are currently known only from southern Africa, and they also occur on native African trees. It has already been suggested that *Chr. austroafricana* is native to South Africa (Wingfield 2003, Heath *et al*. 2006) and the same is probably true for *C. dispersa*. These fungi are virulent pathogens of exotic *Eucalyptus* trees and their accidental introduction into Australia, where *Eucalyptus* spp. and many other *Myrtales* are native, could result in an ecological disaster. This view is based on the fact that similar canker pathogens, such as *Cryphonectria parasitica* (Murrill) M. E. Barr., have caused devastating losses to trees after being introduced into new environments (Anagnostakis 1987, Slippers *et al*. 2005). Both *Chr. austroafricana* and *C. dispersa* also potentially threaten plantation *Eucalyptus* trees wherever they are grown commercially.

Additional surveys are necessary to expand the host and geographic ranges of *Celoporthe* and *Chrysoporthe* spp. on *Myrtales* in South Africa and on other parts of the African continent. The fact that these fungi are almost indistinguishable in the

field will complicate such surveys, and laboratory studies will be required for reliable identifications. New collections and associated isolates of *C. dispersa* could also lead to the sub-division of this species into additional taxa. Additional material will thus add knowledge to the relatively poorly studied fungal biodiversity on the African continent and especially on native African tree species.

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Table 1. Isolates included in this study.

Species identity	Isolate number[a]	Alternative isolate number[b]	Host	Origin	Collector	GenBank accession numbers[c]
Amphilogia gyrosa	CMW10469	CBS 112922	*Elaeocarpus dentatus*	New Zealand	G Samuels	AF452111, AF525707, AF525714
	CMW 10470	CBS 112923	*El. dentatus*	New Zealand	G Samuels	AF452112, AF525708, AF525715
Celoporthe sp.[d]	CMW 10781	CBS 115844	*Syzygium aromaticum*	Kalimantan, Indonesia	MJ Wingfield	AY084009, AY084021, AY084033
	CMW 10779		*S. aromaticum*	Indonesia	MJ Wingfield	AY084007, AY084019, AY084031
	CMW 10780		*S. aromaticum*	Indonesia	MJ Wingfield	AY084008, AY084020, AY084032
Celoporthe dispersa[d]	CMW 9978		*Syzygium cordatum*	Tzaneen, South Africa	M Gryzenhout	AY214316, DQ267135, DQ267141
	CMW 9976		*S. cordatum*	Tzaneen, South Africa	M Gryzenhout	DQ267130, DQ267136, DQ267142
	CMW 13936		*Tibouchina granulosa*	Durban, South Africa	M Gryzenhout	DQ267131, DQ267137, DQ267143
	CMW 13937		*T. granulosa*	Durban, South Africa	M Gryzenhout	DQ267132, DQ267138, DQ267144
	CMW 13646		*Heteropyxis canescens*	Lydenburg South Africa	G Nakabonge, J Roux & M Gryzenhout	DQ267133, DQ267139, DQ267145
	CMW 13645		*H. canescens*	Lydenburg South Africa	G Nakabonge, J Roux & M Gryzenhout	DQ267134, DQ267140, DQ267146
Cryphonectria parasitica	CMW 13749	MAFF 410158	*Castanea mollisima*	Japan	Unknown	AY697927, AY697943, AY697944
	CMW 7048	ATCC 48198	*Quercus virginiana*	USA	FF Lombard	AF368330, AF273076, AF273470
Cryphonectria radicalis	CMW 10455	CBS 238.54	*Castanea dentata*	Italy	A Biraghi	AF452113, AF525705, AF525712
	CMW 10477	CBS 240.54	*Quercus suber*	Italy	M Orsenigo	AF368328, AF368347, AF368346
	CMW 10436	CBS 165.30	*Q. suber*	Portugal	B d'Oliviera	AF452117, AF525703, AF525710
	CMW 10484	CBS 112918	*Castanea sativa*	Italy	A Biraghi	AF368327, AF368349, AF368349
Chrysoporthe austroafricana	CMW 2113	CBS 112916	*Eucalyptus grandis*	South Africa	MJ Wingfield	AF046892, AF273067, AF273462
	CMW 9327	CBS 115843	*Tibouchina granulosa*	South Africa	MJ Wingfield	AF273473, AF273060, AF273455
Chrysoporthe cubensis	CMW 10639	CBS 115747	*E. grandis*	Colombia	CA Rodas	AY263419, AY263420, AY263421
	CMW 10669	CBS 115751	*Eucalyptus* sp.	Republic of Congo	J Roux	AF535122, AF535124, AF535126
	CMW 8651	CBS 115718	*S. aromaticum*	Sulawesi, Indonesia	MJ Wingfield	AY084002, AY084014, AY084026
	CMW 11288	CBS 115736	*S. aromaticum*	Indonesia	MJ Wingfield	AY214302, AY214230, AY214266
Chrysoporthella hodgesiana	CMW 9994	CBS 115729	*T. semidecandra*	Colombia	R Arbelaez	AY956968, AY956975, AY956976
	CMW 10641	CBS 115854	*T. semidecandra*	Colombia	R Arbelaez	AY692322, AY692326, AY692325
Diaporthe ambigua	CMW 5288	CBS 112900	*Malus domestica*	South Africa	WA Smit	AF 543817, AF 543819, AF 543821
	CMW 5587	CBS 112901	*M. domestica*	South Africa	WA Smit	AF 543818, AF 543820, AF 543822
Endothia gyrosa	CMW 2091	ATCC 48192	*Quercus palustris*	USA	RJ Stipes	AF046905, AF368337, AF368336
	CMW 10442		*Q. palustris*	USA	RJ Stipes	AF 368326, AF 368339, AF 368338
Holocryphia eucalypti	CMW 7037	CRY45	*Eucalyptus delegatensis*	Australia	K Old	AF232880, AF368343, AF368342
	CMW 14546	CRY287, CBS115838	*Eucalyptus* sp.	South Africa	H Smith	AF232879[d], **, **
Rostraureum tropicale	CMW 9971	CBS 115725	*Terminalia ivorensis*	Ecuador	MJ Wingfield	AY167426, AY167431, AY167436
	CMW 10796	CBS 115757	*T. ivorensis*	Ecuador	MJ Wingfield	AY167428, AY167433, AY167438

[a] **CMW and CRY**= Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

[b] **ATCC** = American Type Culture Collection, Manassas, USA; **CBS** = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **MAFF**, Microorganisms Section, MAFF GENEBANK, National Institute of Agrobiological Sciences (NIAS), MAFF Gene Bank, Ibaraki, Japan.

[c] Accession numbers refer to sequence data of the ITS, β-tubulin 1 (primers Bt1a/1b) and β-tubulin 2 (primers Bt2a/2b) regions respectively.

[d] Isolates sequenced in this study.

Table 2. Comparison of morphological characteristics between *Celoporthe* and *Chrysoporthe* spp.

Character	*Celoporthe*	*Chrysoporthe*[a]
Perithecia	Black, valsoid, embedded in bark tissue	Similar to *Celoporthe*
Perithecial necks	Short (50 µm)	Long (240 µm)
Stromatic tissue	Limited cinnamon to orange prosenchymatous to pseudoparenchymatous stromatic tissue	Similar to *Celoporthe*
Asci	8-spored, fusoid to ellipsoid	Similar to *Celoporthe*
Ascospores	One septate, hyaline, oblong to elliptical	Similar as *Celoporthe*
Conidiomata	Pulvinate to conical, superficial, without a neck	Pyriform to pulvinate with attenuated necks
Conidia	Oblong to cylindrical to ovoid	Oblong
Conidiophores	Basal cells not prominent	Basal cells prominent
Stromatic tissue	Stromatic tissue of the base of conidiomata is pseudoparenchymatous	Tissue of the base consists of larger cells of *textura globulosa*
Cultures	White with grey patches, eventually becomes umber to hazel to chestnut	White with cinnamon to hazel patches

[a]From Gryzenhout *et al.* 2004

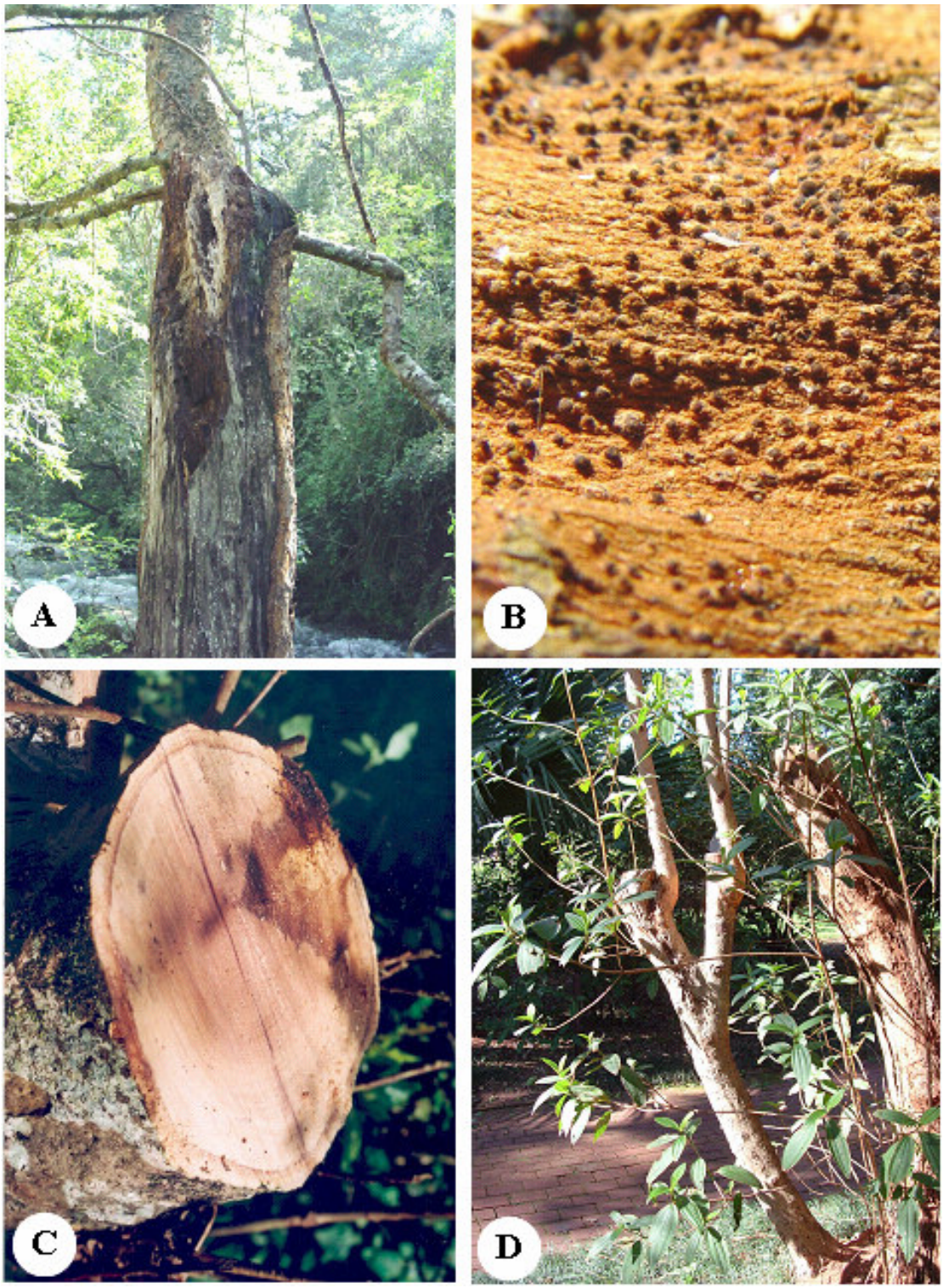

Fig. 1. Symptoms associated with *Celoporthe dispersa* infection. A) Dying *Heteropyxis canescens* B) Fruiting structures of *C. dispersa* on *H. canescens*. C) Cross section through trunk canker on *H. canescens*. D) Cracks and cankers on *Tibouchina granulosa*. (Photographs by Prof J. Roux and Marieka Gryzenhout)

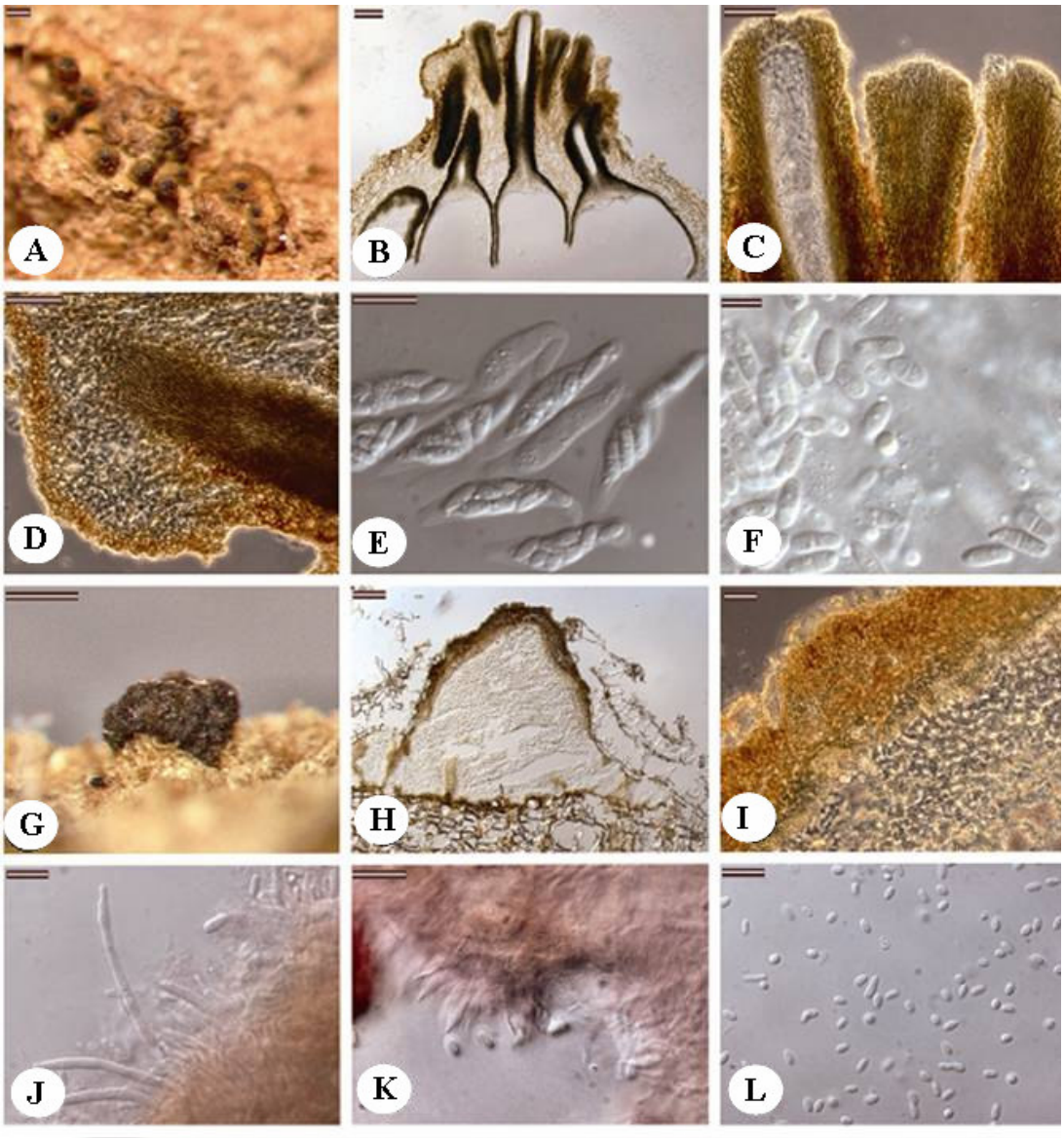

Fig. 2. Fruiting structures of *C. dispersa.* A) Ascoma on bark. B) Longitudinal section through ascoma. C) Perithecial neck tissue. D) Stromatic tissue. E) Asci with ascospores. F) Ascospores. G) Conidioma on the bark. H) Longitudinal section through Conidioma I) Stromatic tissue of conidioma. J) Conidiophores. K) Conidigenous cells. L) Conidia. (Scale bar A–B, G–H= 100 µm; C–D, I = 20µm; E–F, J–K–L= 10µm).

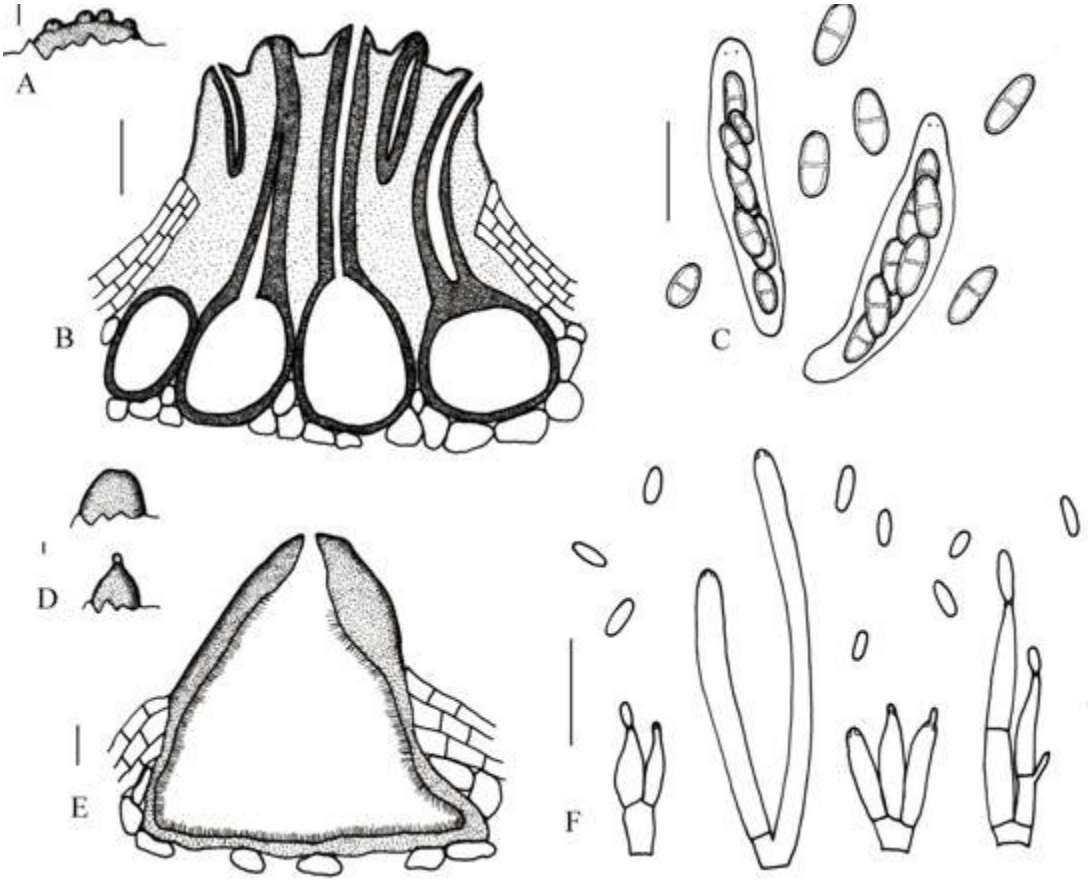

Fig. 3. Line drawings of *Celoporthe dispersa*. A) Shape of ascoma. B) Section through ascoma. C) Asci and ascospores. D) Shapes of conidiomata. E) Section through conidioma. F) Conidiophores and conidia. Bars A–B, D–E = 100 µm; C, F = 10 µm.

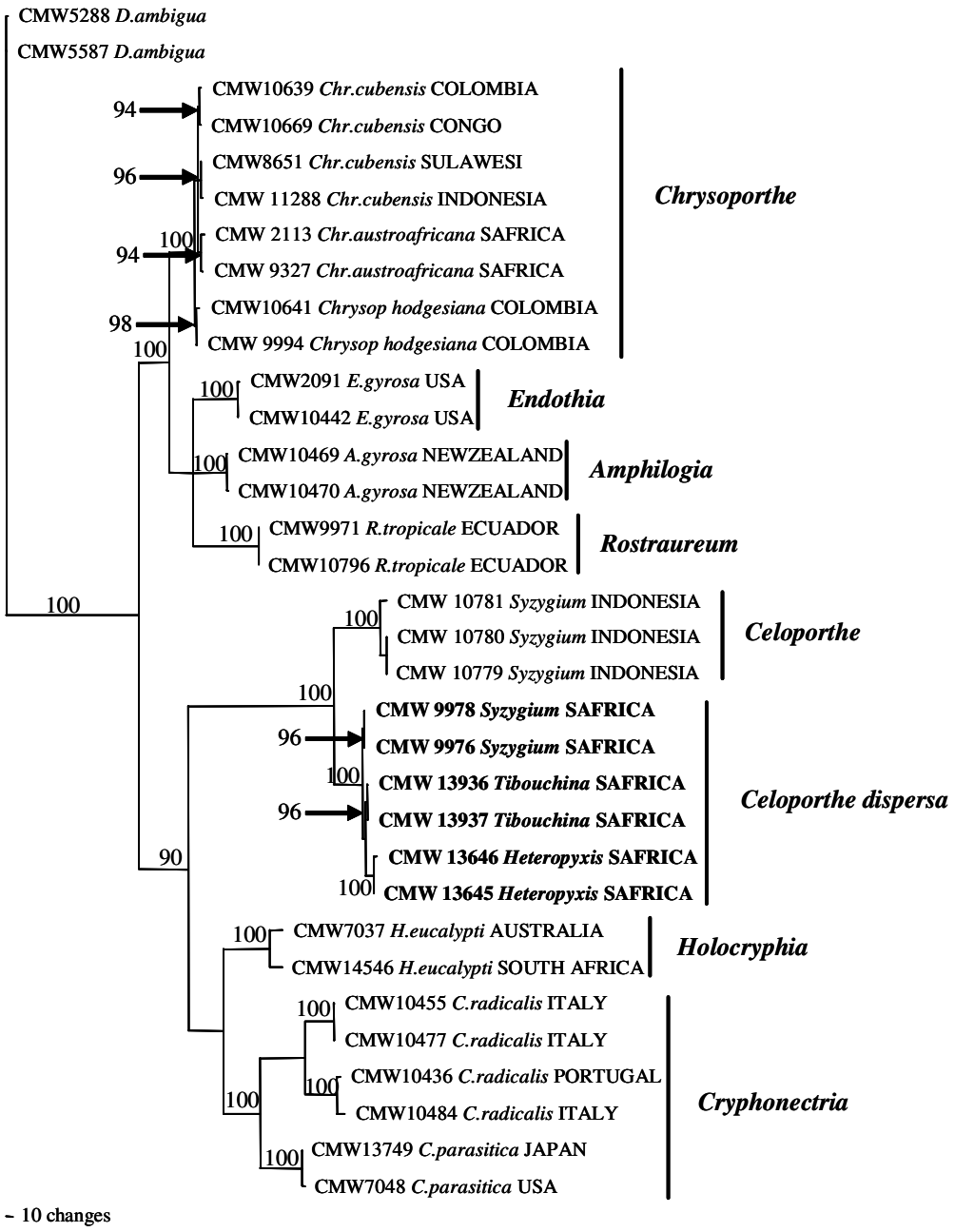

Fig. 4. A Phylogenetic tree generated from combined sequence data of the ITS ribosomal DNA and β-tubulin gene sequence data. One strict consensus tree (Tree length of 1620, CI of 0.740 and RI of 0.920) was generated from heuristic searches performed on the combined data set. Bootstrap values (1000 replicates) above 50% are indicated on the branches. Isolates sequenced in this study are in bold. *Diaporthe ambigua* sequences were used as outgroup.

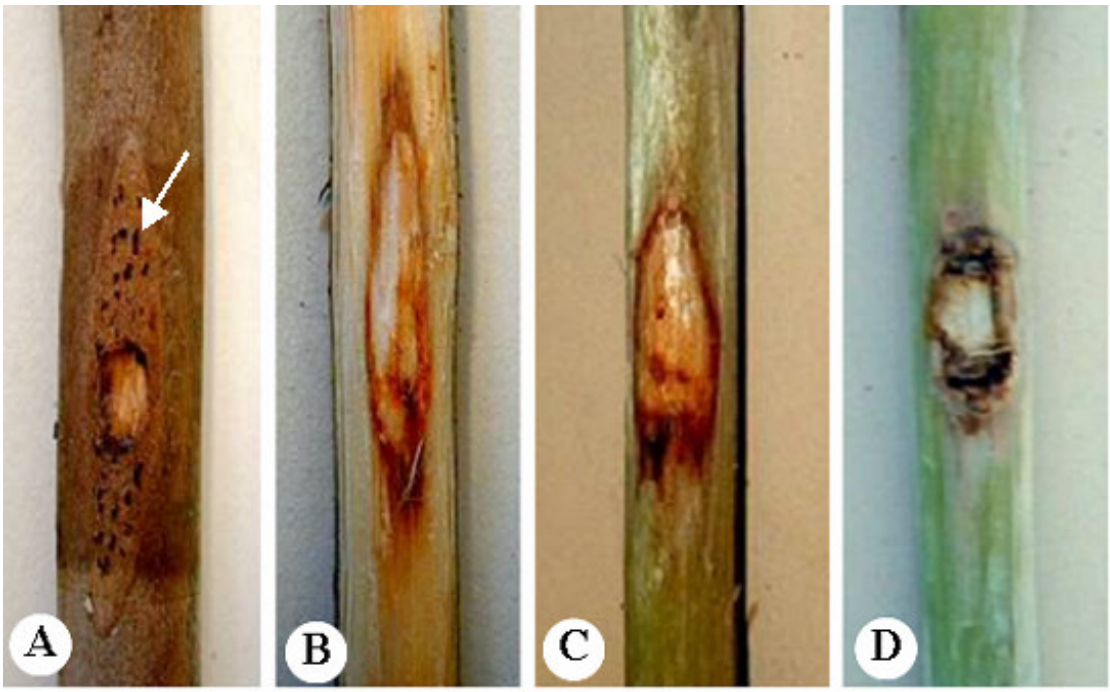

Fig. 5. Lesions associated with inoculation of *C. dispersa* on a clone of *Eucalyptus grandis* (ZG 14) and *T. granulosa*. A) Fruiting structures formed on host as a result of inoculation (Arrow). B) Lesion on *Eucalyptus* sp. C) Lesion formed on *T. granulosa*. D) Control inoculation on *T. granulosa* showing callus formation and the absence of lesion development

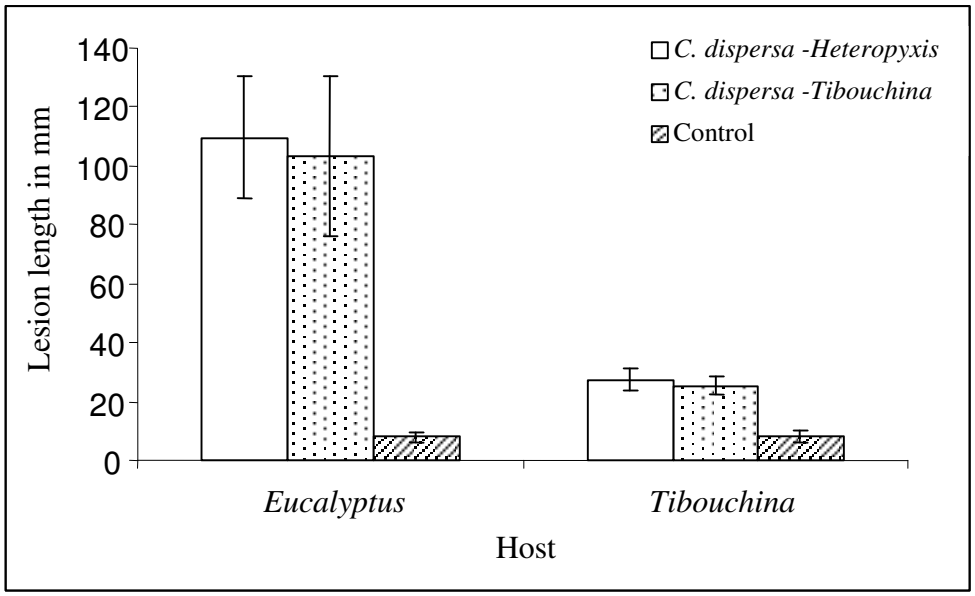

Fig. 6. Comparison of lesion lengths associated with inoculation of *C. dispersa* on *Eucalyptus* (ZG 14) clones and *T. granulosa* plants under greenhouse conditions. The trees were inoculated with *C. dispersa* isolated from *H. canescens* (CMW 13645) and *C. dispersa* isolated from *T. granulosa* (CMW 13936). Mean lesion lengths were determined with 98% confidence limits.

SUMMARY

Considerable changes have occurred in recent years, regarding the taxonomy and ecology of *Eucalyptus* fungal pathogens previously treated in the genera *Cryphonectria* and *Endothia*. *Cryphonectria cubensis* now resides in *Chrysoporthe* with two species, which are very distinct from *Cryphonectria*. The fungus previously known as *E. gyrosa* was moved to *C. eucalypti* and will soon be known as *Holocryphia eucalypti*. It is very likely that *C. eucalypti* and *Chr. cubensis* were introduced onto the African continent, but the hypothesis remains to be tested, while *Chr. austroafricana* seems native to the African continent. The aim of studies contained in this thesis was to consider the distribution, taxonomy and diversity of *Chrysoporthe* spp. and *Cryphonectria eucalypti* on the African continent. This was achieved through surveys in southern and eastern Africa, of both *Eucalyptus* spp. and native tree species belonging to the *Myrtales*. The intention was that the results of the studies in this thesis should aid in a better understanding of the taxonomy, origin, distribution, host range, as well as pathogenicity of various *Cryphonectria* and *Chrysoporthe* species in eastern and southern Africa.

Various new hosts, new areas of occurrence and taxonomic changes have occurred for species of *Cryphonectria sensu lato*, previously known only on *Eucalyptus* spp. Chapter one of this thesis presented an overview of the most recent findings regarding the taxonomy, host range and distribution of *C. cubensis sensu lato* and *C. eucalypti.* The background to the description of a new genus, *Chrysoporthe* Gryzenhout & M.J. Wingf. and three new species namely; *Chr. cubensis*, *Chr. austroafricana* and *Chrysopothella hodgesiana*, previously considered to represent *C. cubensis* was also considered. Furthermore, the wide host range of *Chrysoporthe* spp. has been reviewed. The fungi are known on various genera in the order *Myrtales* in both tropical and subtropical areas, worldwide. Emphasis was placed on these *Eucalyptus* pathogens in Africa.

Chrysoporthe cubensis and *Chr. austroafricana*, collectively known as *Cryphonectria cubensis* in the past, are important canker pathogens of *Eucalyptus* spp. worldwide. In chapter two of this thesis I have shown, for the first time, that *Chr. cubensis* occurs

in Kenya, Malawi and Mozambique on non-native *Eucalyptus* spp. and *Chr. austroafricana* occurs in Mozambique, Malawi and Zambia on non-native *Eucalyptus* spp. and native *S. cordatum*. I was also able to show that *Chr. austroafricana* causes cankers at the base and higher up on stems of *Eucalyptus* trees in South Africa and Malawi, which is contrary to prior knowledge. Likewise, the sexual state of this fungus has been shown to be equally abundant as the asexual state in countries north of South Africa, contrary to the situation in southern Africa where the asexual state predominates. The known distribution range of *Chr. austroafricana* within South Africa was also expanded through this study.

Chrysoporthe cubensis is an important fungal pathogen of *Eucalyptus* spp., worldwide. The fungus is also known on many other hosts all residing in the order *Myrtales*. Previous surveys conducted in eastern and southern Africa to assess the distribution of *Chrysoporthe* spp. in this region, revealed the occurrence of *Chr. cubensis* on *Eucalyptus* spp. in Kenya, Malawi and Mozambique. In chapter three of this thesis, the population structure of *Chr. cubensis* isolates from *Eucalyptus* spp. from Kenya, Malawi and Mozambique was considered for the first time. This represents a first attempt to consider the genetic structure of the fungus from eastern Africa. Results show that there is a very low genetic diversity within the populations of *Chr. cubensis* from Kenya, Malawi and Mozambique, implying that the fungus is probably newly introduced in these areas. Based on phylogenetic analyses, the origin of eastern African *Chr. cubensis* is most likely Asia.

In chapter four of this thesis, polymorphic microsatellite DNA markers were developed from a single spore isolate of *C. eucalypti* collected from *Eucalyptus* stem canker in South Africa. Markers were obtained using the enrichment technique known as FIASCO (Fast Isolation by AFLPs of Sequences Containing Repeats). Ten polymorphic markers were isolated, of which 2 were discarded due to their high polymorphism in the flanking region. These markers will consequently provide useful tools for future investigations considering the population biology and especially the global spread of *C. eucalypti*.

Cryphonectria eucalypti is a fungal pathogen considered opportunistic in South Africa, while in Australia it has been associated with sporadic but serious disease

problems. Chapter five of this thesis presents results on the population structure of *C. eucalypti* from South Africa, eastern and western Australia. Nei's gene diversity (*H*) showed that the eastern Australian population is most genetically diverse and the western Australian populations from *Corymbia* and *Eucalyptus* somewhat less diverse. The South African population displayed the lowest genetic diversity. The high genetic diversity in the Australian populations supports the view that *C. eucalypti* is native to that region. This is consistent with the fact that *Eucalyptus* species are also native to the Australian continent.

In chapter six of this thesis, I have shown that the fungus isolated from *H. canescens, S. cordatum* and *T. granulosa* in South Africa represents a new genus and species related to, but distinctly different from *Chrysoporthe*. *Celoporthe dispersa gen. et sp. nov*. is, therefore, described to accommodate this fungus. This description was supported by both morphological characteristics and DNA sequence data. These have clearly shown that isolates of *C. dispersa* form a clade distinct from *Chrysoporthe*, *Holocryphia* and other taxa, which it resembles morphologically. Pathogenicity tests showed that *C. dispersa* is not pathogenic to *H. natalensis*, but a potential pathogen of *Eucalyptus* and *Tibouchina* spp.

The collection of studies included in this thesis demonstrated that *Chrysoporthe* spp. occur in Malawi, Mozambique, Zambia, Kenya and Tanzania on both *Eucalyptus* and native *Syzygium cordatum* trees. This significantly expands the geographical distribution of these important pathogens. The studies have also shown that *Chrysoporthe cubensis* has recently been introduced on the continent. It is my hope that new knowledge emerging from studies in this thesis will aid in quarantine measure to control the spread of these important fungal pathogens including the new species *Celoporthe dispersa*.