

CHAPTER I

Literature Review

The taxonomic position of *Cryphonectria* and *Endothia* in the Diaporthales.

THE TAXONOMIC POSITION OF *CRYPHONECTRIA* AND *ENDOTHIA* IN THE DIAPORTHALES

1. THE DIAPORTHALES

1.1. Introduction.....	2
1.2. Morphological characterisation.....	2
1.3. Molecular characterisation.....	3

2. *CRYPHONECTRIA* AND *ENDOTHIA*

2.1. Introduction.....	7
2.2. Morphology of <i>Cryphonectria</i> and <i>Endothia</i>	8
2.3. Non-morphological differentiation between <i>Cryphonectria</i> and <i>Endothia</i>	10
2.4. Family status of <i>Cryphonectria</i> and <i>Endothia</i>	11
2.5. Species of <i>Cryphonectria</i> and <i>Endothia</i>	12
2.6. Taxonomic challenges relating to <i>Cryphonectria</i> and <i>Endothia</i> and particularly <i>C. cubensis</i>	13

CONCLUSIONS.....	19
------------------	----

REFERENCES.....	22
-----------------	----

THE TAXONOMIC POSITION OF *CRYPHONECTRIA* AND *ENDOTHIA* IN THE DIAPORTHALES

1. THE DIAPORTHALES

1.1. Introduction

The Diaporthales includes many well-known plant pathogenic fungi. Taxa in this group are causal agents of a number of diseases on economically important fruit and vegetable crops (Farr et al. 2002), while others are known to produce secondary metabolites that lead to toxicoses in animals (Williamson et al. 1994). Some members of this order are also causal agents of serious tree diseases. Of these, the best known is *Cryphonectria parasitica* (Murrill) Barr (Merkel 1905). This fungus causes the devastating tree disease known as chestnut blight that has resulted in the virtual elimination of the American chestnut [*Castanea dentata* (Marsh.) Borkh.] from natural ecosystems in eastern parts of North America (Anagnostakis 1987, Griffin 1986).

1.2. Morphological characterisation

The Diaporthales is delineated by well-defined morphological characteristics (Barr 1978, Cannon 1988). The most distinctive characteristic is the *Diaporthe*-type centrum (Lutrell 1951), which, in the early stages of development, is characterised by thin-walled pseudoparenchymatous tissue and the absence of paraphyses (Alexopoulos and Mims 1978).

Asci develop between collapsing pseudoparenchymatous tissue (Alexopoulos and Mims 1978). Characteristics such as perithecia with long necks situated in pseudostromata and thick-walled asci are also linked with the *Diaporthe*-type centrum (Alexopoulos and Mims 1978). Furthermore, asci have refractive apical rings (Barr 1978).

Anamorphs of the Diaporthales are usually coelomycetous (Barr 1978, Castlebury et al. 2002, Farr et al. 2001, Hawksworth et al. 1995). Fruiting bodies are either acervuli or pycnidia characterised by the presence or absence of well-developed stromatic tissue (Barr 1978, Castlebury et al. 2002, Farr et al. 2001). Conidiogenous cells are phialidic or annellidic (Barr 1978, Castlebury et al. 2002, Farr et al. 2001). Examples of anamorph genera in the Diaporthales are *Phomopsis* (Sacc.) Bubák and *Cytospora* Ehrenb.:Fr.

1.3. Molecular characterisation

A number of studies have focused on family delineation in the Diaporthales (Barr 1978, Barr 1990, Cannon 1988, Castlebury et al. 2002, Farr et al. 2001, Wehmeyer 1975, Zhang and Blackwell 2001). Families that have been recognised in this order in the past include the Diaporthaceae Höhn. ex Wehm., Gnomoniaceae G. Winter, Melanconidaceae G. Winter, Pseudovalsaceae M.E. Barr and the Valsaceae Tul. & C. Tul. (Zhang and Blackwell 2001). Hawksworth et al. (1995) recognised two families, i.e. the Valsaceae and the Melanconidaceae, accommodating all the members of the Diaporthales. Eriksson et al. (2001) recently presented a web-based outline of the Ascomycota and recognised a third family Vialaeaceae P.F. Cannon in the order.

As opposed to the taxonomy at the morphological level, the taxonomy of the group based on DNA is less well-defined. Examples of DNA-based taxonomic studies that have included Diaporthalean fungi are those of Spatafora and Blackwell (1993) and Rehner and Uecker (1994). Spatafora and Blackwell (1993) focused their study on the ordinal level of perithecial ascomycetes including two species of Diaporthales. Rehner and Uecker (1994) considered the phylogeny of *Phomopsis* anamorphs of *Diaporthe*. These studies, however, did not include representatives for all the families necessary to make definitive and comprehensive conclusions regarding the taxonomy of the order as a whole.

A DNA-based phylogenetic study that included a large assemblage of Diaporthalean species was that of Zhang and Blackwell (2001). The primary purpose of this study was to determine the taxonomic position of the mitotic fungus that causes dogwood anthracnose (*Discula Destructiva* Redlin), but family relationships of other diaporthalean species also were evaluated. This assessment was based on the phylogenetic analyses of sequence data from large (LSU) and small (SSU) subunit rDNA as well as RNA polymerase II (RPB2) gene. The phylogenetic results supported the placement of *D. destructiva* in the Diaporthales.

The twenty diaporthalean species included in the study of Zhang and Blackwell (2001) grouped in three major clades in the Diaporthales based on LSU and SSU sequence analyses. Zhang and Blackwell (2001) concluded that the Valsaceae, as defined by Wehmeyer (1975) and Barr (1990), was paraphyletic because it included species with

allantoid ascospores as well as non-allantoid ascospores. Similarly, the Melanconidaceae of Barr (1978, 1990) and Hawksworth (1995) were also found to be paraphyletic. Species representing the Diaporthaceae and Gnomoniaceae were polyphyletic and the characters that defined them were distributed in more than one clade (Zhang and Blackwell 2001).

Representatives of the Magnaporthaceae P.F. Cannon (Cannon 1994), previously considered in the Diaporthales (Alexopoulos et al. 1996), were also studied by Zhang and Blackwell (2001). Based on similarities in pathogenicity, fungi in this family were considered to reside in the Diaporthales (Cannon 1994). The SSU sequence data of Zhang and Blackwell (2001) showed that the Magnaporthaceae might be connected to the Ophiostomatales or the Sordariales rather than the Diaporthales. These conclusions did, however, not have strong statistical support. The authors could, thus, not recommend an ordinal placement for the Magnaporthaceae with any level of confidence.

The most recent and comprehensive phylogenetic study of Diaporthalean fungi was that of Castlebury et al. (2002). In this study, 69 members of the Diaporthales were compared based on LSU nrDNA sequence data. It was concluded that there are at least six lineages in the Diaporthales and not two (Hawksworth 1995) or three (Eriksson et al. 2001). Four of the six lineages have previously been defined. These are the Melanconidaceae (Barr 1978, 1990, Hawksworth et al. 1995), the Gnomoniaceae (Barr 1978, 1990, Wehmeyer 1975), the Valsaceae (Barr 1978, 1990, Hawksworth et al. 1995) and the Diaporthaceae (Wehmeyer 1975).

Two of the six Diaporthalean lineages proposed by Castlebury et al. (2002) have not previously been recognised as distinct families. These lineages are designated as the *Schizoparme* complex and the *Cryphonectria/Endothia* complex. The *Schizoparme* complex also included the anamorph genera *Coniella* and *Pilidiella* (Castlebury et al. 2002), which were previously accommodated in the Melanconidaceae (Samuels et al. 1993). Castlebury et al. (2002) suggested that the *Schizoparme* complex and *Cryphonectria/Endothia* complex, previously incorporated in the Valsaceae (Hawksworth et al. 1995), might ultimately be distinguished in their own families.

DNA sequence analyses have highlighted the fact that families in the Diaporthales require revision (Castlebury et al. 2002). A number of taxa included in the phylogenetic analyses of Castlebury et al. (2002) did not associate with any of the other lineages and likely represent new families or genera. These taxa represent *Wuestneia* and its *Harkenessia* anamorphs as well as the anamorph species *Greeneria uvicola*. Castlebury et al. (2002) suggested that, as their study did not include all members of the Diaporthales, additional families might emerge in future studies on the Diaporthales.

Castlebury et al. (2002) did not include members of the Vialaeaceae and the Sydowiellaceae in their study due to the lack of representative species. The authors, therefore, excluded both these families from their study. Phylogenetic studies of Zhang and Blackwell (2001) and Farr et al (2001) also lacked adequate representatives of the Sydowiellaceae and thus precluded the opportunity to draw conclusions regarding the ordinal position of this family. The ordinal placement of the Vialaeaceae and the Sydowiellaceae, therefore, remain

uncertain and will only be conclusively resolved once appropriate representatives are found and included in phylogenetic comparisons.

The taxonomic relationships of the families in the Diaporthales require further characterisation. Studies such as those of Zhang and Blackwell (2001), Farr et al. (2001), Eriksson et al. (2001) and Castlebury et al. (2002) suggest that the taxonomy of the Diaporthalean lineages will need additional revision and consideration in future. However, these studies already provide a taxonomic framework, based on morphology and phylogenetic data, which provide a firm foundation for future studies.

2. CRYPHONECTRIA AND ENDOTHIA

2.1. Introduction

Endothia and *Cryphonectria* were established in the 1800's, with *Endothia* the older of the two names. This genus was established in 1849 by Fries. Fries (1849) separated *Endothia* from *Sphaeria* Haller and based the description of the newly established *Endothia* on the tubular, red to tawny stromata, pale perithecia and evanescent asci of the *Sphaeria gyrosa* Schw. specimens he had received from Schweinitz. *Endothia gyrosa* was designated as the type species.

Cryphonectria was first established as a sub-genus of *Nectria* in 1883 (Saccardo 1883). Saccardo (1905) gave *Cryphonectria* full generic status (Barr 1978, Kobayashi 1970). In

1909, Von Höhnelt synonymised *Cryphonectria* with *Endothia*. *Cryphonectria gyrosa* (Berk. & Br.) Sacc. was considered as the type of *Cryphonectria* by Von Höhnelt as it was the first species listed in Saccardo's description of *Cryphonectria* (Shear et al. 1917), was thus transferred to *Endothia*. *Cryphonectria gyrosa* was given a new name, *E. tropicalis* Shear & Stevens, as the older name *E. gyrosa* had been used to designate the type of *Endothia* (Barr 1978, Shear et al. 1917).

2.2. Morphology of *Cryphonectria* and *Endothia*

Species of *Cryphonectria* and *Endothia* have superficially similar morphology, both with ascomata in well developed, yellow to orange or orange red stromata, with stromatal wall pigments that turn purple in 3% KOH and yellow in lactic acid (Castlebury et al. 2002). These colour reactions can also be observed in culture (Castlebury et al. 2002). Another morphological feature common to these genera is the dark brown to black perithecial walls (Kobayashi 1970, Shear et al. 1917).

Cryphonectria and *Endothia* species share similar *Endothiella* anamorphs (Barr 1978, Kobayashi 1970, Roane 1986a, Walker et al. 1985). Asexual structures are uni- to multilocular stromata and conidia are minute and aseptate (Kobayashi 1970, Roane 1986a, Shear et al. 1917). Differentiation of *Cryphonectria* and *Endothia* species has been based on ascospore and conidial sizes (Barr 1978, Kobayashi 1970, Roane 1986a), but ranges of spore size usually overlap (Kobayashi 1970, Roane 1986a), complicating conclusive identification and differentiation among the species.

Barr (1978) used stromatal and ascospore morphology to distinguish between *Cryphonectria* and *Endothia*. *Endothia* species were characterised by strongly developed, widely erumpent stromata consisting mainly of pseudoparenchymatous tissue (Barr 1978, Micales and Stipes 1987) with perithecia usually arranged in a diatrypoid fashion (Barr 1978, Micales and Stipes 1987). Species of *Cryphonectria* were characterised by less developed, semi-immersed stromata. The stromatic tissue is mainly prosenchymatous with perithecia arranged in a valsoid fashion (Barr 1978, Micales and Stipes 1987). *Cryphonectria* species were those having one-septate, fusoid to ellipsoid ascospores, while *Endothia* was reserved for species with non-septate, allantoid to cylindrical ascospores (Barr 1978, Micales and Stipes 1987).

Venter et al. (2002) showed that stromatal morphology, rather than ascospore shape and septation is the more useful characteristic to separate *Cryphonectria* from *Endothia*. Ascospore characteristics were found to be an unreliable feature when used to exclusively differentiate between *Cryphonectria* and *Endothia*. In their study, Venter et al. (2002) found that the ascospores of a fungus previously identified as *E. gyrosa* in South Africa and Australia were typical to those of *Endothia*, i.e. unicellular and allantoid (Roane 1986a, Shear et al. 1917) while the stromatal morphology resembled that of *Cryphonectria*. DNA sequence analyses showed that the South African and Australian fungus were the same, should reside in *Cryphonectria* and was described as a new species, *C. eucalypti* Venter and M.J. Wingfield.

2.3. Non-morphological differentiation between *Cryphonectria* and *Endothia*

A number of non-morphological methods have been used to distinguish between *Cryphonectria* and *Endothia* species. These methods include differences in pigment production (Micales et al. 1987, Roane and Stipes 1978), polyacrylamide gel electrophoresis of buffer-soluble proteins (Micales et al. 1987), fungitoxicant tolerance (Micales and Stipes 1986), optimal growth temperatures (Hodges et al. 1986, Stipes and Ratliff 1973) and isozyme analysis (Hodges et al. 1986, Micales et al. 1987). These non-morphological methods were mainly used to differentiate between *C. cubensis*, *C. parasitica* and *E. gyrosa* and could not be used to distinguish between all the species of *Cryphonectria* and *Endothia*. Hodges et al. (1986) also used non-morphological methods to show the conspecificity of *C. cubensis* and *E. eugeniae*.

A number of factors have influenced the use of non-morphological methods to make unambiguous conclusions regarding the taxonomic and phylogenetic relationships of species in *Cryphonectria* and *Endothia*. Hodges et al. (1986) showed that pigment production in culture is variable and is dependant on the formation of pycnidia. Similarly, isozyme metabolism of these fungi changes when they are maintained in culture as opposed to those growing on host material (Micales et al. 1987). Polyacrylamide gel electrophoresis of buffer-soluble proteins and fungitoxicant tolerance allow only for a rapid estimate of the similarity among isolates (Micales et al. 1986) and they allow us only to determine whether the fungal species reside in *Cryphonectria* or *Endothia*.

2.4. Family status of *Cryphonectria* and *Endothia*

Barr (1978) transferred *Endothia* from the Diaporthaceae to the Gnomoniaceae. Differences in ascospore morphology for species in the Diaporthales influenced this decision. The genus *Cryphonectria* was resurrected and accommodated in the Valsaceae (Barr 1978, Micales and Stipes 1987). However, in a subsequent study, Barr (1990) placed greater emphasis on ascospore morphology in these genera and treated *Endothia* in the Valsaceae and *Cryphonectria* in the Gnomoniaceae. Other authors disputed the placement of these genera in two separate families as they were thought too closely related (Chen et al. 1996, Walker et al. 1985). The Gnomoniaceae was reduced to the Valsaceae but the name "Gnomoniaceae" was given *nomen conservandum* status (Hawksworth et al. 1995). Thus *Cryphonectria* and *Endothia* currently reside in the Valsaceae.

Cryphonectria and *Endothia* are closely related. Vasilyeva (1998) recognised this close relationship by placing these genera in the tribe Endothiae M.E. Barr. Castlebury et al. (2002) suggested that *Cryphonectria* and *Endothia* should be considered in a separate family in the Diaporthales. Their results were based in LSU sequence analyses showing that *Cryphonectria* and *Endothia* reside in a discrete lineage. These conclusions were, however, based on the inclusion of only four *Cryphonectria* species (i.e. *C. macrospora*, *C. nitschkei*, *C. parasitica*, *C. cubensis*), one isolate designated as *Endothiella gyrosa* (Castlebury et al. 2002), that probably represented the genus *Endothia*, and an isolate of *E. eugeniae*. Zhang and Blackwell (2001) used LSU and SSU nrDNA sequences to compare species of

Cryphonectria and *Endothia* (i.e. *C. parasitica* and *E. eugeniae*) and also supported the relationship between these genera.

Representatives of two species, not residing in *Cryphonectria* or *Endothia*, were associated with the *Cryphonectria/Endothia* complex designated in Castlebury et al. (2002). These were *Chromendothia citrina* Lar. N. Vasiljeva and *Cryptodiaporthe corni* (Wehm.) Petr. The association of *C. corni* with the *Cryphonectria-Endothia* complex was surprising as *C. corni* grouped separately from the type species of *Cryptodiaporthe*, *C. aesculi*, residing in the Gnomoniaceae. These authors, therefore, suggested that *C. corni* either resides in *Cryphonectria* or *Endothia*.

2.5. Species of *Cryphonectria* and *Endothia*

Cryphonectria and *Endothia* include a number of important fungal pathogens of forest trees as well as less important saprophytic species. Well-documented examples of such pathogens are the chestnut blight fungus *C. parasitica* (Anagnostakis 1987, Merkel 1905, Murrill 1906, Roane 1986a), the *Eucalyptus* canker fungus *C. cubensis* (Bruner) Hodges (Boerboom and Maas 1970, Davison and Coates 1991, Florence et al. 1986, Gibson 1981, Hodges and Reis 1974, Hodges et al. 1979, Sharma et al. 1985a, b, Wingfield et al. 1989) and the pin oak blight fungus, *Endothia gyrosa* (Schw.: Fr.) Fr. (Appel and Stipes 1986, Roane et al. 1974, Stipes and Phipps 1971). The most recent addition to *Cryphonectria* is *C.*

eucalypti M. Venter & M.J. Wingf., a fungal pathogen of *Eucalyptus* spp. in Australia and South Africa (Venter et al. 2001, Venter et al. 2002).

Remaining species of *Endothia* and *Cryphonectria* are considered as saprophytes of woody species in various parts of the world (Roane 1986b). These species include *C. radicalis* (Schw.: Fr.) Barr (Shear et al. 1917), *C. havanensis* (Bruner) Barr (Bruner 1916), *C. gyrosa* (Berk. & Br.) Sacc. (type species) (Berkeley and Broome 1875, Shear et al. 1917), *C. longirostris* (Earle) Micales & Stipes (Earle 1909), *C. coccolobii* (Vizioli) Micales & Stipes (Vizioli 1923), *E. viridistroma* Wehmeyer (Wehmeyer 1936) and *E. singularis* (H. & B. Syd.) Shear & Stevens (Shear et al. 1917).

2.6. Taxonomic challenges relating to *Cryphonectria* and *Endothia* and particularly *C. cubensis*.

Knowledge relating to species of *Cryphonectria* and *Endothia* has increased substantially during the course of the past few years. Acceptance of *Cryphonectria* and *Endothia* as valid genera is now supported by morphological and phylogenetic data (Myburg et al. 1999, Venter et al. 2001, Venter et al. 2002). A firm understanding of the taxonomy of these genera is important, as some of the members are serious pathogens of economically valuable tree species. Knowledge of the taxonomic associations of the species residing in *Cryphonectria* and *Endothia* is essential for the development of disease control strategies and effective identification protocols for this group of fungi. One of the most important of

these is *C. cubensis* and this fungus is the primary topic of the thesis for which this review of the literature is presented.

Cryphonectria cubensis is found in mainly tropical and subtropical areas of the world (Boerboom and Maas 1970, Florence et al. 1986, Hodges and Reis 1974, Wingfield et al. 1989) affecting plantation *Eucalyptus* spp. *Eucalyptus* spp. are propagated in these areas as a source of solid wood products and pulp (Turnbul 2000). These products are important commodities and are valuable to the economic well being of the countries producing them. Damage due to disease is thus recognised as an important threat to the economies of countries propagating exotic plantation species such as *Eucalyptus*.

Cryphonectria cubensis was discovered in South Africa in the late 1980's (Wingfield et al. 1989). At that stage, *Cryphonectria* canker was considered to be the same as the disease known in South America and Southeast Asia. However, there were significant differences in the symptoms found in South Africa and the rest of the world. In South Africa, infection by *C. cubensis* leads to the formation of only basal cankers on young (1 to 2 year-old) *Eucalyptus* trees (Wingfield et al. 1989, Wingfield 2003). In contrast, in South America and Southeast Asia, cankers develop at the base of trees but also higher up on the stems of *Eucalyptus* trees (Florence et al. 1986, Hodges et al. 1979, Sharma et al. 1985).

Another difference between *Cryphonectria* canker in South Africa and that in other parts of the world, concerns the states of the fungus found on cankers. In South Africa, cankers are covered with asexual fruiting structures (pycnidia) and sexual fruiting structures (perithecia)

are seldom present (Wingfield et al. 1989). This is different to cankers in South America and Southeast Asia where both pycnidia and/or perithecia are found in abundance on cankers (Van Heerden et al. 1997, Van Heerden and Wingfield 2001, Wingfield et al. 1997). The only time that perithecia have been found on cankers in South Africa was at the time of the discovery of this pathogen in South Africa (Wingfield et al. 1989). These structures were found on a single tree and at the base of a canker below soil level. This difference between the incidence of sexual and asexual fruiting structures on the cankers suggests that sexual reproduction is the common means of reproduction of *C. cubensis* in South America and Southeast Asia while in South Africa, *C. cubensis* reproduces primarily through asexual reproduction.

Studies on *C. cubensis* in South Africa, South America and Southeast Asia were initiated to investigate the differences in disease symptoms in these countries. The first study considering this issue was a DNA based study by Myburg et al. (1999). This phylogenetic study, based on sequence analyses of the ITS region of the rDNA operon, included *C. cubensis* from South America, Southeast Asia and South Africa. The *C. cubensis* isolates from South America and South Africa resolved into one phylogenetic group, separate from those representing *C. cubensis* in Southeast Asia. Based on these results, Myburg et al. (1999) hypothesised that *C. cubensis* in South Africa might have been introduced from South America. These results were puzzling considering the fact that the differences in disease symptoms suggested that the fungus in South Africa was different from the fungus of the same name in South America.

Careful consideration was needed before conclusions could be made regarding the origin of *C. cubensis* in South America, Southeast Asia and South Africa. Myburg et al. (1999) suggested that inconsistencies between the phylogenetic data and the biological data might be the result of a lack of sequence variation in the ITS region of the ribosomal DNA operon. A similar situation has been reported in ITS based phylogenetic studies done on other fungi. For example, studies on *Fusarium* spp. have resulted in researchers largely abandoning the use of ITS sequence data to differentiate between *Fusarium* species, as this region lacked sufficient sequence variation for species in this genus (O'Donnell et al. 1998, O'Donnell et al. 2000, Steenkamp et al. 1999, Steenkamp et al. 2000, Steenkamp et al. 2002). This lack of variation has been resolved by the discovery of more variable regions in the fungal genome such as the β -tubulin (Glass and Donaldson 1995, Donaldson et al. 1995), histone *H3* (Glass and Donaldson 1995, Donaldson et al. 1995, Steenkamp et al. 1999, Steenkamp et al. 2000, Steenkamp et al. 2002), elongation factor EF 1 α (Carbone and Kohn 1999) and calmodulin gene regions (Carbone and Kohn 1999). These regions have been used effectively as a discriminating tool for *Fusarium* spp. (O'Donnell et al. 1998, O'Donnell et al. 2000, Steenkamp et al. 1999, Steenkamp et al. 2000, Steenkamp et al. 2002) and offer a solution to resolve the inconsistencies seen with the intraspecific differentiation of *C. cubensis*.

Despite the fact that the ITS region has been abandoned as a taxonomic delineator in *Fusarium* (O'Donnell et al. 1998, O'Donnell et al. 2000, Steenkamp et al. 1999, Steenkamp et al. 2000, Steenkamp et al. 2002), this region is still used successfully in other fungal systems. An example of this is the taxonomic impact ITS sequence analyses have had on

the Hypocrealean genus, *Trichoderma* Pers. The ITS region has proven useful to characterize strains of *Trichoderma* to sectional and sometimes species level (Lieckfeldt and Seifert 2000). This region has also been successfully implemented in the delineation of new *Trichoderma* species (Samuels et al. 1999) and the description of neotypes of previously known *Trichoderma* spp. (Lieckfeldt et al. 1998). It is, therefore, worthwhile to initially include the ITS region of the rDNA operon in phylogenetic analyses before considering alternative gene regions.

The occurrence of *C. cubensis* in countries on different continents raises the issue of the origin of this fungus. A possible origin for *C. cubensis* was suggested by Hodges et al. (1986) who showed that *E. eugeniae* (Nutman & Roberts) Reid & Booth, associated with die-back on clove species in Indonesia, and *C. cubensis* are conspecific (Hodges et al. 1986). Micales et al. (1987) also confirmed this conspecificity. Hodges et al. (1986) suggested that *C. cubensis* could have had an origin in Indonesia where clove is native and that it has spread via the spice trade since the Middle Ages (Gibbs 1909). The fungus would then have cross-infected *Eucalyptus* spp., which was propagated in close proximity to clove. Although this hypothesis might be feasible to explain the occurrence of *C. cubensis* in Southeast Asian countries, DNA sequence data (Myburg et al. 1999) have suggested that an alternative origin should be considered for *C. cubensis* in South American countries.

An origin for *C. cubensis* in South America has recently been proposed. In a study by Wingfield et al. (2001), phylogenetic analyses, pathogenicity trials and morphological comparisons showed that *C. cubensis* causes a canker disease on native *Tibouchina* spp. in

Colombia. In this study, the authors suggested that *C. cubensis* in South America might have originated from native South American *Tibouchina* spp. and other Melastomataceae, rather than having an Indonesian origin. The idea of a South American origin is also supported by a population study on Brazilian *C. cubensis*, suggesting that *C. cubensis* has been present in South America for a long time (Van Zyl et al. 1998).

The study of Wingfield et al. (2001) reporting the occurrence of *C. cubensis* on *Tibouchina* spp. in Colombia was also the first report of *C. cubensis* occurring on host species that resided in a family (Melastomataceae) other than that of *Eucalyptus* (Myrtaceae). What is important in this regard is the fact that the Melastomataceae and Myrtaceae are members of the Myrtales and are now recognised to be relatively closely related (Conti et al. 1996, Conti et al. 1997, Dahlgren and Thorne 1984). The dual occurrence of *C. cubensis* on native trees and exotic plantation tree species in South America further suggest that *C. cubensis* in this country might have originated from native Melastomataceae. In addition, the possibility that *C. cubensis* has passed from native Melastomataceae to clove species or that the fungus has crossed from clove to Melastomataceae will need to be considered in future studies.

The origin of *C. cubensis* in South Africa has been the subject of considerable study and consideration (Van der Merwe 2000, Van Heerden 1999, Van Heerden et al. 2001). Studies in this thesis and some others associated with it have been conducted to address the question of differences between *C. cubensis* in South Africa, South America and Southeast Asia. Because of these differences and the fact that origins have been proposed to explain *C.*

cubensis in South America and Southeast Asia, an origin for the South African *C. cubensis* needs to be considered.

In a study of Venter et al (2001), although aimed at the molecular characterisation of *E. gyrosa* in South Africa and Australia, preliminary observations were made regarding the taxonomic position of *C. cubensis* in *Cryphonectria*. Based on the phylogenetic results, the authors proposed that *C. cubensis* might be segregated from *Cryphonectria* in future. The suggestion of separating *C. cubensis* from *Cryphonectria* clearly required investigation as Venter et al. (2001) included only two taxa representing *C. cubensis*, and limited species of *Cryphonectria*. Investigations pertaining to this question have been included in chapters of the thesis for which this review is presented.

Venter et al. (2002) demonstrated the value of using morphological together with DNA-based phylogenetic data when assessing taxonomic relationships in *Cryphonectria sensu lato*. These combined data sets provide a means to re-consider taxonomic relationships at DNA level and to re-evaluate morphological characteristics that have been used to describe the fungal groups in *Cryphonectria* and *Endothia*. No comprehensive study has as yet been done that includes *C. cubensis* and all available *Cryphonectria* and *Endothia* species. The information from these data sets will also be useful as a reliable identification tool to distinguish currently known and new species in *Cryphonectria* and related genera.

CONCLUSIONS

The Diaporthales represents an important group of fungi that includes a number of important plant pathogenic fungal genera. Examples of such genera are *Cryphonectria* and *Endothia*. These genera and others in the Diaporthales have been described based on distinct morphological characteristics. Advances in DNA sequence comparisons and the phylogenetic species concept have provided taxonomists, morphologists and plant pathologists with new and objective characteristics to identify fungi, including those in the Diaporthales. Using traditional morphological criteria in combination with DNA sequence data has already been useful and will certainly be most valuable in the future.

This thesis presents a series of studies relating to questions regarding the taxonomy of the Diaporthales and more specifically species of *Cryphonectria* that are tree pathogens. Some of the key questions that will be considered are as follows:

- Is *Cryphonectria cubensis* in South Africa different from the fungus of the same name found in other parts of the world? (**Chapter 2**)
- Is *Cryphonectria cubensis* present on South African *Tibouchina* spp? (**Chapter 3**)
- Is the conspecificity of *C. cubensis* and *E. eugeniae* valid? (**Chapter 4**)
- What are the phylogenetic relationships between all described species of *Cryphonectria* and *Endothia*? (**Chapter 5**)
- What species of *Cryphonectria* are present on Fagaceous hosts in Europe and Asia? (**Chapter 6**)

- What are the taxonomic positions of *Cryphonectria* and *Endothia*, as well as other undescribed taxa closely related to these genera, within the Diaporthales? (**Chapter 7**)

REFERENCES

- Alexopoulos, C.J. and Mims, C.W.** 1978. *Introductory Mycology*. 3rd edition. John Wiley, New York. pp. 632.
- Anagnostakis, S.L.** 1987. Chestnut blight: The classical problem of an introduced pathogen. *Mycologia* **79**: 23-37.
- Appel, D.N. and Stipes, R.J.** 1986. A description of declining and blighted pin oak in eastern Virginia. *Journal of Arboriculture* **12**: 155-158.
- Barr, M.E.** 1978. The Diaporthales in North America with emphasis on *Gnomonia* and its segregates. *Mycological Memoirs* **7**: 1-232.
- Barr, M.E.** 1990. Prodrumus to nonlichenized, pyrenomycetous members of class hymenoascomycetes. *Mycotaxon* **39**: 43-184.
- Berkeley, M.J. and Broome, C.E.** 1875. Enumeration of the Fungi of Ceylon. *Journal of the Linnean Society* **14**: 29-140.
- Biggs, A.R.** 1989. Integrated approach to controlling *Leucostoma* canker of peach in Ontario. *Plant Disease* **73**: 869-874.
- Boerboom, J.H.A. and Maas, P.W.T.** 1970. Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**: 94-99.
- Bruner, S.C.** 1917. Una enfermedad gangrenosa de los eucaliptos. *Estacion Experimental Agronomica, Santiago de las Viegas, Cuba Bolletin* **37**: 1-33.
- Cannon, P.F.** 1988. Proposal to merge the Phyllachorales with the Diaporthales, with a new family structure. *Systema Ascomycetum* **7**: 23-43.

- Cannon, P.F.** 1994. The newly recognized family Magnaporthaceae and its relationships. *Systema Ascomycetum* **13**: 25-42.
- Carbone, I. and Kohn, L.M.** 1999. A method for designing primer sets for speciation in filamentous ascomycetes. *Mycologia* **91**: 553-556.
- Castlebury, L.A., Rossman, A.Y., Jaklitsch, W.J. and Vasilyeva, L.N.** 2002. A preliminary overview of the Diaporthales based on large subunit nuclear DNA sequences. *Mycologia* **94**: 1017-1031.
- Chen, B., Chen, C-H., Bowman, B.H. and Nuss, D.L.** 1996. Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogenic fungi phylogenetically related to *Cryphonectria parasitica*. *Phytopathology* **86**: 301-310.
- Conti, E., Litt, A. and Sytsma, K.** 1996. Circumscription of Myrtales and their relationships with other rosids: Evidence from RBCL sequence data. *American Journal of Botany* **83**: 221-233.
- Conti, E., Litt, A., Wilson, P.G., Graham, S.A., Briggs, B.G., Johnson, L.A.S., and Sytsma, K.** 1997. Interfamilial relationships in Myrtales: Molecular phylogeny and patterns of morphological evolution. *Systematic Botany* **22**: 629-647.
- Dahlgren, R. and Thorne, R.F.** 1984. The order Myrtales: Circumscription, variation and relationships. *Annals of the Missouri Botanical Garden* **71**: 633-699.
- Davison, E.M. and Coates, D.J.** 1991. Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**: 157-160.

- Donaldson, G.C., Ball, L.A., Axelrood, P.E. and Glass, N.L.** 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating *Fusarium* species associated with conifers. *Applied and Environmental Microbiology* **61**: 1331-1340.
- Earle, F. S.** 1909. Some fungi from Porto Rico. *Muehlenbergia* **1**: 10-17.
- Eriksson, O.E., Baral, H-O., Currah, R.S., Hansen, K., Kurtzman, C.P., Rambold, G. and Laess, E.T.** 2001. Outline of the Ascomycota – 2001. Myconet. <http://www.umu.se/myconet/Myconet.html>
- Farr, D.F., Castlebury, L.A. and Pardo-Schultheiss, R.A.** 1999. *Phomopsis amygdali* causes peach shoot blight of cultivated peach trees in the southern United States. *Mycologia* **91**: 1008-1015.
- Farr, D.F., Castlebury, L.A., Rossman, A.Y. and Erincik, O.** 2001. *Greeneria uvicola*, cause of bitter rot of grapes, belongs in the Diaporthales. *Sydowia* **53**: 185-199.
- Farr, D.F., Castlebury, L.A., Rossman, A.Y.** 2002. Morphological and molecular characterisation of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States. *Mycologia* **94**: 494-504.
- Florence, E.J.M., Sharma, J.K. and Mohanan, C.** 1986. A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forestry Research Institute Scientific Paper* **66**: 384-387.
- Fries, E.M.** 1849. *Summa Vegetabilium Scandinaviae*. Sectio posterior. Holmiae & Lipsiae, Uppsala. pp. 385-386.
- Gibson, I.A.S.** 1981. A canker disease of *Eucalyptus* new to Africa. *FAO, Forest Genetic Resources Information* **10**: 23-24.

- Gibbs, W.M.** 1909. Spices and how to know them. Matthews-Northup Works, Buffalo, New York. pp. 179.
- Glass, N.L. and Donaldson, G.C.** 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied Environmental Microbiology* **61**: 1323-1330.
- Griffin, G.J.** 1986. Chestnut blight and its control. *Horticulture Review* **8**: 291-336.
- Grove, W.B.** 1935. British stem and leaf fungi (Coelomycetes). Vol. 1. Cambridge, Massachusetts. Cambridge University Press. pp. 488.
- Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.M.** 1995. Ainsworth & Bisby's Dictionary of the fungi. 8th edition. CAB International, Egham, UK. pp. 616.
- Hodges, C.S. and Reis, M.S.** 1974. Identificação do fungo causador de cancro de *Eucalyptus* spp. no Brasil. *Brazil Florestal* **5**: 19.
- Hodges, C.S., Geary, T.F. and Cordell, C.E.** 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. *Plant Disease Reporter* **63**: 216-220.
- Hodges, C.S., Alfenas, A.C. and Cordell, C.E.** 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**: 343-350.
- Kobayashi, T.** 1970. Taxonomic studies of Japanese Diaporthaceae with special reference to their life histories. *Bulletin of the Government Forest Experiment Station* **226**: 132-147.

- Lieckfeldt, E., Samuels, G.J., Borner, T. and Gams, W. 1998. *Trichoderma koningii*, neotypification and *Hypocrea* teleomorph. *Canadian Journal of Botany* **76**: 1507-1522.
- Lieckfeldt, E. and Seifert, K.A. 2000. An evaluation of the use of ITS sequences in the taxonomy of the *Hypocreales*. *Studies in Mycology* **45**: 35-44.
- Luttrell, E.S. 1951. Taxonomy of the pyrenomycetes. *University of Missouri Studies* **24**: 1-120.
- Merkel, H.W. 1905. A deadly fungus on the American chestnut. New York Zoological Society, 10th Annual Report, pp. 97-103.
- Micales, J.A. and Stipes, R.J. 1986. The differentiation of *Endothia* and *Cryphonectria* species by exposure to selected fungitoxicants. *Mycotaxon* **26**: 99-117.
- Micales, J.A. and Stipes, R.J. 1987. A re-examination of the fungal genera *Cryphonectria* and *Endothia*. *Phytopathology* **77**: 650-654.
- Micales, J.A., Stipes, R.J. and Bonde M.R. 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. *Mycologia* **79**: 707-720.
- Mostert, L., Crous, P.W., Kang, J-C. and Phillips, A.J.L. 2001. Species of *Phomopsis* and a *Libertella* sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterization. *Mycologia* **93**: 146-167.
- Murrill, W.A. 1906. A serious chestnut disease. *Journal of the New York Botanical Garden* **7**: 143-153.
- Myburg, H., Wingfield, B.D. and Wingfield, M.J. 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**: 243-250.

- O'Donnell, K., Cigelnik, E. and Nirenberg, H.I.** 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 465-493.
- O'Donnell, K., Nirenberg, H.I., Aoki, T. and Cigelnik, E.** 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. *Mycoscience* **41**: 61-78.
- Rehner, S.A. and Uecker, F.A.** 1994. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis*. *Canadian Journal of Botany* **72**: 1666-1674.
- Roane, M.K, Stipes, R.J., Phipps, P.M. and Miller, O.K. Jr.** 1974. *Endothia gyrosa*, causal pathogen of pin oak blight. *Mycologia* **66**: 1042-1047.
- Roane, M.K. and Stipes, R.J.** 1978. Pigments in the fungal genus *Endothia*. *Virginia Journal of Science* **29**:137-141.
- Roane, M.K.** 1986a. Taxonomy of the genus *Endothia*. In: Roane, M.K., Griffin, G.J. and Elkins, J.R., eds. Chestnut blight, other *Endothia* diseases, and the genus *Endothia*. APS Press, St. Paul, Minnesota, USA. pp. 28-39.
- Roane, M.K.** 1986b. Other diseases caused by *Endothia* species. In: Roane, M.K., Griffin, G.J. and Elkins, J.R., eds. Chestnut blight, other *Endothia* diseases, and the genus *Endothia*. APS Press, St. Paul, Minnesota, USA. p. 27.
- Saccardo, P.A.** 1883. *Sylloge fungorum*, vol. II. p. 507. Patavii.
- Saccardo, P.A.** 1905. *Sylloge Fungorum* **42**: 783-781. Patavii
- Samuels, G.J., Barr, M.E. and Lowen, R.** 1993. Revision of *Shizoparme* (Diaporthales, Melanconidaceae). *Mycotaxon* **46**: 459-483.

- Samuels, G.J., Lieckfeldt, E. and Nirenberg, H.I.** 1999. *Trichoderma asperellum*, a new species with warted conidia, and redescription of *T. viride*. *Sydowia* **51**: 71-88.
- Sharma, J.K., Mohanan, C. and Florence, E.J.M.** 1985a. Disease survey in nurseries and plantations of forest tree species grown in Kerala. KFRI Research Report 36. Kerala Forest Research Institute, Kerala, India.
- Sharma, J.K., Mohanan, C. and Florence, E.J.M.** 1985b. Occurrence of *Cryphonectria* canker disease of *Eucalyptus* in Kerala, India. *Annual Applied Biology* **106**: 265-276.
- Shear, C.L., Stevens, N.E. and Tiller, R.J.** 1917. *Endothia parasitica* and related species. *United States Department of Agriculture Bulletin* **380**: 1-82.
- Spatafora, J.W. and Blackwell, M.** 1993. Molecular systematics of unitunicate perithecial ascomycetes: the *Clavicipitales-Hypocreales* connection. *Mycologia* **85**: 912-922.
- Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J., and Marasas, W.F.O.** 1999. Differentiation of *Fusarium subglutinans* f.sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**: 3401-2406.
- Steenkamp, E.T., Britz, H., Coutinho, T.A., Wingfield, B.D., Marasas, W.F.O. and Wingfield, M.J.** 2000. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. *Molecular Plant Pathology* **1**: 187-193.
- Steenkamp, E.T., Wingfield, B.D., Desjardin, A.E., Marasas, W.F.O. and Wingfield, M.J.** 2002. Cryptic speciation in *Fusarium subglutinans*. *Mycologia* **94**: 1032-1043.

- Stipes, R.J. and Phipps, P.M.** 1971. A species of *Endothia* associated with a canker disease of pin oak (*Quercus palustris*) in Virginia. *Plant Disease Reporter* **55**: 467-469.
- Stipes, R.J. and Ratliff, J.L.** 1973. Effect of temperature on linear growth of *Endothia gyrosa* and *E. parasitica*. *Virginia Journal of Science* **24**: 136 (abstract).
- Turnbul, J.W.** 2000. Economic and social importance of eucalypts. In: Keane, P.J., Kile, G.A., Podger, F.D. and Brown, B.N., eds. Diseases and pathogens of eucalypts. CSIRO Publishing, Australia. pp. 1-9.
- Van der Merwe, N.A.** 2000. Molecular phylogeny and population biology studies on the *Eucalyptus* canker pathogen *Cryphonectria cubensis*. M. Sc thesis. Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.
- Van Heerden, S.W., Wingfield, M.J., Coutinho, T., Van Zyl, L.M. and Wright, J.A.** 1997. Diversity of *Cryphonectria cubensis* isolates in Venezuela and Indonesia. Proceedings of IUFRO Conference on Silviculture and Improvement of Eucalypts, Salvador, Brasil, August 24-29, 1997. Embrapa, Colombia. pp. 142-146.
- Van Heerden, S.W.** 1999. Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. M.Sc. thesis. Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.
- Van Heerden, S.W. and Wingfield, M.J.** 2001. Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycological Research* **105**: 94-99.
- Van Zyl, L.M., Wingfield, M.J., Alfenas, A.C. and Crous, P.W.** 1998. Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**: 41-47.

- Vasilyeva, L.N.** 1998. Pyrenomycetidae et Laculoascomycetidae. Plantae non Vasculares, Fungi et Bryopsidae. Orientis extremi Rossica. *Fungi* **4**: 1-418 (in Russian).
- Venter, M., Wingfield, M.J., Coutinho, T.A. and Wingfield, B.D.** 2001. Molecular characterization of *Endothia gyrosa* isolates from *Eucalyptus* in South Africa and Australia. *Plant Pathology* **50**: 211-217.
- Venter, M., Myburg, H., Wingfield, B.D., Coutinho, T.A. and Wingfield, M.J.** 2002. A new species of *Cryphonectria* from South Africa and Australia, pathogenic on *Eucalyptus*. *Sydowia* **54**: 98-117.
- Vizioli, J.** 1923. Some Pyrenomycetes of Bermuda. *Mycologia* **15**: 107-119.
- Walker, J., Old, K.M. and Murray, D.I.L.** 1985. *Endothia gyrosa* on *Eucalyptus* in Australia with notes on some other species of *Endothia* and *Cryphonectria*. *Mycotaxon* **23**: 353-370.
- Wehmeyer, L.E.** 1936. Cultural studies of three new pyrenomycetes. *Mycologia* **28**: 23-46.
- Wehmeyer, L.E.** 1975. The pyrenomycetous fungi. *Mycological Memoirs* **6**: 1-250.
- Williamson, P.M., Highet, A.S., Gams, W., Sivasithamparam, K. and Cowling, W.A.** 1994. *Diaporthe toxica* sp. nov., the cause of lupinosis in sheep. *Mycological Research* **98**: 1364-1368.
- Wingfield, M.J., Swart, W.J. and Ahear, B.** 1989. First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**: 311-313.
- Wingfield, M.J., van Zyl, L.M., van Heerden, S.W., Myburg, H. and Wingfield, B.D.** 1997. Virulence and genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In: Lieutier, F., Mattson, W.J. and Wagner, M.R., eds.

Physiology and Genetics of Tree-Phytophage Interactions. Les Colloques, N°90,INRA Editions, Gujan. pp. 163-172.

Wingfield, M.J., Rodas, C., Myburg, H., Venter, M., Wright, J. and Wingfield, B.D.

2001. Cryphonectria canker on *Tibouchina* in Colombia. *Forest Pathology* **31**: 297-306.

Wingfield, M.J. 2003. Increasing threat of diseases to exotic plantation forests in the

Southern Hemisphere: lessons from Cryphonectria canker. *Australasian Plant Pathology* **32**: 133-139.

Zhang, N. and Blackwell, M. 2001. Molecular phylogeny of dogwood anthracnose fungus

(*Discula destructiva*) and the Diaporthales. *Mycologia* **93**: 355-365.

CHAPTER 2

β -Tubulin and histone *H3* gene sequences distinguish *Cryphonectria* *cubensis* from South Africa, Asia and South America.

Henrietta Myburg¹, Marieka Venter², Brenda D. Wingfield¹, Michael J.
Wingfield¹

¹Department of Genetics, ²Department of Microbiology and Plant Pathology, Forestry
and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002,
South Africa.

Published: Myburg et al. (2002). *Canadian Journal of Botany* 80: 590-596.

**β -TUBULIN AND HISTONE *H3* GENE SEQUENCES
DISTINGUISH *CRYPHONECTRIA CUBENSIS* FROM SOUTH
AFRICA, ASIA AND SOUTH AMERICA.**

ABSTRACT

Cryphonectria cubensis is the causal agent of an important stem canker disease of *Eucalyptus*. Previous phylogenetic studies based on sequence data have shown that *C. cubensis* is distinct from other species of *Cryphonectria*, and that *C. cubensis* isolates reside in two distinct groups, consistent with geographical origin. Thus, isolates of *C. cubensis* from South America and South Africa grouped together but apart from those originating from South East Asia and Australia. These results were in contrast to the symptoms of *Cryphonectria* canker in South Africa, which are different to those observed elsewhere in the world. The aim of this study was to use more variable regions of the fungal genome to test whether South African isolates of *C. cubensis* are genetically distinct from those from other parts of the world. For this comparison, β -tubulin and histone *H3* gene sequences were used. Specimens from South America, Southeast Asia, Australia and South Africa were also compared morphologically. The phylogram emerging from the analysis indicated that South American and Southeast Asian/Australian isolates resided in two, well resolved but closely related clades. However, isolates from South Africa were distinct from other groups. This is

consistent with ecological aspects of the South African fungus, although no obvious morphological differences between the fungi from the various regions could be found. Our results suggest that the South African fungus represents a species distinct from *C. cubensis* occurring elsewhere in the world.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is an important stem canker pathogen of *Eucalyptus* trees in plantations. It causes a disease known as Cryphonectria canker that is common in many tropical and sub-tropical parts of the world where *Eucalyptus* spp. are propagated commercially (Boerboom and Maas 1970, Davison and Coates 1991, Florence et al. 1986, Gibson 1981, Hodges and Reis 1974, Hodges et al. 1979, Sharma et al. 1985a, b, Wingfield et al. 1989). *Eucalyptus* species are primarily cultivated as a source of fibre to produce pulp and to a lesser extent, solid wood products. In South Africa, *Eucalyptus* species are widely grown in plantations and Cryphonectria canker is of major concern to local forestry companies (Wingfield et al. 1989).

Cryphonectria cubensis was first observed in South Africa in 1988 (Wingfield et al. 1989). Surveys for this fungus in the late 1970's did not detect the pathogen, and it thus appeared that the pathogen had been introduced recently into the country. Consistent with this view, Van Heerden and Wingfield (2001) showed that *C. cubensis* in South Africa is represented by a relatively low number of genets and thus, a narrow genetic base. This is in contrast to

the high level of genetic variation that is found in *C. cubensis* populations elsewhere (Van Heerden et al. 1997).

Cryphonectria canker in South Africa is different from the disease occurring elsewhere in the world. While cankers in South America and Southeast Asia are generally at various heights on stems, those in South Africa form exclusively at the bases of trees (Conradie et al. 1990). Another unusual aspect of the disease in South Africa is that only asexual fruiting structures are found on cankers. This is in contrast to those in Southeast Asia and South America where sexual and/or asexual fruiting bodies are the predominant structures present on cankers (Van Heerden and Wingfield 2001).

In a previous phylogenetic study, *C. cubensis* isolates from various parts of the world were compared using ribosomal RNA gene sequence data (Myburg et al. 1999). Results showed that isolates from South Africa and South America group together and separately from those originating in Southeast Asia and Australia. This result was inconsistent with pathological data, which suggested that *C. cubensis* from South Africa might be different from the fungus occurring elsewhere in the world.

Previous studies have shown that β -tubulin and histone *H3* gene regions are polymorphic (Donaldson et al. 1995, Glass and Donaldson 1995, Steenkamp et al. 1999, Steenkamp et al. 2000) and useful in phylogenetic studies on fungi. The aim of this study was, therefore, to compare isolates of *C. cubensis* from South Africa with those from other parts of the world

using DNA sequences likely to have higher resolution than the ITS region of the ribosomal RNA operon. Morphological comparisons of asexual fruiting structures were also made to determine whether specimens from South Africa could be distinguished from those originating in Southeast Asia and South America.

MATERIALS AND METHODS

Fungal isolates

Isolates used in this study included *Cryphonectria parasitica* (Murr.) Barr, the causal agent of chestnut blight (Elliston, 1981), and *C. cubensis* isolates from Southeast Asia, Australia, South America and South Africa (Table 1). *Diaporthe ambigua* Nits., a canker pathogen of stone and pome fruit trees (Smit et al. 1996, Smit et al. 1997) was included as outgroup. All isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA isolation and amplification

DNA isolations were performed as described by Myburg et al. (1999). PCR amplifications were performed as described by Glass and Donaldson (1995). Amplification reactions of the β -tubulin gene and the histone *H3* gene were done using primer pairs Bt1a/1b, Bt2a/2b and H3 1a/1b respectively. Each 50 μ l amplification reaction consisted of the following: 1mM dNTPs (0.25mM of each), 1 \times reaction buffer (supplied with the enzyme), 2.5mM MgCl₂,

0.1 μ M of each primer, 5 units of Expand *Taq* polymerase (Roche Biochemicals, Mannheim, Germany) and DNA template. Amplifications were done on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied BioSystems, Inc., Foster City, California). Amplification of the histone *H3* gene was done using the following reaction conditions: an initial denaturing step at 94 °C (1 min), followed by 30 cycles of denaturing at 94 °C (1 min), annealing at 68 °C (1 min), and elongation at 72 °C (1 min). Amplification of the Bt1a/1b region of the β -tubulin gene was done using the same reaction conditions except that the annealing temperature was adjusted to 60 °C. Amplification of the Bt2a/2b region was done over a range of annealing temperatures (55 °C-68 °C) because the Bt2a/2b primers annealed at different temperatures for the respective isolates used in this study. PCR products were visualised on 1% agarose (Promega, Madison, Wisconsin) gels containing ethidium bromide.

DNA sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The PCR products were sequenced in both directions using the same primers used in the amplification reactions. Sequencing reactions were conducted using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom). DNA sequences were determined using an ABI PRISM 377™ automated DNA sequencer.

Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) was used to translate the β -tubulin and histone *H3* DNA sequences into putative amino-acid sequences. The amino-acid sequence of the respective β -tubulin gene was compared with β -tubulin amino-acid sequence of *Neurospora crassa* Shear and B.O. Dodge (Genbank accession no. M13630, Orbach et al. 1986). The amino-acid sequence of the histone *H3* gene was compared to that of *N. crassa* (Genbank accession no. CAA25761, Woudt et al. 1983, Glass and Donaldson 1995). Conserved exon and variable intron sites were determined for *C. cubensis*, *C. parasitica* and *D. ambigua*. DNA sequences were aligned using CLUSTAL W (Thompson et al. 1997) and the alignment was checked manually.

Phylogenetic analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford, 1998). A partition-homogeneity test was performed on a combined data set including β -tubulin and histone *H3* gene sequences. Analyses were done using heuristic searches with no branch swapping and MULTREES (saving all optimal trees) options effective. Gaps were treated as fifth characters (NEWSTATE) in the heuristic searches. The confidence levels of the branching points were determined by a bootstrap analysis (1000 replications). *Diaporthe ambigua* was used as outgroup taxon to root trees. Sequences were deposited in Genbank and accession numbers are listed in Table 1.

Morphological comparisons

Anamorph structures of *C. cubensis* occurring on *E. grandis* bark from South Africa, were examined microscopically. These specimens, PREM 49377, PREM 49378, PREM 49379 (Table 2), were those deposited as part of the study by Wingfield et al. (1989). Specimens of bark with pycnidia from Colombia, Mexico, Vietnam, Indonesia and Hong Kong, were also examined (Table 2). Pycnidia were sectioned in length using a Leitz 1310K freezing microtome and KRYOMAT 1700 generator, following a method described by Venter et al. (2001a). Sections of pycnidia, as well as other anamorph structures, were subsequently examined using a Zeiss Axioskop compound microscope. Measurements were presented as (min)-(x bar -sd) - (x bar +sd)(-max).

RESULTS

DNA amplification and sequencing

The β -tubulin gene fragments (1a/1b and 2a/2b) were both approximately 550 bp (base pairs) in size. Amplification of the histone *H3* gene generated a 550 bp fragment. Manual alignment of the β -tubulin gene and histone *H3* gene sequence data resulted in a total of 1365 characters (Appendix 1). Positions of introns and exons of the β -tubulin and histone *H3* genes amplified in this study, were the same as those of *N. crassa* (Genbank accession no. M13630 and CAA25761 respectively). The coding regions were highly conserved with

sequence variation limited to the third codon position. No insertions or deletions of coding regions were observed in the gene regions considered in this study.

The partition-homogeneity test generated a P-value of 0.01. This indicated that the β -tubulin gene and the histone *H3* gene sequence data sets could be combined into one phylogenetic analysis. The aligned β -tubulin and histone *H3* gene sequences were, therefore, analysed as one data set. For each taxon 1365 characters were included in the heuristic search. Among these, 966 characters were constant and 136 characters parsimony-uninformative. One most parsimonious tree (length of tree = 499 steps, consistency index (CI) = 0.9579 and retention index (RI) = 0.9651) was produced from 263 parsimony-informative characters (Fig. 1).

The phylogenetic tree (Fig. 1) generated from the combined β -tubulin and histone *H3* gene sequence data showed that all of the *C. cubensis* isolates grouped within one clade (bootstrap = 100%), separately from the *C. parasitica* isolates and the outgroup, *D. ambigua*. Within the greater *C. cubensis* clade, three distinct groups were obvious. These included a South American (bootstrap = 90%), a Southeast Asian/Australian (bootstrap = 80%) and a South African sub-clade (bootstrap = 95%). The South American and Southeast Asian/Australian clades were more closely related to each other (bootstrap value = 55%) than they were to the South African clade (Fig. 1).

Morphological comparisons

Pycnidia from the South African specimens differed slightly in morphology from structures collected in South America and Southeast Asia. Pycnidia from South Africa had an obvious eustromatic appearance where the layer of cells giving rise to the conidiophores was convoluted (Fig. 2a) and tissues in the pycnidial walls were prosenchymatous. Furthermore, sections through the edge of the pycnidia often revealed more than one cavity (Fig. 2b), while those near the middle revealed a single cavity (Fig. 2a). In contrast, pycnidial cavities from South American and Southeast Asian collections (Fig. 2c, 2d) were seldom as strongly convoluted, and longitudinal sections rarely revealed more than one pycnidial cavity. Where additional conidial cavities were observed, these were usually small (Fig. 2d).

Conidia from specimens representing different geographical areas were similar in size and shape (Fig. 2e-h). Conidia from Southeast Asian were 3-4(-4.5) x 1-1.5 μm (Fig. 2e), South American conidia were 3-4(-4.5) x 1-1.5 μm (Fig. 2f), and conidia from South Africa were (3-)3.5-4.5(-5) x 1-1.5 μm (Fig. 2g). Although rarely so, some conidia from the South African collections had papillate apices (Fig. 2h). These have not previously been noted in *C. cubensis* and were not found in the specimens from other parts of the world.

DISCUSSION

Using comparisons of histone *H3* and β -tubulin gene sequences, we have been able to show conclusively that *C. cubensis* from South Africa is phylogenetically distinct from the fungus

of the same name occurring in Southeast Asia/Australia and South America. This finding is consistent with the fact that the fungus in South Africa is associated with different symptoms from those found in the latter areas. Our results suggest that the South African fungus has an origin different from that of *C. cubensis* from other parts of the world.

In a previous study, Myburg et al. (1999) examined the relationships between *C. cubensis* and other *Cryphonectria* spp. as well as between *C. cubensis* from different hosts and areas. Results of the present study confirm results from Myburg et al. (1999) that the fungus from Southeast Asia/Australia and South America forms two distinct, yet closely related groups. However, ITS sequences presented by Myburg et al. (1999), indicated that South African isolates were most closely related to the fungus from South America. However, the sequences from the ITS1/ITS2 regions are insufficiently variable to resolve the taxonomic questions relating to *C. cubensis sensu lato*.

Cryphonectria cubensis cankers in South America and Southeast Asia are typically covered with perithecia. Pycnidia are present but rare (M.J. Wingfield, data not shown). This is in contrast to the situation in South Africa where structures thought to be perithecia of *C. cubensis* have been observed only once (Wingfield et al. 1989) and pycnidia are the dominant structures on cankers (Van Heerden and Wingfield 2001). These differences, and the unique nature of cankers in South Africa, are consistent with findings in this study, showing that the South African fungus is unique.

Cankers caused by *C. cubensis* in Australia have only been reported once (Davison and Coates 1991) and are different from cankers observed in Southeast Asia. These cankers on *E. marginata* Sm. were on the roots and only pycnidia were observed (Davison and Coates 1991). This could indicate that the Australian fungus is also different. Molecular data reported in the present study, however, clearly show that the Australian fungus is part of the Southeast Asian subclade.

The overall morphology of pycnidia found on cankers in South Africa, Southeast Asia and South America is very similar. In this study, however, slight differences between pycnidia from South Africa and those from elsewhere in the world have been detected. Small differences were also noted in conidial shape, although the papillate apices of a small number of South African conidia are an insufficiently consistent feature to note with any confidence.

Previous descriptions of *C. cubensis* have treated the anamorph fruiting structures as pycnidia (Bruner 1917, Hodges et al. 1976, Hodges 1980). This is possibly due to the fact that the anamorph has the typical shape and appearance of a pycnidium when viewed on the bark. However, when sectioned, the anamorph structures closely resemble convoluted eustromata (Hawksworth et al. 1996). We suggest that the term eustroma should be used in future to describe the anamorph structure of *C. cubensis*.

Although not an objective of the present study, our results confirm those of previous studies (Myburg et al. 1999, Venter et al. 2001b) that *C. cubensis* is very distantly related to *C. parasitica*. This is also consistent with the fact that the two fungi can be distinguished by a number of clear morphological characteristics. The most obvious of these are that *C. cubensis* has loosely aggregated perithecia embedded in a weakly developed basal stroma. This is different from *C. parasitica* where perithecial bases are embedded in a well developed stroma. These differences have led Venter et al. (2001b) to conclude that the fungi probably reside in distinct genera. This conclusion is supported by SSU and LSU sequence data, where *C. cubensis* (= *E. eugeniae*) does not group in the same clade as *C. parasitica* (Zhang and Blackwell 2001). Results of the present study have also shown that β -tubulin and histone *H3* gene sequence data should be useful in future investigations aimed at providing better resolution to differentiate between various species of *Cryphonectria* and related fungi.

Substantial sequence data are now available to support the view that *C. cubensis* in South Africa is different from the fungus with the same name occurring elsewhere in the world. Available data also suggest that the South African fungus and *C. cubensis* elsewhere in the world have different origins. One commonly held hypothesis is that *C. cubensis* originated from native clove trees (*Syzygium aromaticum* (L.) Merr. and Perry, Myrtaceae) in Indonesia (Hodges et al. 1986). There is equally strong evidence to suggest that *C. cubensis* originated on *Tibouchina* trees (Melastomataceae) in South America (Wingfield et al. 2001). This raises the intriguing question of where the South African fungus might have originated.

Based on results of this study, the origin of the South African fungus is likely to be different from the origin of the fungus in South America, Southeast Asia and Australia. Resolution of this question is likely to emerge from collections of fungi similar to *C. cubensis* on native Myrtaceae and Melastomataceae, both in Africa and elsewhere in the world.

ACKNOWLEDGEMENTS

We are grateful to Dr. J.C. David, CABI Bioscience and to various colleagues listed in Table 2 for loans of specimens and providing cultures used in this study. We also thank the Biosystematics Division of the Agricultural Research Council, Pretoria for providing the microtome facilities and anonymous reviewers who provided important suggestions to improved the original manuscript. This study was supported by grants from the National Research Foundation (NRF), members of the Tree Pathology Co-operative Programme (TPCP), and the THRIP support programme of the Department of Trade and Industry, South Africa.

REFERENCES

- Boerboom, J.H.A. and Maas, P.W.T.** 1970. Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**: 94-99.
- Bruner, S.C.** 1917. Una enfermedad gangrenosa de los eucaliptos. *Estacion Experimental Agronomica Bulletin* **37**: 1-33.
- Conradie, E., Swart, W.J. and Wingfield, M.J.** 1990. Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**: 43-49.
- Davison, E.M. and Coates, D.J.** 1991. Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**: 157-160.
- Donaldson, G.C., Ball, L.A., Axelrood, P.E. and Glass, N.L.** 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating *Fusarium* species associated with conifers. *Applied and Environmental Microbiology* **61**: 1331-1340.
- Elliston, J.E.** 1981. Hypovirulence and chestnut blight research: fighting disease with disease. *Journal of Forestry* **79**: 657-660.
- Florence, E.J.M., Sharma, J.K. and Mohanan, C.** 1986. A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**: 384-387.

- Gibson, I.A.S.** 1981. A canker disease of *Eucalyptus* new to Africa. *FAO, Forest Genetic Resources Information* **10**: 23-24.
- Glass, N.L. and Donaldson, G.C.** 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied Environmental Microbiology* **61**: 1323-1330.
- Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N.** 1995. Ainsworth and Bisby's Dictionary of the Fungi, 8th edition. Oxford University Press, Oxford, U.K.
- Hodges, C.S. and Reis, M.S.** 1974. Identificação do fungo causador de cancro de *Eucalyptus* spp. no Brasil. *Brazil Florestal* **5**: 19.
- Hodges, C.S., Reis, M.S., Ferreira, F.A. and Henfling, J.D.M.** 1976. O cancro do eucalipto causado por *Diaporthe cubensis*. *Fitopatologia Brasileira* **1**: 129-162.
- Hodges, C.S., Geary, T.F. and Cordell, C.E.** 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. *Plant Disease Reporter* **63**: 216-220.
- Hodges, C.S.** 1980. The taxonomy of *Diaporthe cubensis*. *Mycologia* **72**: 542-548.
- Hodges, C.S., Alfenas, A.C. and Cordell, C.E.** 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**: 334-350.
- Myburg, H., Wingfield, B.D. and Wingfield, M.J.** 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**: 243-250.
- Orbach, M.J., Porro, E.B. and Yanofsky, C.** 1986. Cloning and characterisation of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as dominant marker. *Molecular and Cellular Biology* **6**: 2452-2461.

- Sharma, J.K., Mohanan, C. and Florence, E.J.M.** 1985a. Disease survey in nurseries and plantations of forest tree species grown in Kerala. KFRI Research Report 36. Kerala Forest Research Institute, Kerala, India.
- Sharma, J.K., Mohanan, C. and Florence, E.J.M.** 1985b. Occurrence of *Cryphonectria* canker disease of *Eucalyptus* in Kerala, India. *Annals of Applied Biology* **106**: 265-276.
- Smit, W.A., Viljoen, C.D., Wingfield, B.D., Wingfield, M.J. and Calitz, F.J.** 1996. A new canker disease of apple, pear, and plum rootstocks caused by *Diaporthe ambigua* in South Africa. *Plant Disease* **80**: 1331-1335.
- Smit, W.A., Wingfield, B.D. and Wingfield, M.J.** 1997. Vegetative incompatibility in *Diaporthe ambigua*. *Plant Pathology* **46**: 366-372.
- Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J. and Marasas, W.F.O.** 1999. Differentiation of *Fusarium subglutinans* f.sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**: 3401-2406.
- Steenkamp, E.T., Britz, H., Coutinho, T.A., Wingfield, B.D., Marasas, W.F.O. and Wingfield, M.J.** 2000. Molecular characterization of *Fusarium subglutinans* associated with mango malformation. *Molecular Plant Pathology* **1**: 187-193.
- Swofford, D.L.** 1998. PAUP. Phylogenetic Analysis Using Parsimony, version 4.0b1. Sinauer Associates, Sunderland, Massachusetts.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G.** 1997. The CLUSTAL W windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

- Van Heerden, S.W., Wingfield, M.J., Coutinho, T., Van Zyl, L.M. and Wright, J.A.** 1997. Diversity of *Cryphonectria cubensis* isolates in Venezuela and Indonesia. Proceedings of IUFRO Conference on Silviculture and Improvement of Eucalypts, Salvador, Brasil, August 24-29, 1997. Embrapa, Colombia. pp. 142-146.
- Van Heerden, S.W. and Wingfield, M.J.** 2001. Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycological Research* **105**: 94-99.
- Venter, M., Myburg, H., Wingfield, B.D., Coutinho, T.A. and Wingfield, M.J.** 2001a. A new species of *Cryphonectria* from South Africa and Australia, pathogenic on *Eucalyptus*. *Sydowia* **54**: 98-119.
- Venter, M., Wingfield, M.J., Coutinho, T.A. and Wingfield, B.D.** 2001b. Molecular characterization of *Endothia gyrosa* isolates from *Eucalyptus* in South Africa and Australia. *Plant Pathology* **50**: 211-217.
- Wingfield, M.J., Swart, W.J. and Abear, B.** 1989. First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**: 311-313.
- Wingfield, M.J., Rodas, C., Wright, J., Myburg, H., Venter, M. and Wingfield, B.D.** 2001. First report of *Cryphonectria* canker on *Tibouchina* in Colombia. *Forest Pathology* **31**: 297-306.
- Woudt, L.P., Pastink, A., Kempers-Veenstra, E., Jansen, A.E.M., Mager, W.H. and Planta, R.J.** 1983. The genes encoding histone *H3* and *H4* in *Neurospora crassa* are unique and contain intervening sequences. *Nucleic Acids Research* **14**: 5347-5360.

Zhang, N. and Blackwell, M. 2001. Molecular phylogeny of dogwood anthracnose fungus (*Discula destructiva*) and the Diaporthales. *Mycologia* **93**: 355-365.

Table 1. Taxa used in this study.

Species	Culture no*	Origin	Genbank accession number
<i>Cryphonectria cubensis</i>	CMW 2113	South Africa	AF273067 [¶] , AF273462 [¶] , AF281805 [#]
<i>Cryphonectria cubensis</i>	CMW 62	South Africa	AF273063 [¶] , AF273458 [¶] , AF281806 [#]
<i>Cryphonectria cubensis</i>	CMW 8755	South Africa	AF273064 [¶] , AF273459 [¶] , AF281807 [#]
<i>Cryphonectria cubensis</i>	CMW 8757	Venezuela	AF273069 [¶] , AF273464 [¶] , AF281810 [#]
<i>Cryphonectria cubensis</i>	CMW 8758	Venezuela	AF273068 [¶] , AF273463 [¶] , AF281243 [#]
<i>Cryphonectria cubensis</i>	CMW 1853	Brazil	AF273070 [¶] , AF273465 [¶] , AF281808 [#]
<i>Cryphonectria cubensis</i>	CMW 8756	Indonesia	AF273077 [¶] , AF375606 [¶] , AF285165 [#]
<i>Cryphonectria cubensis</i>	CMW 1840	China	AF273071 [¶] , AF273466 [¶] , AF281814 [#]
<i>Cryphonectria cubensis</i>	CMW 2632	Australia	AF273078 [¶] , AF375607 [¶] , AF466697 [¶]
<i>Cryphonectria parasitica</i>	CMW 1652	USA	AF273468 [§] , AF273075 [§] , AF281802 [#]
<i>Cryphonectria parasitica</i>	CMW 7047	USA	AF273469 [§] , AF273073 [§] , AF281803 [#]
<i>Cryphonectria parasitica</i>	CMW 7048	USA	AF273470 [§] , AF273076 [§] , AF281804 [#]
<i>Diaporthe ambigua</i>	CMW 2498	Netherlands	AF273471 [§] , AF273072 [§] , AF281815 [#]

* Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

[§] β -tubulin 1a/1b and 2a/2b sequence data obtained from Venter et al. (2001a).

[¶] β -tubulin 1a/1b and 2a/2b sequence data generated in this study.

[#] Histone *H3* sequence data generated in this study.

Table 2. Specimens used in morphological comparisons.

Herbarium no.*	Identity	Host	Origin	Date	Collector
PREM 49379	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	1988	M.J. Wingfield
PREM 49377	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	1986	M.J. Wingfield
PREM 49378	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	1987	M.J. Wingfield
PREM 57293	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	2001	M. Venter
PREM 57294	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	Colombia	2000	M.J. Wingfield
PREM 57295	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Mexico	2000	M.J. Wingfield
IMI 263717	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Hong Kong	1981	C.S. Hodges
PREM 57296	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i> X <i>urophylla</i> clone	Vietnam	2000	M.J. Wingfield
PREM 57297	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Indonesia	2001	M.J. Wingfield

* **PREM**, National Collection of Fungi, Pretoria, South Africa.

* **IMI**, Herbarium, CABI Bioscience, Bakeham Lane, Egham, Surrey TW20 9TY, UK.

Fig. 1. One most parsimonious tree (tree length = 499 steps, CI = 0.9579, RI = 0.9651) generated from sequence variation within a combined β -tubulin and histone *H3* gene sequence data set. Bootstrap values (1000 replicates) are indicated.

Tree length = 499 steps

CI = 0.9579

RI = 0.9651

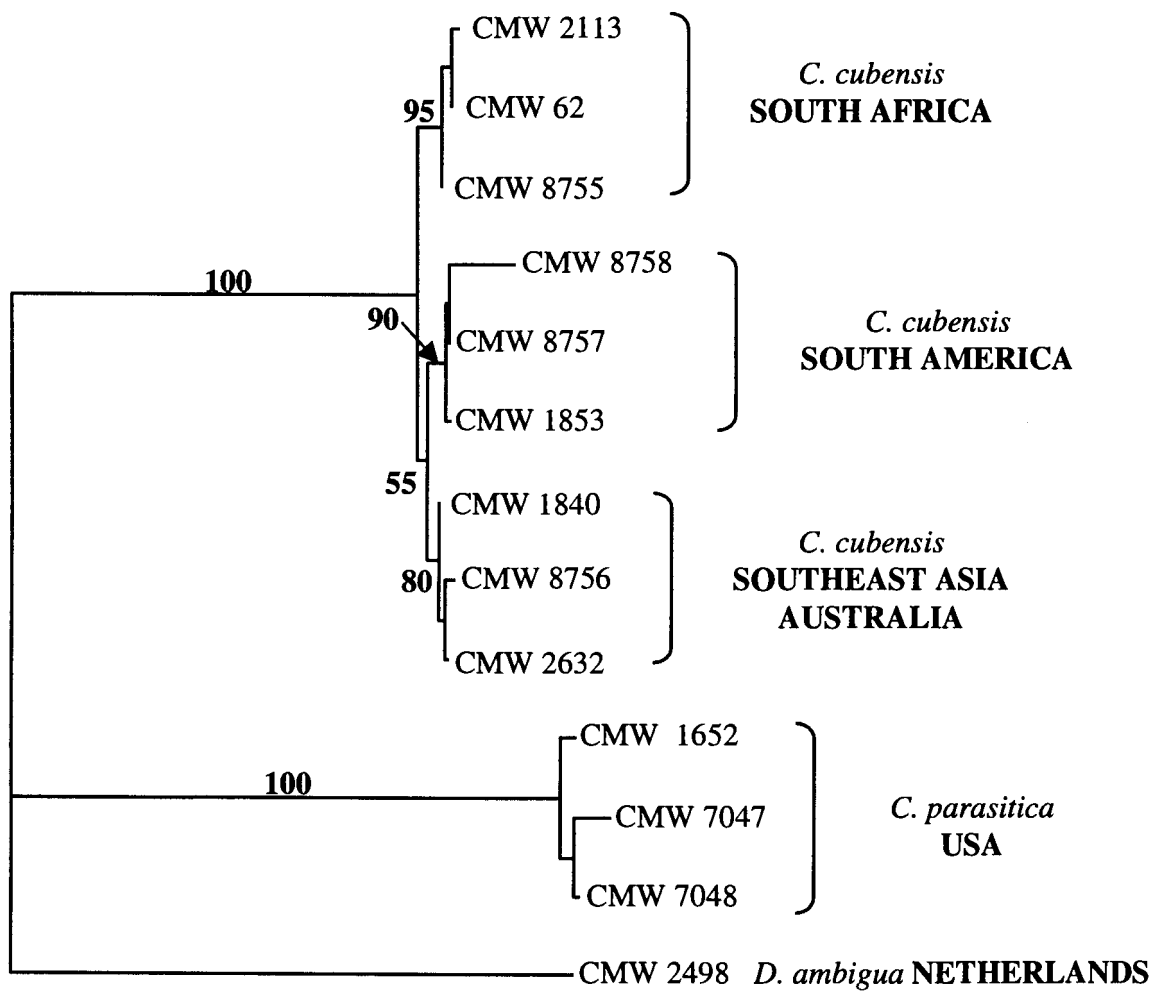
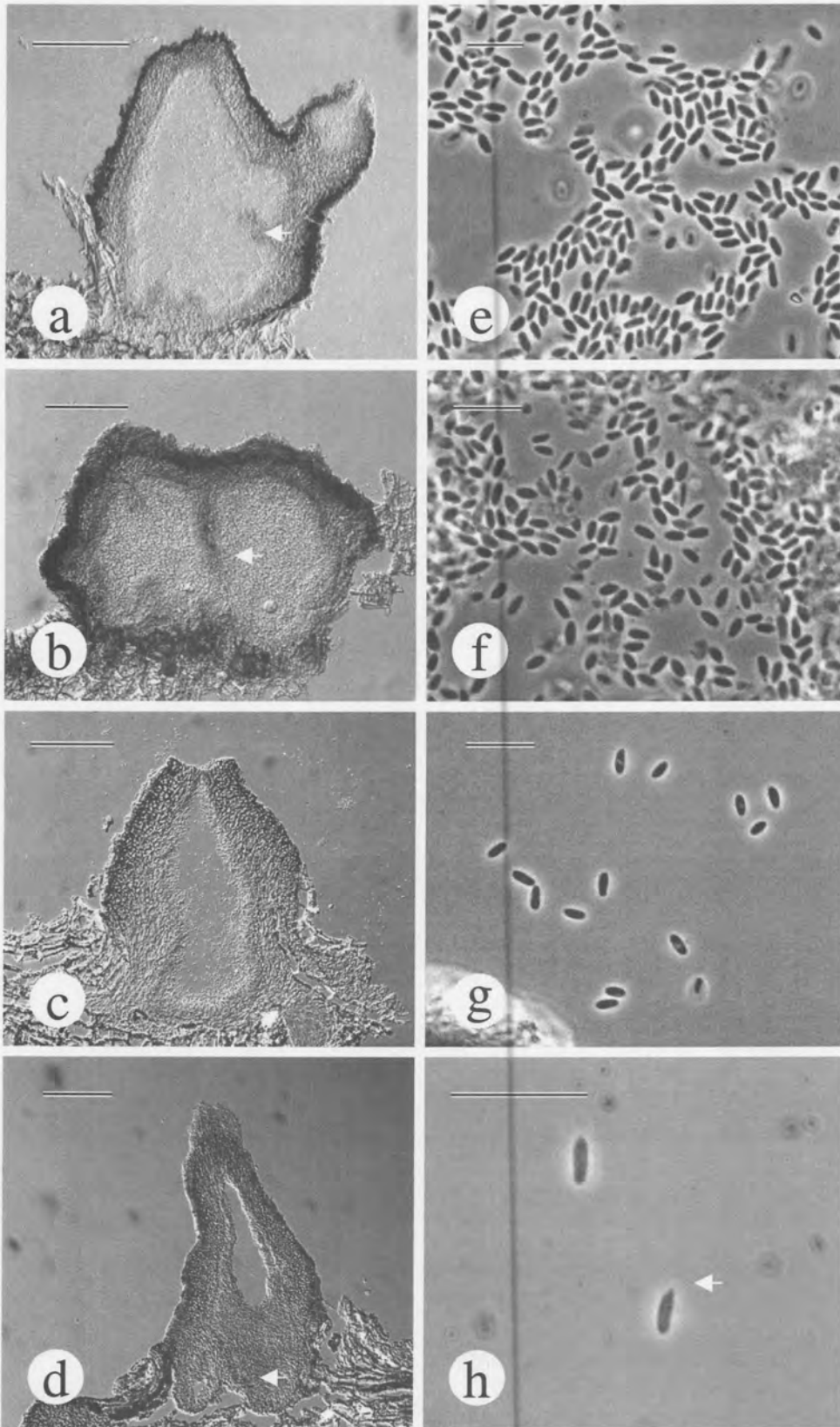


Fig. 2. Conidiomata and conidia of *Cryphonectria cubensis* from various parts of the world. **a, b.** Cross section of a conidiomata from South Africa showing convoluted basal layer (arrow in **a**) and cross wall (arrow in **b**) (bar = 100 μm). **c.** Cross section of a conidioma from Hong Kong (bar = 100 μm). **d.** Cross section of a conidioma from Colombia with second, small cavity (arrow) (bar = 100 μm). **e, f, g.** Conidia from Hong Kong, Mexico and South Africa (bars = 10 μm). **h.** Papillate apex (arrow) on a conidium from South Africa (bar = 10 μm).



CHAPTER 3

Cryphonectria canker on *Tibouchina* in South Africa.

Henrietta Myburg¹, Marieka Gryzenhout², Ronald Heath², Jolanda Roux³,
Brenda D. Wingfield¹ and Michael J. Wingfield³

¹Department of Genetics, University of Pretoria, Pretoria, 0002, South Africa.

²Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002,
South Africa. ³Forestry and Agricultural Biotechnology Institute (FABI), University of

Pretoria, Pretoria, 0002, South Africa.

Published: Myburg et al. (2002). *Mycological Research* 106: 1299-1306.

CRYPTHONECTRIA CANKER ON *TIBOUCHINA* IN SOUTH AFRICA.

ABSTRACT

Cryphonectria cubensis is an important canker pathogen of plantation *Eucalyptus* spp. in tropical and sub-tropical areas of the world, including South Africa. It is best known on *Eucalyptus* spp., but it also occurs on *Syzigium aromaticum* (clove). In 1998, *C. cubensis* was found to cause cankers on the non-myrtaceous hosts *Tibouchina urvilleana* and *T. lepidota* in Colombia. In this study, we report on a similar canker disease that has recently been found in South Africa on *T. granulosa*, commonly grown as an ornamental tree. The identity of the pathogen was determined through morphological comparisons and phylogenetic analyses of ITS and β -tubulin gene sequences. The pathogenicity of the fungus was also tested on *T. granulosa* and *E. grandis*. Morphological as well as DNA sequence comparisons showed that the fungus on *T. granulosa* is the same as *C. cubensis* occurring on *Eucalyptus* spp. in South Africa. Pathogenicity tests on *T. granulosa* and *E. grandis* clones showed that the fungus from *T. granulosa* is able to cause cankers on both hosts.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is one of the most important pathogens in *Eucalyptus* plantations in tropical and sub-tropical areas of the world (Boerboom and Maas 1970, Hodges 1980, Wingfield et al. 1989). High temperatures and rainfall favor the occurrence of this disease (Hodges et al. 1976, Hodges, Geary and Cordell 1979, Sharma et al. 1985a, b). Reduction of losses due to *Cryphonectria* canker is usually achieved by the vegetative propagation of selected disease tolerant *Eucalyptus* clones and hybrids (Alfenas et al. 1983, Conradie et al. 1990).

Cryphonectria cubensis is known to occur naturally on two host genera other than *Eucalyptus*. In 1986, the fungus was reported from clove [*Syzigium aromaticum* (L.) Merr. & Perry] (Hodges et al. 1986) in Brazil. Subsequently the opportunistic pathogen of clove, *Endothia eugeniae* (Nutman & Roberts) Reid & Booth, was reduced to synonymy with *C. cubensis*. Micales et al. (1987) confirmed the synonymy of *C. cubensis* and *E. eugeniae* using isozyme analysis, total protein banding patterns and fungal pigments. More recently, *C. cubensis* was reported on native *Tibouchina urvilleana* Cogn. and *T. lepidota* Baill. in Colombia (Wingfield et al. 2001). This was an intriguing discovery because it was the first time that the fungus had been found on trees outside the Myrtaceae.

Species of *Tibouchina* are widely grown in the warmer parts of South Africa as ornamentals in gardens, parks and along roadsides. In January 1999, a fungus had been found on *T. granulosa* Cogn and *T. granulosa* var. *rosea* in KwaMbonambi, South Africa. This disease

resulted in the death of branches and die-back of mature trees. Fruiting bodies on the surface of cankers resembled those of the anamorph of *C. cubensis* found on *Eucalyptus* spp. in South Africa (Wingfield et al. 1989). The aim of this study was to identify the causal agent of the disease found on *T. granulosa* in South Africa using morphological and molecular tools, and to test its pathogenicity.

MATERIALS AND METHODS

Disease symptoms and collection of samples

In 1999, *T. granulosa* trees showing branch die-back with girdling cankers were identified in the town of KwaMbonambi, South Africa (28°22'S 32°19'E). Bark samples from the surface of cankers bearing fruiting bodies were incubated in moist chambers for 2-3 days to induce production of spores. Single conidial isolations were made onto 2% Malt Extract Agar (MEA) (20 g Biolab Malt Extract, 15g Biolab Agar, 1L water) and incubated at 25 °C. These cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Morphological comparisons

Bark specimens from *T. granulosa*, with fruiting structures of the fungus, were collected from natural cankers and deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (Table 1). Bark material from field pathogenicity trials (see

Pathogenicity section), was also examined (Table 1). These specimens were compared with specimens of *C. cubensis* from *Eucalyptus* spp. used in a previous study (Wingfield et al. 1989) (Table 1). Comparisons were made using standard light microscopic techniques.

Stems (~10-15 mm diam) of an unknown *E. grandis* W. Hill ex Maiden clone were inoculated with isolate CMW 9343 from *T. granulosa* and isolate CMW 2113 from *E. grandis*. This was done to induce the formation of fresh fruiting structures. These stems were cut into segments (5-8 cm long) and the ends sealed with melted paraffin wax. Bark plugs 6 mm in diam were removed from the segments with a cork borer and replaced with mycelial plugs of the same size. Wounds were covered with parafilm and incubated in a moisture chamber until fruiting structures were produced.

Fruiting structures were sectioned using a Leitz 1310K freezing microtome with a KRYOMAT 1700 generator (Setpoint Technologies, Johannesburg) as described in Venter et al. (2002). Sections were executed at $-30\text{ }^{\circ}\text{C}$ and were 14-20 μm thick. Ten measurements each was taken of relevant structures and are presented as (min)-(mean - sd) - (mean + sd)(-max). For stromata, a range was obtained from three structures. Colour notations described by Rayner (1970) were used to standardise colour annotations.

DNA isolation and amplification

Isolates used to confirm the identity of the fungus from *T. granulosa* are listed in Table 2. DNA was isolated as described by Myburg et al. (1999). The internal transcribed spacer

(ITS) regions of the ribosomal RNA operon were amplified using the primer sets ITS1 and ITS4 (White et al. 1990). Amplification of two β -tubulin gene regions was done using primer pairs Bt1a/Bt1b and Bt2a/Bt2b (Glass and Donaldson 1995). Amplification of the ITS1 and ITS2 and the two β -tubulin gene regions were as described by Myburg et al. (1999) and Myburg et al. (2002) respectively. Amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, Inc. Foster City, California). PCR products were visualised on a 1 % agarose-ethidium bromide gel using an ultraviolet light source.

Sequencing and analysis of sequence data

PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The respective DNA fragments were sequenced in both directions with the same primer pairs used in the amplification reactions. An ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington) was used for sequencing. DNA sequences were determined with an ABI PRISM 377™ automated DNA sequencer. Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Foster City, CA) was used to translate the DNA sequences to amino-acid sequences. The DNA sequences were aligned using CLUSTAL W (Thompson et al. 1997) and verified manually.

Phylogenetic analyses were performed using PAUP* version 4.0b (Swofford 1998). Heuristic searches with branch swapping (no swapping) and MULTREES (saving all

optimal trees) effective were used in parsimony analyses. Gaps inserted during sequence alignment were treated as fifth characters (NEWSTATE). A partition-homogeneity test (PHT) was conducted (500 replicates, heuristic search type) to assess the combinability of the ITS and β -tubulin data sets. Bootstrap analyses (1000 replications) were done to determine the confidence levels of the tree branching points. Previously published sequences for *Cryphonectria parasitica*, the causal agent of chestnut blight (Elliston 1981), were included in the study for comparative purposes. *Diaporthe ambigua* Nitschke was used as the outgroup taxon to root the phylogenetic tree. All sequences obtained in this study have been deposited in GenBank (accession nos. are listed in Table 2).

Pathogenicity

Tibouchina granulosa trees (~10-15 mm diam) were kept under greenhouse conditions for two weeks to acclimatise prior to inoculation. The greenhouse was subjected to natural day/night conditions and a temperature setting of ~25 °C. Mycelial plugs (9 mm diam) were taken from the edges of actively growing cultures and placed, mycelium facing the cambium, into wounds made with a cork borer (9 mm diam). Wounds were sealed with parafilm to protect the inoculated fungus and cambium from desiccation. Ten trees were inoculated for each of the ten selected isolates. Sterile plugs were used for control inoculations on ten *Tibouchina* trees. To determine the relative pathogenicity, lesion lengths were measured on each tree after four weeks and the averages computed. The most virulent isolate was selected and used in a subsequent inoculation study on *T. granulosa* and *E. grandis*.

To assess reciprocal pathogenicity of *Tibouchina* and *Eucalyptus* isolates, the most virulent isolate (CMW 9343) from *Tibouchina* and a previously identified virulent isolate (CMW 2113) of *C. cubensis* from *E. grandis* (Van Zyl and Wingfield 1999), were inoculated on 20 *T. granulosa* and 20 *E. grandis* (clone ZG 14) trees (~10 mm diam) respectively. The same inoculation technique as described above was used and results based on lesion length were assessed after 4 weeks. Ten *E. grandis* and ten *T. granulosa* trees were inoculated with sterile MEA plugs to serve as controls. This trial was repeated once. To determine the variance between isolates and among trees, the inoculation data were subjected to analysis of variance using the General Linear Model procedure of SAS (SAS/STAT Users guide, Version 6).

To investigate the pathogenicity of the *Tibouchina* and *Eucalyptus* isolates under field conditions, inoculations were performed on trees (~1 yr old) of an established *E. grandis* x *E. camaldulensis* clone (GC 747). These inoculations were performed at KwaMbonambi (KwaZulu-Natal) (28°22'S 32°19'E) where *C. cubensis* is known to occur in *Eucalyptus* plantations. Twenty trees (100-150 mm diam) each were inoculated with the three most virulent isolates from *Tibouchina* (CMW 9343, CMW 9346, CMW 9358) and one *C. cubensis* isolate from *E. grandis* (CMW 2113). Twenty trees were also inoculated with sterile MEA plugs to serve as controls. Results based on lesion length were measured after 6 months and the entire trial was repeated once. Statistical significance of results was determined as described above.

RESULTS

Disease symptoms and collection of samples

During the first disease survey of *T. granulosa* trees, ten trees showing branch die-back were found in KwaMbonambi. One infected tree was also found in Richards Bay during a preliminary survey. Disease symptoms included branch die-back, cracking of the bark (Fig. 1, Fig. 2), and the development of girdling stem cankers. Fruiting bodies of a fungus resembling the anamorph of *C. cubensis* were found between the cracks and on dead areas of the stem.

Morphological comparisons

Anamorph structures of the fungus occurring on *T. granulosa* were superficial to slightly immersed on the bark, pyriform to clavate (Fig. 3a). Longitudinal sections revealed a unilocular to occasionally multilocular, convoluted eustroma (Fig. 3b) similar to those observed in Myburg et al. (2002). Structures were blackened with a luteous (colour 17) interior, 192-310 μm wide; base 248-477 μm long, base above the bark surface 167-477 μm long (Fig. 3a). One to four necks, either originating from a single locule or from more than one locule, occurred on a single superficial eustroma, and were up to 240 μm long, 105-124 μm wide. Conidiophores were hyaline, septate, with or without branching underneath the septum, 9.5-14(-15.5) x 1.5 μm (Fig. 3c). Conidia were exuded as bright luteous (colour 17) spore tendrils or drops (Fig. 3a). Conidia were hyaline, non-septate, cylindrical to oblong to oval, 3-4.5(-5.5) x (1.5-)1-1.5 μm (Fig. 3d).

Perithecia were rarely seen, and formed either below the conidioma (Fig. 3e) or separately (Fig. 3f). When perithecia developed on their own, they were semi-immersed and surrounded with weakly developed, orange (colour 15), predominantly prosenchymatous stromatic tissue, which was often slightly erumpent (Fig. 3f). When the perithecia were produced below the blackened eustroma of the conidioma, the same type of stromatic tissue as that observed for the single ascomata was produced around the bases of the perithecial necks (Fig. 3e). This tissue was different from that of the conidiomata, which was umber (colour 15m) and composed of *textura globulosa*. Perithecia were black, globose, 267-310 μm diam. Black, periphysate perithecial necks protruded through the stromatal surface for up to 500 μm . Necks were 62-105 μm wide and covered in brown, *textura porrecta* tissue as it extends beyond the stroma, with the width of the extended parts being 112-200 μm . Ascospores were hyaline, septate, fusoid to oblong, sometimes slightly curved, (5-)5.5-7.5(-8) μm long, 1.5-2 μm wide (Fig. 3g). No asci were seen in the available material.

The anamorph of the fungus from *T. granulosa* is similar to *C. cubensis* from *Eucalyptus* spp. in South Africa (Myburg et al. 2002, Wingfield et al. 1989). The presence of the teleomorph of this fungus in South Africa has been reported only once (Wingfield et al. 1989). The teleomorph material observed in this study conforms to the description provided by these authors. The anamorph and teleomorph of the fungus on *T. granulosa* were also similar to those described for *C. cubensis* from other parts of the world (Hodges et al. 1976, Hodges et al. 1979, Hodges 1980, Wingfield et al. 2001).

Identification of the fungus on *T. granulosa* as *C. cubensis* is supported by the morphology of the structures produced on plants used in the inoculation studies. Fruiting structures formed by isolates from *T. granulosa* (PREM 57362, PREM 57363, PREM 57364, PREM 57365, PREM 57367) on the *Eucalyptus* clone (GC 747) were similar to those formed by isolate CMW 2113 (isolated from *E. grandis*) on the same host (PREM 57366). Only anamorph structures were produced in these inoculations.

Morphology of conidia from conidiomata formed on the artificially inoculated stems and field inoculations (PREM 57362, PREM 57363, PREM 57364, PREM 57365, PREM 57366, PREM 57367) (Table 1) was variable. Conidia produced by isolates CMW 9343 and CMW 2113 on the *Eucalyptus* stems varied from clavate to cylindrical to allantoid (Fig. 3h). The conidia were also variable in size and longer (3.5-5.5(-8) x 1-1.5(-2) μm , than those found on material collected from natural infections. Such variation in spore morphology was not observed for pycnidia from natural infections. Many of the conidia in the inoculated material also had papillate apices (Fig. 3h), although these were rarely seen in the natural material on *T. granulosa*. The papillate apices on conidia of South African material have been noted before, but were thought inordinately rare and not taxonomically significant (Myburg et al. 2002).

Sequencing and analysis of sequence data

A PHT value of $P = 0.2$ showed that the data partitions (ITS1, 5.8S, ITS2, Bt1a/1b and Bt2a/2b) could be combined as one data set in the phylogenetic analyses. Manual alignment

of the combined sequences resulted in a data set of 1530 characters (Appendix 2), consisting of 996 constant characters, 338 parsimony informative characters and 196 variable characters that were parsimony uninformative. A strict consensus tree (50% majority rule) (tree length = 671 steps, consistency index/CI = 0.96 and retention index/RI = 0.95) was generated from the 196 variable characters (Fig. 4).

The phylogenetic tree based on the combined DNA sequences (Fig. 3a) showed that the fungi from *T. granulosa* and *Eucalyptus* in South Africa, South America and Southeast Asia/Australia resided in separate groups to *C. parasitica* and the outgroup *D. ambigua* (bootstrap support = 100%). The *C. cubensis/Tibouchina* clade is subdivided into subgroups, consistent with geographical origins of the isolates. The South African isolates thus formed a clade separate (bootstrap support = 93%) from the Southeast Asian and South American isolates. The South American *C. cubensis* strains isolated from *Tibouchina* spp. grouped together with the South American *C. cubensis* isolates from *Eucalyptus* spp. The *C. cubensis* isolates from Southeast Asia/Australia grouped together (bootstrap support = 63%). The bootstrap support for the branch node separating the South American and Southeast Asian/Australian groups is 64%, suggesting that these two groups are closely related.

Pathogenicity

Greenhouse inoculation of *T. granulosa* seedlings and the *E. grandis* clone (ZG 14) resulted in the formation of extensive lesions on both the hosts within 4 wks (Table 3). Some of the seedlings were already dropping their leaves, dying and producing epicormic shoots on the

stems within this time period. The control inoculations showed no lesions. Both isolates from *Tibouchina* and *Eucalyptus* were more pathogenic to *Eucalyptus* than to *Tibouchina* ($P > 0.0001$) (Table 3).

Field inoculations resulted in the formation of girdling cankers, cracking bark and fungal sporulation around the lesions. The same symptoms were seen on the naturally infected *Tibouchina* trees (Fig. 1, Fig. 2). The analysis of data from the field inoculations, however, showed no significant differences in pathogenicity between isolates ($P > 0.1464$).

DISCUSSION

This study represents the first report of a serious canker and die-back disease of ornamental *Tibouchina* trees in South Africa. To the best of our knowledge, this is the first disease to be recorded on this important ornamental tree in South Africa. Our results clearly show that the disease is caused by the well-known *Eucalyptus* canker pathogen, *C. cubensis*. The fungus has recently been reported as a serious pathogen of native *Tibouchina* spp. in Colombia (Wingfield et al. 2001) and the current study represents the first report of the disease on a species of *Tibouchina* outside South America.

In South Africa, *C. cubensis* on *Eucalyptus* is mostly characterised by the occurrence of the asexual fruiting structures. The sexual state on *Eucalyptus* has been found only once (Wingfield et al. 1989, Van Heerden et al. 1997). It was, therefore interesting that perithecia of *C. cubensis* were occasionally found on *T. granulosa* in South Africa. This is in contrast

to the situation in Colombia where only asexual fruiting structures were found on *T. urvilleana* and *T. lepidota* (Wingfield et al. 2001), while the sexual state is the dominant form on *Eucalyptus* spp. in that country (Van der Merwe et al. 2001). Wingfield et al. (2001) suggested that the differences in occurrence of the sexual state could be the result of differences in biology of *C. cubensis* on different hosts. This hypothesis is supported by our data, where the South African *C. cubensis* rarely produces sexual structures on *Eucalyptus*.

Sequence data generated in this study confirm the fact that the fungus from *T. granulosa* in South Africa is *C. cubensis*. Our results further support a recent report that *C. cubensis* isolates from South Africa reside in a well resolved and strongly supported group different from that accommodating South American and Southeast Asian *C. cubensis* isolates (Myburg et al. 2002). Isolates from *T. granulosa* in South Africa cluster together with the South African *C. cubensis* isolates, separate from the *Tibouchina* isolates in Colombia. These data suggest that South African *C. cubensis* on *Tibouchina* and *Eucalyptus* has a similar, but probably different origin to *C. cubensis* found in other parts of the world.

Greenhouse inoculation trials conducted in this study suggest that the *Eucalyptus* clone used is more susceptible to infection by the South African *C. cubensis* than is *T. granulosa*. After four weeks, both tree species were producing epicormic shoots and had begun to die. This is in contrast to Colombian trials (Wingfield et al. 2001), where none of the *Eucalyptus* trees died and the *Tibouchina* trees were dead or dying after four months. It also appears that the South African *C. cubensis* isolates are more virulent than *C. cubensis* from *Tibouchina* in Colombia, since trees had begun to die within four weeks of inoculation.

At the present time, the origin of the South African form of *C. cubensis*, now known to occur on *Eucalyptus* and *Tibouchina*, is uncertain. Although morphologically almost identical to the fungus from Asia and South America (Myburg et al. 2002), biological and molecular evidence provide robust support for the notion that the South African fungus represents a discrete taxon. This leads us to speculate that the fungus on *Eucalyptus* and *Tibouchina* in South Africa will have an origin different to that of the fungus in Asia and South America. A likely origin of the fungus would be on native Myrtaceae in South Africa and surveys have been initiated to consider this hypothesis.

ACKNOWLEDGEMENTS

The authors thank the staff of the Biosystematics Division of the Agricultural Research Council (ARC), Pretoria, for the microtome facilities. We thank the National Research Foundation (NRF), members of the Tree Pathology Co-operative Programme (TPCP), and the THRIP support programme of the Department of Trade and Industry, South Africa for financial support. We are also grateful to Mr. Paul Viero of Mondi forests for assistance with the field inoculations.

REFERENCES

- Alfenas, A.C., Jeng, R. and Hubbes, M.** 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**: 197-205.
- Boerboom, J.H.A. and Maas, P.W.T.** 1970. Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**: 94-99.
- Conradie, E., Swart, W.J. and Wingfield, M.J.** 1990. *Cryphonectria* canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**: 43-49.
- Conradie, E., Swart, W.J. and Wingfield, M.J.** 1992. Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. *European Journal of Forest Pathology* **22**: 312-315.
- Elliston, J.E.** 1981. Hypovirulence and chestnut blight research: fighting disease with disease. *Journal of Forestry* **79**: 657-660.
- Glass N.L. and Donaldson, G.C.** 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.
- Hodges, C.S., Reis, M.S., Ferreira, F.A. and Henfling, J.D.M.** 1976. O cancro do eucalipto causada por *Diaporthe cubensis*. *Fitopatologia Brasileira* **1**: 129-162.
- Hodges, C.S., Geary, T.F. and Cordell, C.E.** 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. *Plant Disease Reporter* **63**: 216-220.

- Hodges, C.S.** 1980. The taxonomy of *Diaporthe cubensis*. *Mycologia* **72**: 542-548.
- Hodges, C.S., Alfenas, A.C. and Ferreira, F.A.** 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**: 343-350.
- Micales, J.A., Stipes, R.J. and Bonde, M.R.** 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. *Mycologia* **79**: 707-720.
- Myburg, H., Wingfield, B.D. and Wingfield, M.J.** 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**: 243-250.
- Myburg, H., Gryzenhout, M., Wingfield, B.D. and Wingfield, M.J.** 2002. β -tubulin and Histone *H3* gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia and South America. *Canadian Journal of Botany* **80**: 590-596.
- Rayner, R.W.** 1970. *A Mycological Colour Chart*. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey, U.K.
- Roux, J., Wingfield, M.J., Coutinho, T.A., Bouillett, J.P. and Leigh, P.** 2000. Diseases of plantation *Eucalyptus* in the Republic of the Congo. *South African Journal of Science* **96**: 454-456.
- SAS Statistical Software.** 1989. SAS/STAT User's Guide, Version 6, Fourth Edition, Vol. 1 and 2. SAS Institute Inc., Cary, NC, U.S.A.
- Sharma, J.K., Mohanan, C., and Florence, E.J.M.** 1985a. Disease survey in nurseries and plantations of forest tree species grown in Kerala. Research report 36. Kerala, India: Kerala Forest Research Institute. 268p.
- Sharma, J.K., Mohanan, C., and Florence, E.J.M.** 1985b. Occurrence of *Cryphonectria* canker disease of *Eucalyptus* in Kerala, India. *Annals of Applied Biology* **106**: 265-276.

- Swofford, D.L.** 1998. *PAUP: Phylogenetic Analysis Using Parsimony (*and Other Methods)* version 4. Sinauer Assoc. Inc.: Sunderland, MA, U.S.A.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G.** 1997. The CLUSTAL W windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.
- Van der Merwe, N.A., Myburg, H., Wingfield, B.D., Rodas, C. and Wingfield, M.J.** 2001. Identification of *Cryphonectria cubensis* from Colombia based on rDNA sequence data. *South African Journal of Science* **97**: 295-296.
- Van Heerden S.W., Wingfield, M.J., Coutinho, T., van Zyl, L.M. and Wright, J.A.** 1997. Diversity of *Cryphonectria cubensis* isolates in Venezuela and Indonesia. Proceedings of IUFRO Conference on Silviculture and Improvement of Eucalypts. Salvador, Bahia, Brazil. Pp. 142-146.
- Van Zyl, L.M., Wingfield, M.J., Alfenas, A.C. and Crous, P.W.** 1998. Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**: 41-47.
- Van Zyl, L.M. and Wingfield, M.J.** 1999. Wound response of *Eucalyptus* clones after inoculation with *Cryphonectria cubensis*. *European Journal of Forest Pathology* **29**: 161-167.
- Venter, M., Myburg, H., Wingfield, B.D., Coutinho, T.A. and Wingfield, M.J.** 2001. A new species of *Cryphonectria* from South Africa and Australia, pathogenic on *Eucalyptus*. *Sydowia* **54**: 98-117.
- White, T.J., Bruns, T., Lee, S. and Taylor, J.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H.,

Sninsky, J.J. and White, T.J., eds. PCR Protocols: a guide to methods and applications. Academic Press, San Diego. pp. 315-322.

Wingfield, M.J., Swart, W.J. and Abear, B.J. 1989. First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**: 311-313.

Wingfield, M.J., van Zyl, L.M., van Heerden, S.W., Myburg, H. and Wingfield, B.D. 1997. Virulence and genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In: Lieutier, F., Mattson, W.J. and Wagner, M.R., eds. Physiology and Genetics of Tree-Phytophage Interactions. Les Colloques, N°90, INRA Editions, Gujan. pp. 163-172.

Wingfield, M.J., Rodas, C., Myburg, H., Venter, M., Wright, J. and Wingfield, B.D. 2001. *Cryphonectria* canker on *Tibouchina* in Colombia. *Forest Pathology* **31**: 297-306.

Table 1. Specimens of *Cryphonectria cubensis* used in morphological comparisons.

Reference no. ^a	Host	Origin	Date	Collector
PREM 49379	<i>Eucalyptus grandis</i>	South Africa	1988	M.J. Wingfield
PREM 49377	<i>E. grandis</i>	South Africa	1986	M.J. Wingfield
PREM 49378	<i>E. grandis</i>	South Africa	1987	M.J. Wingfield
PREM 57357	<i>Tibouchina granulosa</i>	South Africa	1999	J. Roux
PREM 57358	<i>T. granulosa</i>	South Africa	1999	J. Roux
PREM 57359	<i>T. granulosa</i>	South Africa	1999	J. Roux
PREM 57360	<i>T. granulosa</i>	South Africa	2000	J. Roux, R. Heath and L. Lombaard
PREM 57361	<i>T. granulosa</i>	South Africa	2000	J. Roux, R. Heath and L. Lombaard
PREM 57362	CMW 9343 inoculation of GC 747 ^b	South Africa	2000	J. Roux and R. Heath
PREM 57363	CMW 9343 inoculation of GC 747 ^b	South Africa	2001	M. Gryzenhout and R. Heath
PREM 57364	CMW 9346 inoculation of GC 747 ^b	South Africa	2001	M. Gryzenhout and R. Heath
PREM 57365	CMW 9358 inoculation of GC 747 ^b	South Africa	2001	M. Gryzenhout and R. Heath
PREM 57366	CMW 2113 inoculation of GC 747 ^b	South Africa	2001	M. Gryzenhout and R. Heath
PREM 57367	CMW 9343 stick inoculation ^c	South Africa	2002	R. Heath

^a PREM, National Collection of Fungi, Pretoria, South Africa.

^b An *E. grandis* X *camaldulensis* clone (GC747) inoculated with *C. cubensis* isolate in the field.

^c Cut stems of an unknown *E. grandis* clone inoculated in the lab.

Table 2. Isolates used for molecular comparison and pathogenicity trials^a.

Isolate no.	Isolate identity	Host	Origin	GenBank accession numbers
CMW 8757	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	Venezuela	AF046897 ^b , AF273069 ^d , AF273464 ^d
CMW 1853	<i>C. cubensis</i>	<i>Eugenia caryophyllus</i>	Brazil	AF046891 ^b , AF273070 ^d , AF273465 ^d
CMW 9927	<i>C. cubensis</i>	<i>Tibouchina urvilleana</i>	Colombia	AF265653 ^c , AF292034 ^f , AF292037 ^g
CMW 9928	<i>C. cubensis</i>	<i>T. urvilleana</i>	Colombia	AF265654 ^c , AF292036 ^f , AF292039 ^g
CMW 9929	<i>C. cubensis</i>	<i>T. urvilleana</i>	Colombia	AF265655 ^c , AF292035 ^f , AF292038 ^g
CMW 2113	<i>C. cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	AF046892 ^b , AF273067 ^d , AF273462 ^d
CMW 62	<i>C. cubensis</i>	<i>E. grandis</i>	South Africa	AF292041 ^d , AF273063 ^d , AF273458 ^d
CMW 8755	<i>C. cubensis</i>	<i>E. grandis</i>	South Africa	AF292040 ^d , AF273064 ^d , AF273459 ^d
CMW 9932	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	AF273472 ^e , AF273062 ^f , AF273457 ^g
CMW 9327	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	AF273473 ^e , AF273060 ^f , AF273455 ^g
CMW 9328	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	AF273474 ^e , AF273061 ^f , AF273456 ^g
CMW 8756	<i>C. cubensis</i>	<i>E. grandis</i>	Indonesia	AF046896 ^b , AF273077 ^d , AF285165 ^d
CMW 9903	<i>C. cubensis</i>	<i>Eucalyptus</i> sp.	Kalimantan	AF292044 ^e , AF273066 ^f , AF273461 ^g
CMW 9906	<i>C. cubensis</i>	<i>Eucalyptus</i> sp.	Kalimantan	AF292045 ^e , AF273065 ^f , AF273460 ^g
CMW 1651	<i>C. parasitica</i>	<i>Castanea dentata</i>	USA	AF046901 ^b , AF273074 ^d , AF273467 ^d
CMW 1652	<i>C. parasitica</i>	<i>C. dentata</i>	USA	AF046902 ^b , AF273075 ^d , AF273468 ^d
CMW 2498	<i>Diaporthe ambigua</i>	<i>Malus sylvestris</i>	Netherlands	AF046906 ^b , AF273072 ^d , AF273471 ^d
CMW 9343	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	
CMW 9346	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	
CMW 9358	<i>C. cubensis</i>	<i>T. granulosa</i>	South Africa	

^a Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. CMW refer to the general and *Cryphonectria* culture collections.

^b Ribosomal DNA sequences obtained from Myburg et al. (1999).

^c Ribosomal DNA sequences obtained from Wingfield et al. (2001).

^d Sequence data obtained from Myburg et al. (2002).

^e Ribosomal DNA sequences generated in this study.

^f Beta-tubulin 1a/1b sequences generated in this study.

^g Beta-tubulin 2a/2b sequences generated in this study.

Table 3. Lesion lengths on 1 yr-old *Eucalyptus grandis* (clone ZG 14) and *Tibouchina granulosa* four wks after inoculation in the greenhouse.

Isolate	^a Lesion length (mm)		^a Lesion length (mm)		^a Lesion length (mm)	
	(1 st trial)		(2 nd trial)		(3 rd trial)	
	<i>Tibouchina</i>	ZG 14	<i>Tibouchina</i>	ZG 14	<i>Tibouchina</i>	ZG 14
CMW 2113	49.9	146.5	65.6	975	867	155.5
CMW 9343	108.9	133.0	34.2	104.1	90.9	129.3
Control	10	10	9.7	9.6	10	10

^a Each value is the average of 20 measurements for each isolate.

P > 0.0001

CV = 43.23666

R-Square = 0.806038

F = 7.45

Table 4. Lesion length on 18 month-old GC 747 trees grown in the plantation after inoculation with *Cryphonectria cubensis* isolates from *Tibouchina* and *Eucalyptus*.

Isolate	^aLesion length (mm) (1st trial)	^aLesion length (mm) (2nd trial)
CMW 2113	77.9	56.1
CMW 9343	83.4	45.9
CMW 9346	50.8	92.5
CMW 9358	68	54
Control	10	10

^a Each value is the average of 20 measurements for each isolate.

P > 0.1464

CV = 77.84968

R-Square = 0.555225

F = 1.24

Figs. 1. Disease symptoms. **a.** *Tibouchina* tree showing branch die-back caused by *C. cubensis*. **b.** Girdling canker on the main stem of a *Tibouchina* tree infected by *C. cubensis*.



Fig. 2. Light micrographs of the anamorph and teleomorph of *Cryphonectria cubensis* from *Tibouchina granulosa* in South Africa. **a.** Conidioma on bark. **b.** Vertical section through conidioma. **c.** Conidiophores. **d.** Conidia. **e.** Perithecia (pe) with stromatic tissue (arrow) below a conidioma (co). **f.** Ascoma. **g.** Ascospores. **h.** Conidia produced by isolate CMW 9343 after inoculation into a cut stem of an unknown *Eucalyptus* clone. Bars Figs. **a, b, e, f** = 100 μm ; Figs. **c, d, g, h** = 10 μm .

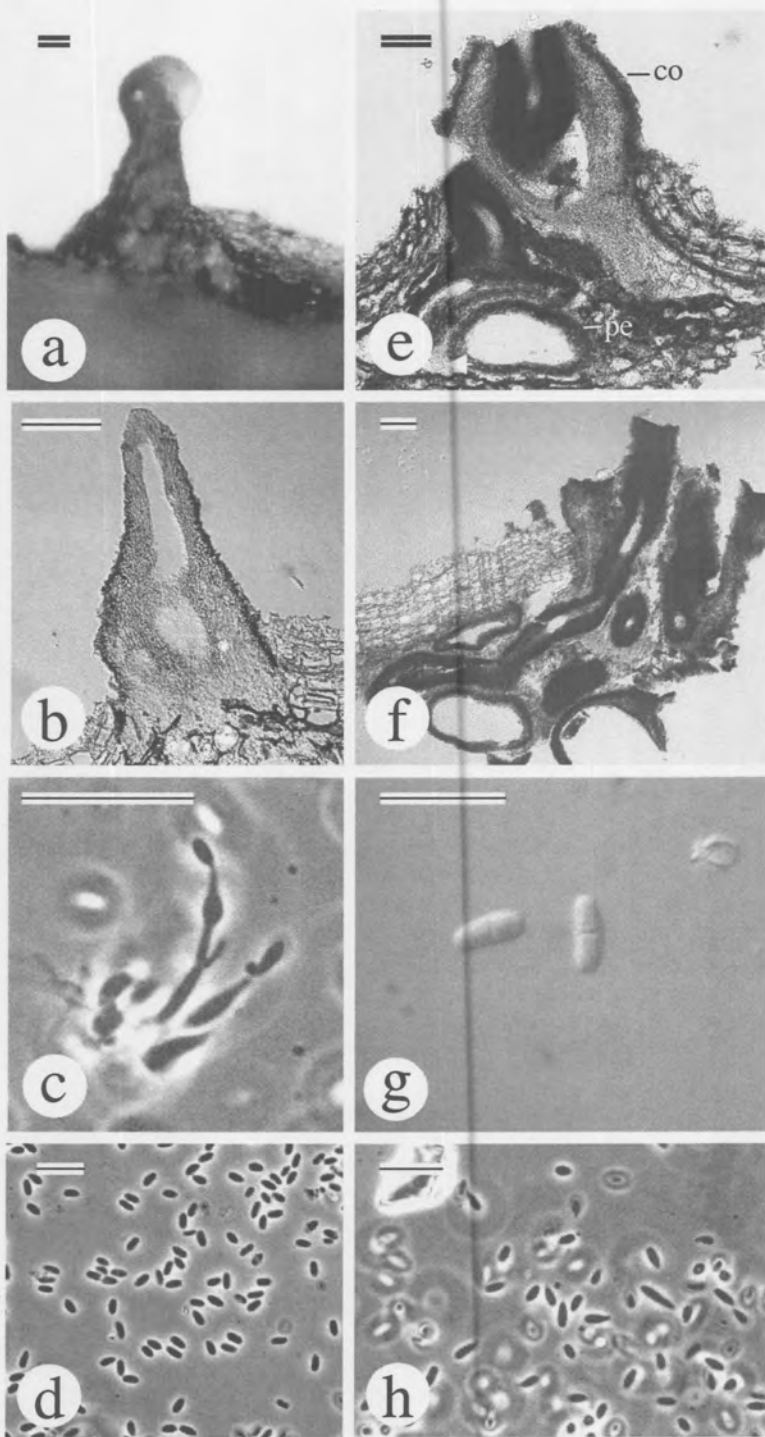
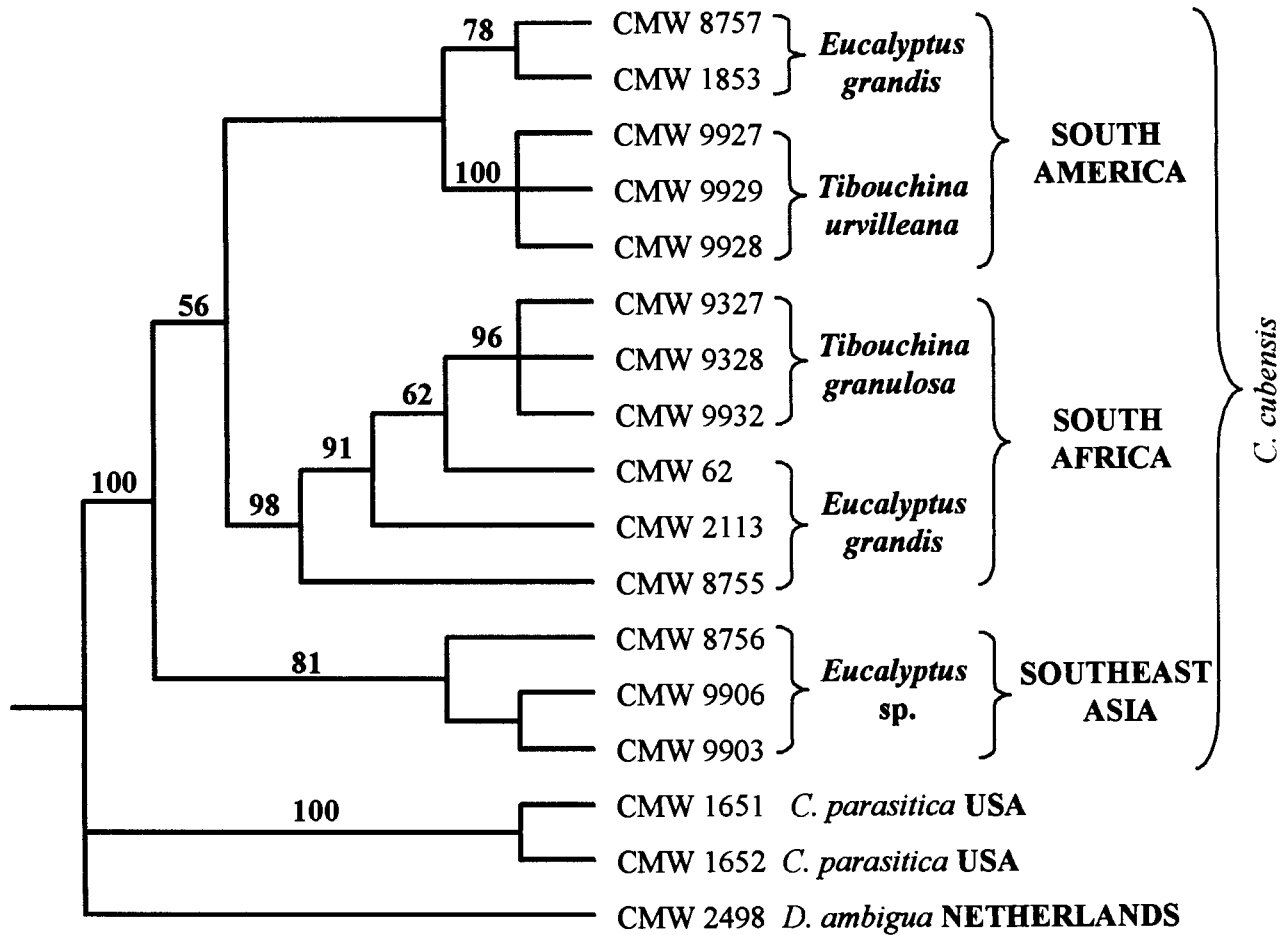


Fig. 3. Phylogenetic tree generated from a combined data set including ribosomal DNA (ITS1/ITS2) and β -tubulin gene sequence data. One strict consensus tree (tree length = 671 steps, CI = 0.96 and RI = 0.95) was generated from heuristic searches performed on the combined data set. Bootstrap values (1000 replicates) are indicated above the branches and those lower than 50 % are not shown. *Diaporthe ambigua* was used to root the tree.

Tree length = 671 steps

CI = 0.96

RI = 0.95



CHAPTER 4

Conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*: A re- evaluation based on morphology and DNA sequence data.

Henrietta Myburg¹, Marieka Gryzenhout², Brenda D. Wingfield¹, Michael J.
Wingfield¹

¹Department of Genetics, ²Department of Microbiology and Plant Pathology, Forestry
and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002,
South Africa.

In press: Myburg et al. (2003). *Mycoscience* 44(3): 187-196.

**CONSPECIFICITY OF *ENDOTHIA EUGENIAE* AND
CRYPHONECTRIA CUBENSIS: A RE-EVALUATION BASED
ON MORPHOLOGY AND DNA SEQUENCE DATA.**

ABSTRACT

Cryphonectria cubensis and *Endothia eugeniae* are fungal pathogens of *Eucalyptus* and clove respectively that were reduced to synonymy based on the results of cross-inoculation studies, isozyme analysis, cultural studies and morphology. A previous phylogenetic study on *Cryphonectria*, based on sequence variation in the ITS region of the ribosomal RNA operon, also supported the conspecificity of *C. cubensis* and *E. eugeniae*, but was based on only one *E. eugeniae* isolate. New collections from clove in Brazil and Indonesia have become available, providing the opportunity to reconsider the conspecificity of *C. cubensis* and *E. eugeniae*. The occurrence of *C. cubensis* on clove was confirmed based on morphological comparisons and phylogenetic analyses of ribosomal DNA and β -tubulin gene sequence data. In addition to *C. cubensis*, other fungi morphologically similar to *Cryphonectria* species based on their orange stromata were also present on some clove specimens. These isolates, for which no herbarium material exists, grouped separately from the *C. cubensis* clade and closer to the *Cryphonectria* clade. The presence of more than one closely related fungus on clove raises questions relating to the legitimacy of the synonymy of *E. eugeniae* and *C. cubensis*. However, based on the presence of *C. cubensis* on the type specimen of *E. eugeniae*, we retain the synonymy of the two fungi but provide evidence that other fungi, closer related to *Cryphonectria* spp. than to *C. cubensis*, are present on clove.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is a well-known and important canker pathogen of *Eucalyptus* species (Boerboom and Maas 1970, Hodges 1980, Wingfield et al. 1989). The fungus is present in tropical and subtropical areas of the world, where high temperatures and rainfall favor infection and disease development (Alfenas et al. 1982). Management of *Cryphonectria* canker is primarily achieved by the vegetative propagation of disease-tolerant *Eucalyptus* clones and *Eucalyptus* hybrids (Alfenas et al. 1983, Van Zyl and Wingfield 1999).

Endothia eugeniae (Nutman & Roberts) Reid & Booth was first reported from Zanzibar, Tanzania, causing acute die-back of clove [*Syzygium aromaticum* (L.) Merr. & Perry] (Nutman and Roberts 1952). The pathogen infected trees through wounds and caused die-back of branches or death of whole trees by girdling of trunks. At the point of infection, the wood was stained reddish-brown (Nutman and Roberts 1952). The disease has also been reported from Malaysia (Anonymous 1954, Heath 1956, Reid and Booth 1969), which is the region of native cloves (Hodges et al. 1986).

The clove pathogen, now known as *C. cubensis*, was first described as *Cryptosporella eugeniae* Nutman & Roberts (1952), but was later transferred to the genus *Endothia* (Reid and Booth 1969). Hodges et al. (1986) reduced *E. eugeniae* to synonymy with *C. cubensis*. This synonymy was based on morphological comparisons, cultural characteristics, inoculation studies as well as analysis of isozyme banding patterns. Micales et al. (1987) confirmed this synonymy using additional isozyme analyses, general protein patterns and pigment identification.

Previous descriptions of *E. eugeniae* describe a fungus with brown pycnidia, immersed in the bark and emerging through the periderm, to assume a flattened conical shape (Nutman and Roberts 1952). Reid and Booth (1969) and Booth and Gibson (1973) describe immersed, becoming erumpent, conical and orange to rust brown stromata containing more than one convoluted to irregular conidial cavity. This is in contrast to *C. cubensis* that has superficial to slightly immersed, cylindrical to pyriform pycnidia-like eustromata with attenuated necks (Bruner 1917, Hodges 1980, Myburg et al. 2002a). These pycnidia are reddish-brown when young, but turn black with age (Bruner 1917, Hodges et al. 1979, Hodges 1980). These inconsistencies in the descriptions of the two fungi continue to raise questions pertaining to the validity of their synonymy.

Descriptions suggest that a fungus, morphologically similar but different to *C. cubensis*, could be present on clove. The possibility thus exists that the second fungus on clove, and not *C. cubensis*, might represent the originally described *E. eugeniae*. A phylogenetic study of isolates of *C. cubensis* based on sequence variation within the internal transcribed spacer (ITS) regions of the ribosomal RNA operon (Myburg et al. 1999), provided support for the synonymy of *C. cubensis* and *E. eugeniae*. These authors, however, noted that their conclusion was based on a single isolate of *E. eugeniae* and that this question should be addressed more closely using additional isolates from clove. Recently, a larger collection of isolates from clove has become available to us. The objective of the present study was, therefore, to reconsider the conspecificity of *E. eugeniae* with *C. cubensis*, based on DNA sequence data from two different gene regions. In addition, a comprehensive morphological study was

undertaken on the original herbarium specimens from clove, as well as newly obtained, fresh specimens from this host.

MATERIALS AND METHODS

Fungal isolates

Isolates used in this study were obtained from culture collections, supplied by colleagues or collected during field studies by the last author (Table 1). These include *C. cubensis* isolated from *Eucalyptus* and *S. aromaticum* from various parts of the world. Sequence data generated for other members of *Cryphonectria* (Myburg et al. 2002b, Venter et al. 2002), i.e. *C. parasitica* (Murr.) Barr, *C. macrospora* (Kobayashi & Ito) Barr, *C. nitschkei* (Oth.) Barr and *C. radicalis* (Schw.: Fr.) Barr, were also included in this study. *Diaporthe ambigua* Nitschke, the causal agent of stem cankers on stone fruit trees (Smit et al. 1996, 1997), was used as the outgroup taxon to root the phylogenetic trees (Table 1). Cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002.

Morphological comparisons

Herbarium specimens from clove listed in the original descriptions (Nutman and Roberts 1952, Reid and Booth 1969) were studied (Table 2). These specimens originated from Zanzibar and Malaysia. New clove material was also collected from Sulawesi, Indonesia (Table 2) and has been deposited in the herbarium of the National Collection of Fungi,

Pretoria, South Africa (PREM). Isolates CMW 8649, CMW 8650 and CMW 8651 (Table 1) originated from these specimens. Specimens of *C. cubensis* from *Eucalyptus* spp., used in the study of Myburg et al. (2002a), were also included (Table 2).

Some isolates originating from clove (CMW 10779, CMW 10780, CMW 10781) had culture morphology different from clove isolates that were thought to represent *C. cubensis*. The cultures were buff (colour 19''f) to hazel (colour 11'k) in contrast to those of *C. cubensis* that were creamy white with cinnamon (colour 15'') patches. Unfortunately no vouchered specimens exist for these isolates.

Fruiting structures of *Cryphonectria* spp. are infrequently produced in culture and are not representative of fruiting structures occurring naturally on bark. Isolate CMW 10781 from clove was, therefore, inoculated into wax-sealed sticks of another member of the Myrtaceae, *Eucalyptus grandis* W. Hill: Maiden clone (ZG 14), to gain additional information on its morphology. Isolates CMW 8649, CMW 8650 and CMW 8651 from clove, known to be *C. cubensis*, were also inoculated into *E. grandis* sticks for comparative purposes. These inoculations were done using the technique described by Van Heerden and Wingfield (2001). Specimens resulting from these inoculations (Table 2) have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

Hodges et al. (1986) performed inoculations on *E. saligna* and clove sticks using *C. cubensis* isolates from *Eucalyptus* and clove. The aim of that study was to consider the effect of clove and *Eucalyptus* bark on the morphology of the infecting fungus. The

specimens from these inoculations were made available to us by Dr. C.S. Hodges, Department of Plant Pathology, North Carolina State University, Raleigh, USA. These specimens (Table 2) have also been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

Structures for morphological study were mounted in Leica mountant (Setpoint Premier, Johannesburg, South Africa) after boiling in water for 1 minute. Specimens were sectioned 12–16 μm using a Leica CM1100 cryostat (Setpoint Premier) at -20°C . Sections were mounted in lacto-phenol and examined microscopically. Ten measurements were taken for conidia and ascospores and are presented as (min-) (mean - SD) - (mean + SD) (-max). The colour notations of Rayner (1970) were used throughout this study.

DNA isolations and PCR

DNA was isolated as previously described by Myburg et al. (1999). Amplification of the ITS region of the ribosomal RNA operon, as well as the two regions within the β -tubulin gene were carried out as described in Myburg et al. (1999) and Myburg et al. (2002a) respectively. The primer pairs used to amplify the two β -tubulin regions were Bt1a with Bt1b and Bt2a with Bt2b (Glass and Donaldson 1995), while ITS 1 and ITS 4 (White et al. 1990) were used to amplify the ITS 1 and ITS 2 region of the ribosomal RNA operon. PCR products were separated on 1% agarose (Promega, Madison, Wisconsin) gels containing ethidium bromide and visualised using an UV light.

Sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The PCR products were sequenced in both directions using the same primers mentioned above. Sequencing reactions were carried out using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom). DNA sequences were determined using an ABI PRISM 3100™ automated DNA sequencer. DNA sequences were verified with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) and aligned using CLUSTAL X (Thompson et al. 1997). The resulting alignment was checked manually.

A Templeton nonparametric Wilcoxon Signed Ranked (WSR) test (Kellogg et al. 1996) was done on a combined sequence data set including aligned β -tubulin and ITS sequences. Results from this test indicated that the data sets could be combined and considered as one data set in subsequent to phylogenetic analyses.

A heuristic search was executed on the aligned data set using PAUP* version 4.0b (Swofford 1998). The TBR (tree-bisection-reconnection) algorithm of the heuristic search (MulTrees option effective, saving all optimal trees) was chosen. Seventeen trees were generated and a strict consensus tree was computed. Gaps were treated as fifth characters (Newstate) and characters were unordered and equally weighted. A bootstrap analysis of 1000 replicates was done to assess the confidence levels of the internodes. The consensus tree was rooted with the two *D. ambigua* isolates. Sequences generated in this study were deposited in GenBank and accession numbers are listed in Table 1.

Sequence alignments are available from TreeBase (Study accession number = S874, Matrix accession number = M1419). Accession numbers of sequence data obtained from previous studies (Myburg et al. 1999, Myburg et al. 2002a ,b, Roux et al. 2003) are also listed in Table 1.

RESULTS

Morphological comparisons

More than one fungus residing in *Cryphonectria* was observed on the various clove specimens included in this study. These fungi had conidia and ascospores similar in size and shape and were difficult to distinguish on the bark, but differed based on position relative to the bark, stromatic tissue types and internal morphology of the stroma. *Cryphonectria cubensis* occurred on bark specimens from Zanzibar, Malaysia and Indonesia. A fungus with orange (colour 15) to sienna (colour 15i) stromata was found on the material from Zanzibar. Furthermore, herbarium materials, originating from inoculation with isolate CMW 10781 from Indonesia, contained fruiting structures with different characteristics to *C. cubensis* or the other fungus with the orange to sienna coloured stromata. These different fungi are discussed in greater detail under the following sections and morphological features are summarised in Table 3.

Cryphonectria cubensis on clove

Structures typical of *C. cubensis* (Table 3) were found on the clove specimens from Zanzibar (IMI 45449, IMI 45450, IMI 45440), Malaysia (IMI 56425a, IMI 58569, IMI

58388, IMI 58567, IMI 58568) and Indonesia (PREM 57470). Conidiomata were either pyriform with attenuated necks (Figs. 1a, 1c), or pulvinate since necks were broken or the structures were not fully developed (Figs. 1b, 1d). The tissue type in these stromata was characteristic of *C. cubensis* (Table 3) with base tissue *textura globulosa* (Fig. 1e) and neck tissue *textura porrecta* (Fig. 1f). Structures with a tissue type resembling that of *C. cubensis* were found on the type specimen of *E. eugeniae* (IMI 44954), but these were too brittle for thorough examination. Conidia (Fig. 1g, Table 3) were similar to those on specimens of *C. cubensis* on *Eucalyptus* (IMI 279614, IMI 304273, PREM 57297, IMI 284438, PREM 57294) and those previously described for *C. cubensis* (Bruner 1917, Hodges 1980, Myburg et al. 2002a, b).

The internal structure of conidiomata of *C. cubensis* was variable on clove. Pulvinate, blackened multilocular structures with convoluted and multilocular conidial chambers below the bark (Fig. 1d) were observed on clove tissue from Zanzibar (IMI 45440). The tissue type of the erumpent parts, as well as the spore shape and size [3.5-4(-4.5) x 1-1.5 µm] were similar to those of *C. cubensis*. The same extent of differences was observed for the clove and *Eucalyptus* material inoculated with *C. cubensis* (PREM 57469) and studied by Hodges et al. (1986). A small number of conidiomata were observed on this material, with structures on clove semi-immersed and conidial locules strongly convoluted and occurring underneath the bark.

The teleomorph of *C. cubensis* (Table 3, Figs. 1 h, 1i) on specimens IMI 45450 and IMI 45440 was frequently observed developing underneath anamorph structures (Fig. 1i). Stromatal development (Table 3) was prosenchymatous, orange (15) to luteous (17) and

restricted to the area around the base of the perithecial necks (Fig. 1j). Ascospores (Fig. 1k) were similar to those of *C. cubensis* (Table 3) as previously observed (Bruner 1917, Hodges 1980).

Other fungi on clove specimens

A fungus with stromatal structure and colour different from that of *C. cubensis* (Table 3) was found on some specimens from Zanzibar (IMI 45452, IMI 44951), studied in the original descriptions of *E. eugeniae* (Nutman and Roberts 1952; Reid and Booth 1969). These structures were only conidiomatal and occurred between structures of *C. cubensis*. They were erumpent, pulvinate (Fig. 2a) with several convoluted locules beneath the bark (Fig. 2b). Stromatic tissue (Table 3) was densely prosenchymatous (Fig. 2c), different from that of *C. cubensis* that is *textura globulosa* (Fig. 1e). Conidia from the orange structures (Fig. 2d) were similar in size and shape to those of *C. cubensis* (Table 3). No isolates exist for these specimens and it is impossible to study them further.

Specimens from Indonesia (PREM 57470) that gave rise to isolates of *C. cubensis* (CMW 8649, CMW 8650, CMW 8651) also contained ascomata different from those of *C. cubensis*. These ascomata superficially resembled the teleomorph of *C. cubensis* and also had one-septate, fusoid ascospores. They differed from *C. cubensis* in that stromatic tissue was densely prosenchymatous and orange to sienna. The latter characteristics were similar to those of the orange to sienna fungus on specimens from Zanzibar, but thorough comparisons between the fungus from Zanzibar and the Indonesian structures were hindered by the fact that stromata of the Indonesian specimens were very few in number. Furthermore, no isolates exist for these structures and for the present, we are

unable to draw a definitive conclusion regarding the identity of the fungus associated with these structures on the Indonesian material.

Eucalyptus sticks (PREM 57473) that had been inoculated with isolate CMW 10781 from Indonesia, showed structures different to those arising from the *C. cubensis* isolates (CMW 8649, CMW 8650, CMW 8651). Conidiomata (Table 3) were blackened (Fig. 3a), sometimes with a luteous (19) apex, with pseudoparenchymatous tissue (Figs. 3b, 3c). These structures and the tissues associated with them were also different from those of the other fungus (IMI 45452, IMI 44951) with orange to sienna stromata from Zanzibar (Figs. 2b, 2c). Conidia were also distinct in being more cylindrical (Fig. 3d), but length measurements were in the same size range as *C. cubensis* (Table 3). The shape and internal structure of the fruiting bodies were too variable to draw any definite conclusions on the identity of this fungus. The *C. cubensis* isolates, however, produced ascomata and conidiomata that were similar to those of *C. cubensis* on the clove specimens from nature and showed little variation amongst each other.

Sequencing

Amplification products for the DNA regions considered in this study were approximately 600 bp (ITS) and 550 bp (β -tubulin) in size. A combined sequence data set, comprising of ITS ribosomal and β -tubulin gene sequences included 1505 aligned sequence (Appendix 3) characters, of which 879 were constant, 40 parsimony-uninformative and 586 parsimony-informative. A strict consensus tree (tree length = 1198 steps,

consistency index/CI = 0.8 and retention index/RI = 0.9) was computed (Fig. 4) from the seventeen trees generated in the heuristic search.

The phylogram generated for the combined sequence data set (Fig. 4) showed three groups of fungi, clustering separately from the outgroup taxon represented by the *D. ambigua* isolates. The first clade (bootstrap support 100%) represents *C. cubensis* isolated from *Eucalyptus* species and clove originating from Southeast Asia (bootstrap support 98%), South America (bootstrap support 80%) and South Africa (bootstrap support 62%). The second group represents isolates that also originated on clove in Indonesia (bootstrap support 100%). A third group (bootstrap support 100%) is characterised by *C. parasitica*, *C. radicalis*, *C. macrospora* and *C. nitschkei* and represents species that characterise the genus *Cryphonectria sensu stricto* (Myburg et al. 2002a).

Three sub-groups of fungi make up the *C. cubensis* clade. These groups, previously identified by Myburg et al. (2002a), represent three geographical areas, where *C. cubensis* is known to occur. In the present study, one group represents *C. cubensis* isolated from *Eucalyptus* and clove originating from countries in Southeast Asia and Australia (bootstrap support 98%). The clove isolates (CMW 8649, CMW 8650, CMW 8651) from Indonesia as well as the Indonesian clove isolate (CMW 3839) used in the study of Myburg et al. (1999) clustered within this Southeast Asian/Australian clade. A clove isolate from Zanzibar (CMW 10774) also grouped in the Southeast Asian clade.

The second group within the *C. cubensis* clade (bootstrap support 80%) included Brazilian isolates from clove (CMW 10775, CMW 10776, CMW 10777, CMW 10778) as well as Brazilian (CMW 1853) and Venezuelan (CMW 8757) *C. cubensis* isolates from *Eucalyptus*. This clade also contained *C. cubensis* isolates from the Congo (CMW 10667, CMW 10668) that have been reported previously to group within the South American sub-clade (Roux et al. 2003).

The third sub-clade in the larger *C. cubensis* group included isolates originating from South Africa (bootstrap support 62%). This clade grouped separately from the South American and Southeast Asian *C. cubensis* group (bootstrap support 100%) and appears to represent a distinct taxon as previously shown by Myburg et al. (2002a).

A group of isolates originating on clove in Indonesia (CMW 10779, CMW 10780, CMW 10781) formed a separate and discrete clade, separately from the other clove isolates in the *C. cubensis* clade (bootstrap support 100%). This group was also separate from *C. parasitica*, *C. radicalis*, *C. macrospora* and *C. nitschkei* (bootstrap support 100%). The isolates in this clade were those that had cultural and morphological characteristics different to those of *C. cubensis*.

DISCUSSION

In this study, we have been able to confirm unequivocally that *C. cubensis* occurs on clove. This was based on ribosomal ITS and β -tubulin gene sequence data for fungi isolated from clove originating from South America, Indonesia and central Africa. We have linked these results to morphological characteristics for relevant herbarium

specimens collected from clove in Indonesia, Malaysia and Zanzibar. However, morphological and phylogenetic data from this study also indicate the presence of other fungi related to *Cryphonectria* occurring on clove.

The presence of a fungus other than *C. cubensis* on the clove specimens used in the original description of *E. eugeniae* raises doubt as to which fungus was referred to in the original description of *E. eugeniae*. *Cryphonectria cubensis* and the second fungus with orange stromata are similar and their conidia are undistinguishable. It is, therefore, likely that previous workers could have unwittingly assumed that these fungi represented a single taxon. The teleomorph description of *E. eugeniae* clearly refers to *C. cubensis*, since it describes perithecia developing below the conidiomata (Nutman and Roberts 1952, Reid and Booth 1969). The description of the anamorph of *E. eugeniae*, however, could relate to either *C. cubensis*, or the fungus with the orange anamorph, which we have found on specimens. The identity of *E. eugeniae* is connected to the type specimen of this fungus (IMI 44954), which contains structures with the same tissue type as *C. cubensis*. The synonymy of *E. eugeniae* with *C. cubensis* is, therefore, valid and the other fungi occurring on clove will require independent names.

A fungus, represented by isolates CMW 10779, CMW 10780 and CMW 10781, different from both *C. cubensis* and the fungus with orange to sienna stromata from Zanzibar, was isolated from cankers on clove in northern Sumatra and Kalimantan, Indonesia. DNA sequence data clearly show that this fungus is different from *C. cubensis* and the other *Cryphonectria* sp., yet it is closely related. These isolates could not be connected to morphological structures on host tissue. Bark inoculations on *Eucalyptus* yielded

information on conidial and tissue morphology, but structural morphology was too variable to be used in descriptions. Additional specimens and isolates of this third fungus on clove will be necessary before a name can be provided for it.

Results of this study have shown that *C. cubensis* occurs on clove in South America, Southeast Asia and central Africa. Isolates from clove reside in two phylogenetic groups that were previously defined by Myburg et al. (1999, 2002a) for *C. cubensis* isolates from *Eucalyptus*. It was interesting to discover that *C. cubensis* isolates from central Africa included those from both the Southeast Asian and South American phylogenetic lineages. These phylogenetic data suggest that *C. cubensis* has been introduced into Africa on two separate occasions. South African *C. cubensis* isolates, however, clearly reside in a separate lineage with a distinct origin as recently shown by Myburg et al. (2002a).

The presence of *Cryphonectria* spp. on clove appears to be considerably more complex than previously known. Based on detailed comparisons of DNA sequence and morphological characteristics, we have found that at least two closely related and similar fungi can occur on a single clove specimen. The lack of cultures linked to herbarium specimens has made conclusive identifications of these fungi difficult. However, there is good evidence to show that at least three different species of *Cryphonectria* occur on clove and future collections should make it possible to provide names for the two unidentified species.

ACKNOWLEDGEMENTS

We are grateful to Dr. C.S. Hodges who provided a wide range of collections used in this study and for his valuable advice. We also thank many colleagues including Dr. Brett Summerell, Dr. Dan Sembel, Dr. Edward Lieuw, Mr. Paul Clegg, Dr. Elaine Davison and Dr. Ken Old for either supplying cultures or specimens, or for making it possible to collect material used in this study. We are also grateful to the curators of many herbaria listed in Table 2 for loans of specimens. This study was made possible by grants from the National Research Foundation (NRF), members of the Tree Pathology Co-operative Programme (TPCP) and the THRIP support programme of the Department of Trade and Industry, South Africa.

REFERENCES

- Alfenas, A.C., Hubbes, M. and Conto, L.** 1982. Effects of phenolic compounds from *Eucalyptus* on the mycelial growth and conidial germination of *Cryphonectria cubensis*. *Canadian Journal of Botany* **60**: 2535-2541.
- Alfenas, A.C., Jeng, R. and Hubbes, M.** 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**: 197-205.
- Anonymous.** 1954. Notes on current investigations, research July to September, 1954. *Reviews in Applied Mycology* **34**: 577-578.
- Boerboom, J.H.A. and Maas, P.W.T.** 1970. Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**: 94-99.
- Booth, C. and Gibson, I.A.S.** 1973. *Endothia eugeniae*. CMI Descriptions of Pathogenic Fungi and Bacteria No. 363, Commonwealth Mycological Institute, Kew, England.
- Bruner, S.C.** 1917. Una enfermedad gangrenosa de los eucaliptos. *Estacion Experimental Agronomica, Santiago de las Viegas, Cuba Bolletin* **37**: 1-33.
- Glass, N.L. and Donaldson, G.C.** 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.
- Heath, R.G.** 1956. Annual report of the Department of Agriculture, Malaya, for the year 1955. *Reviews in Applied Mycology* **37**: 7-8.
- Hodges, C.S., Geary, T.F. and Cordell, C.E.** 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. *Plant Disease Reporter* **63**: 216-220.

- Hodges, C.S.** 1980. The taxonomy of *Diaporthe cubensis*. *Mycologia* **72**: 542-548.
- Hodges, C.S., Alfenas, A.C. and Cordell, C.E.** 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**: 334-350.
- Kellogg, E.A., Appels, R. and Mason-Gamer, R.J.** 1996. When genes tell different stories: the diploid genera of *Triticeae* (Gramineae). *Systematic Botany* **21**: 321-347.
- Micales, J.A., Stipes, R.J. and Bonde, M.R.** 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. *Mycologia* **79**: 70-720.
- Myburg, H., Wingfield, B.D. and Wingfield, M.J.** 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**: 243-250.
- Myburg, H., Gryzenhout, M., Wingfield, B.D. and Wingfield, M.J.** 2002a. β -tubulin and Histone *H3* gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia and South America. *Canadian Journal of Botany* **80**: 590-596.
- Myburg, H., Gryzenhout, M., Heath, R., Roux, J., Wingfield, B.D. and Wingfield, M.J.** 2002b. *Cryphonectria* canker on *Tibouchina* in South Africa. *Mycological Research* **106**: 1299-1306.
- Nutman, F.J. and Roberts, F.M.** 1952. Acute die-back of clove trees in the Zanzibar Protectorate. *Annals in Applied Biology* **39**: 599-607.
- Rayner, R.W.** 1970. *A Mycological Colour Chart*. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey, U.K.
- Reid, J. and Booth, C.** 1969. Some species segregated from the genera *Cryptospora*, *Cryptosporella*, and *Sillia*. *Canadian Journal and Botany* **47**: 1055-1060.

- Roux, J., Myburg, H., Wingfield, B.D. and Wingfield, M.J.** 2003. Two *Cryphonectria* species causing economically important diseases of *Eucalyptus* in Africa. *Plant Disease* (in press).
- Smit, W.A., Viljoen, C.D., Wingfield, B.D., Wingfield, M.J. and Calitz, F.J.** 1996. A new canker disease of apple, pear, and plum rootstocks caused by *Diaporthe ambigua* in South Africa. *Plant Disease* **80**: 1331-1335.
- Smit, W.A., Wingfield, B.D. and Wingfield, M.J.** 1997. Vegetative incompatibility in *Diaporthe ambigua*. *Plant Pathology* **46**: 366-372.
- Swofford, D.L.** 1998. PAUP. Phylogenetic Analysis Using Parsimony, version 4.0b1. Sinauer Associates, Sunderland, Massachusetts.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G.** 1997. The CLUSTAL W windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.
- Van Heerden, S.W. and Wingfield, M.J.** 2001. Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycological Research* **105**: 94-99.
- Van Zyl, L.M. and Wingfield, M.J.** 1999. Wound response of *Eucalyptus* clones after inoculation with *Cryphonectria cubensis*. *European Journal of Forest Pathology* **29**: 161-167.
- Venter, M., Myburg, H., Wingfield, B.D., Coutinho, T.A. and Wingfield M.J.** 2002. A new species of *Cryphonectria* from South Africa and Australia, pathogenic on *Eucalyptus*. *Sydowia* **54**: 98-117.
- White, T.J., Bruns, T., Lee, S. and Taylor, J.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A.,

Gelfand, D.H., Sninsky, J.J. and White, T.J., eds. PCR Protocols: a guide to methods and applications. Academic Press, San Diego. pp. 315-322.

Wingfield, M.J., Swart, W.J. and Abear, B. 1989. First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**: 311-313.

Table 1. Isolates used in sequencing analyses.

Culture no. ^a	Species	Host	Origin	Collector	GenBank accession numbers for ITS and β -tubulin sequence data
CMW 10774	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Tanzania, Zanzibar	n.a	AF 492130, AF 492131, AF 492132
CMW 10775	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	C.S. Hodges	AY 084003, AY 084015, AY 084027
CMW 10776	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	C.S. Hodges	AY 084004, AY 084016, AY 084028
CMW 10777	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	C.S. Hodges	AY 084005, AY 084017, AY 084029
CMW 10778	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	C.S. Hodges	AY 084006, AY 084018, AY 084030
CMW 3839	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Indonesia	M.J. Wingfield	AF 046904, AY 084011, AY 084023
CMW 8649	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Sulawesi, Indonesia	M.J. Wingfield	AY 084000, AY 084012, AY 084025
CMW 8650	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Sulawesi, Indonesia	M.J. Wingfield	AY 084001, AY 084013, AY 084024
CMW 8651	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Sulawesi, Indonesia	M.J. Wingfield	AY 084002, AY 084014, AY 084026
CMW 8756	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Indonesia	M.J. Wingfield	AF 046896, AF 273077, AF 285165
CMW 9903	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Kalimantan, Indonesia	C.S. Hodges	AF 292044, AF 273066, AF 273461
CMW 9906	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Kalimantan, Indonesia	C.S. Hodges	AF 292045, AF 273065, AF 273460
CMW 1853	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	n.a	AF 046891, AF 273070, AF 273465
CMW 8757	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Venezuela	M.J. Wingfield	AF 046897, AF 273069, AF 273464
CMW 10667	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Republic of Congo	J. Roux	AY 063477, AY 063479, AY 063481
CMW 10668	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Republic of Congo	J. Roux	AF 535121, AF 535123, AF 535125
CMW 1856	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Hawaii	n.a	AY 083999, AY 084010, AY 084022
CMW 2631	<i>Cryphonectria cubensis</i>	<i>Eucalyptus marginata</i>	Australia	E. Davison	AF 543823, AF543824, AF523825
CMW 2632	<i>Cryphonectria cubensis</i>	<i>Eucalyptus marginata</i>	Australia	E. Davison	AF 046893, AF 273078, AF 375607
CMW 2113	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
CMW 62	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	AF 292041, AF 273063, AF 273458
CMW 8755	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	AF 292040, AF 273064, AF 273458
CMW 10463	<i>Cryphonectria macrospora</i>	<i>Castanopsis cuspidata</i>	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350
CMW 10518	<i>Cryphonectria nitschkei</i>	<i>Quercus</i> sp.	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
CMW 10455	<i>Cryphonectria radicalis</i>	<i>Quercus suber</i>	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	<i>Cryphonectria radicalis</i>	<i>Quercus suber</i>	Italy	A. Biraghi	AF 368328, AF 368347, AF 368346
CMW 7047	<i>Cryphonectria parasitica</i>	<i>Quercus virginiana</i>	USA	R.J. Stipes	AF 368329, AF 273073, AF 273469
CMW 7048	<i>Cryphonectria parasitica</i>	<i>Quercus virginiana</i>	USA	R.J. Stipes	AF 368330, AF 273076, AF 273470
CMW 10779	<i>Cryphonectria</i> sp.	<i>Syzygium aromaticum</i>	Somosir, Indonesia	M.J. Wingfield	AY 084007, AY 084019, AY 084031
CMW 10780	<i>Cryphonectria</i> sp.	<i>Syzygium aromaticum</i>	Somosir, Indonesia	M.J. Wingfield	AY 084008, AY 084020, AY 084032
CMW 10781	<i>Cryphonectria</i> sp.	<i>Syzygium aromaticum</i>	Kalimantan, Indonesia	M.J. Wingfield	AY 084009, AY 084021, AY 084033
CMW 5288	<i>Diaporthe ambigua</i>	<i>Malus domestica</i>	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821
CMW 5587	<i>Diaporthe ambigua</i>	<i>Malus domestica</i>	South Africa	W.A. Smit	AF 543818, AF 543820, AF 543822

^aCulture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

Table 2. Specimens used in morphological comparisons.

Herbarium no.*	Identity	Host	Origin	Date	Collector
IMI 44954 (holotype)	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44945	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45440	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45445	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45443	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45448a	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	n.a.
IMI 45446	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44953	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44951	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45452	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 49266	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	n.a.	J. Nutman & F.M. Roberts
IMI 45449	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	n.a.
IMI 45450	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	1951	n.a.
IMI 279614	<i>Cryphonectria cubensis</i>	<i>Eucalyptus urophylla</i>	Cameroon	1983	I.A.S. Gibson
IMI 56425a	<i>Endothia eugeniae</i>	Isolate ex <i>Eugenia</i> sp. on elm twigs	Malaysia	1954	W.J. Cherewick
IMI 58569	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Malaysia	1954	A. Johnston
IMI 58388	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Malaysia	1954	A. Johnston
IMI 58567	<i>Endothia eugeniae</i>	<i>Eugenia</i> sp.	Malaysia	n.a.	A. Johnston
IMI 58568	<i>Endothia eugeniae</i>	<i>Eugenia</i> sp.	Malaysia	1954	A. Johnston
IMI 350626	<i>Cryphonectria cubensis</i>	<i>Syzygium aromaticum</i>	Singapore	1991	C.P. Yik
PREM 57469	<i>Cryphonectria cubensis</i>	Inoculations into <i>E. saligna</i> and <i>S. aromaticum</i>	n.a.	1986	C.S. Hodges
PREM 57470 [§]	<i>Cryphonectria cubensis</i> and unknown fungus	<i>Syzygium aromaticum</i>	Sulawesi, Indonesia	2001	M.J. Wingfield
PREM 57471	<i>Cryphonectria cubensis</i>	Inoculation of CMW 8649 into <i>E. grandis</i>	n.a.	2002	M. Gryzenhout
PREM 57472	<i>Cryphonectria cubensis</i>	Inoculation of CMW 8650 into <i>E. grandis</i>	n.a.	2001	M. Gryzenhout
PREM 57473	unknown	Inoculation of isolate CMW 10781 into <i>E. grandis</i>	n.a.	2001	M. Gryzenhout
IMI 304273	<i>Cryphonectria cubensis</i>	<i>Eucalyptus aromatica</i>	Malaysia	1986	Low Chow Fong
PREM 57297	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Indonesia	2001	M.J. Wingfield
IMI 284438	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i> / <i>Eugenia</i> sp.	Venezuela	1983	C.S. Hodges
PREM 57294	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	Colombia	2000	M.J. Wingfield

* PREM, National Collection of Fungi, Pretoria, South Africa.

* IMI, Herbarium, CABI Bioscience, Bakeham Lane, Egham, Surrey TW20 9TY, UK.

[§] Vouchered specimens linked to isolates CMW 8649 (PREM 57471), CMW 8650 (PREM 57472) and CMW 8651.

Table 3. Key morphological characteristics of the different fungi found on herbarium material of clove.

Fungus	Origin	Conidioma				Ascoma			
		External colour	Structure	Stromatic tissue	Conidia	Stroma colour	Structure	Stromatic tissue	Ascospores
<i>Cryphonectria cubensis</i>	Zanzibar, Malaysia, Indonesia	Dark mouse grey (15''''k)	Pyriform with attenuated neck, or pulvinate, unilocular occasionally multilocular, convoluted	Umber (15m), base <i>textura globulosa</i> , neck <i>textura porrecta</i>	Oval to ovoid, aseptate, 3-4 (-4.5) x 1-1.5 (-2) µm	Orange (15) to pale luteous (19d) stroma blackened perithecial necks	Semi-immersed, slightly erumpent, frequently formed underneath conidioma	Limited, diffuse, prosenchymatous	Fusoid, one-septate, (5-) 6-7.5 (-8) x 1.5-2.5 µm
Orange to sienna fungus	Zanzibar	Orange (15) to sienna (15i)	Erumpent, elongated pulvinate, convoluted multilocular	Orange (15) to sienna (15i), lower part often lighter, dense, prosenchymatous	Oval to ovoid, aseptate, (3-) 3.5-4 (-4.5) x 1-1.5 (-2) µm	-	-	-	-
CMW 10781 artificial inoculation	Indonesia	Blackened with luteous (19) apex	Generally ovoid	Umber (15m), pseudoparenchymatous	Cylindrical aseptate, (2.5-) 3-3.5 (-4) x 1 µm	n.a.	n.a.	n.a.	n.a.

Fig. 1. Light micrographs of *Cryphonectria cubensis* occurring on clove. **a.** Pyriform conidiomata. **b.** Pulvinate conidiomata without necks. **c.** Longitudinal section through conidioma with neck attached. **d.** Longitudinal section through multilocular, pulvinate conidioma. **e.** Base tissue. **f.** Neck tissue. **g.** Conidia. **h.** Ascomata. **i.** Longitudinal section through perithecia occurring underneath conidioma (arrow). **j.** Stromatic tissue of ascoma. **k.** Ascospores. (Scale bars: **a, h** 200 μ m; **b-d, i**; 100 μ m; **e-g, j, k** 10 μ m.).

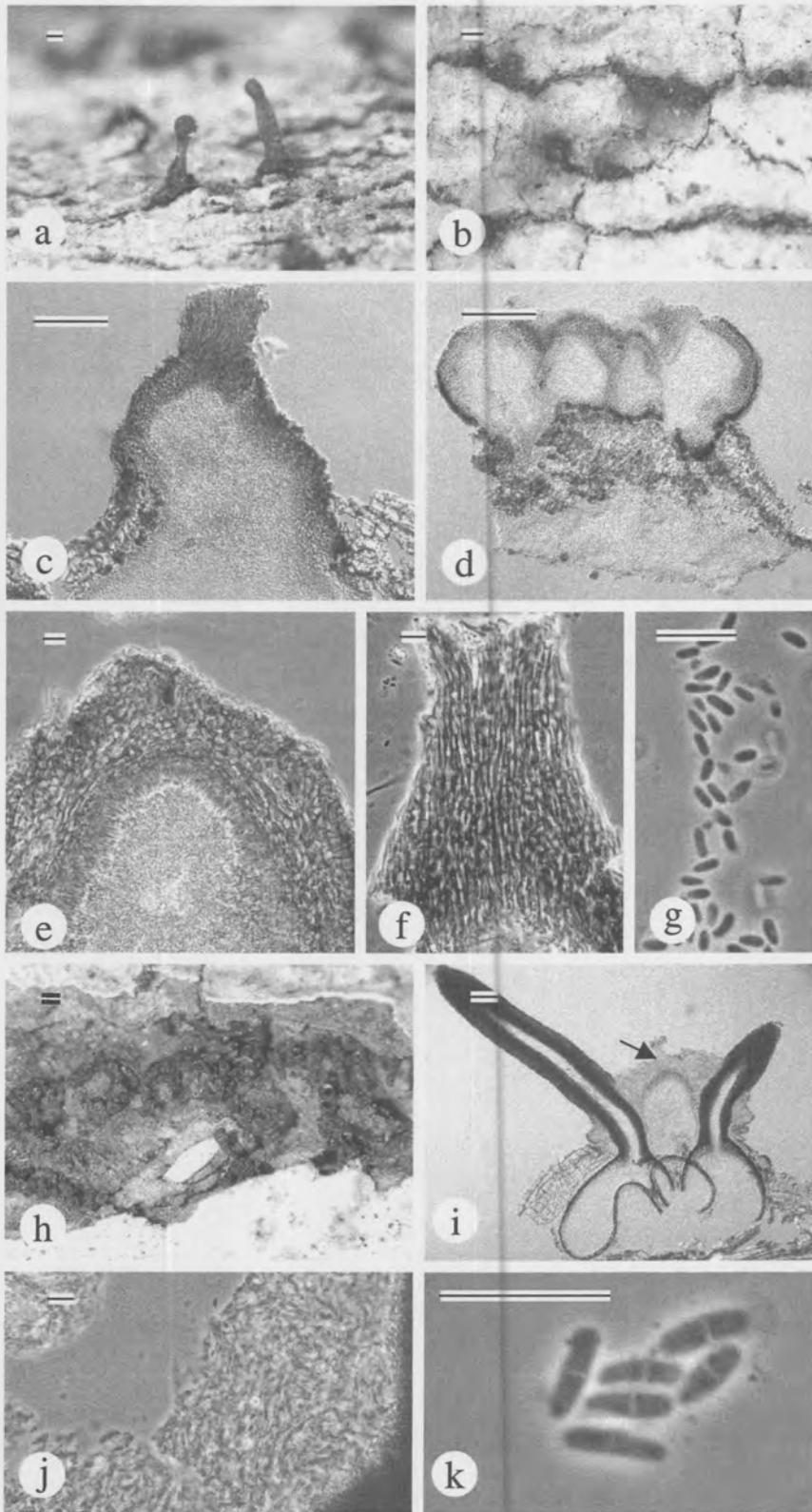


Fig. 2. Light micrographs of the unknown fungus with an orange to sienna anamorph occurring on clove in Zanzibar. **a.** Conidioma. **b.** Longitudinal section through conidioma. **c.** Stromatic tissue. **d.** Conidia. (Scale bars: **a, b**, 100 μm ; **c, d** 10 μm .)

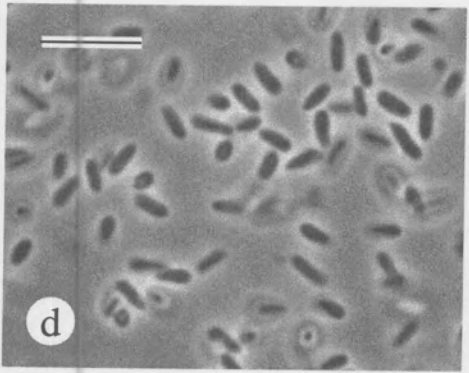
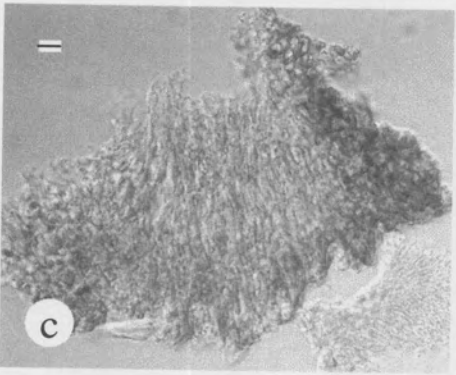
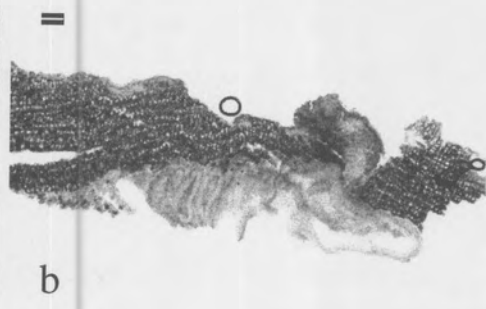
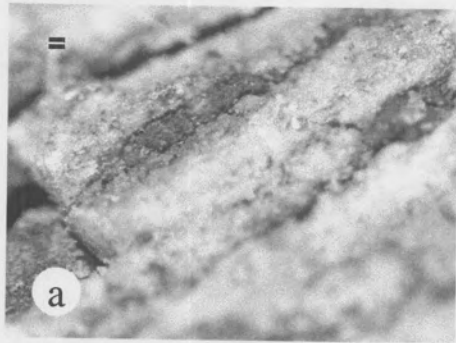


Fig. 3. Light micrographs of fruiting structures produced by isolate CMW 10781 in artificial inoculations on *Eucalyptus* sticks. **a.** Longitudinal section through superficial conidioma. **b-c.** Stromatic tissue. **d.** Conidia. (Scale bars: **a** 100 μm ; **b, c, d** 10 μm).

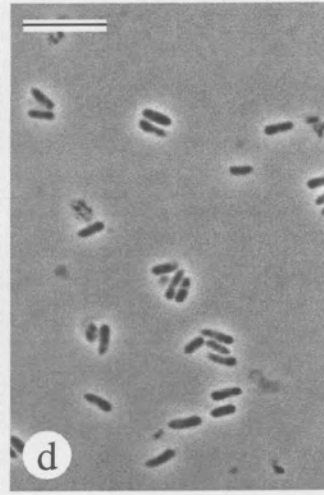
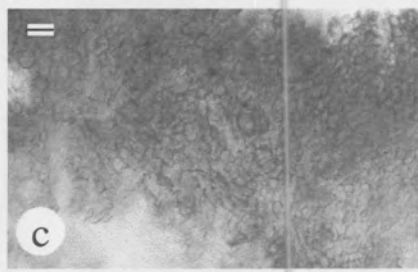
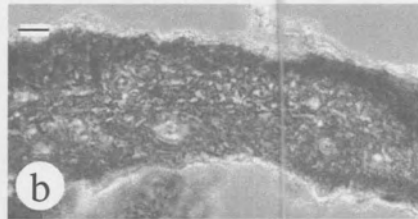


Fig. 4. Strict consensus tree (tree length = 1198 steps, CI = 0.8, RI = 0.9) computed from seventeen trees generated after heuristic search of a combined data set including ribosomal DNA and β -tubulin gene sequences. Bootstrap values (1000 replicates) are indicated above the inter nodes. Taxa in bold represent those sequenced in the present study. The *Diaporthe ambigua* isolates included in this study were used as outgroups to root the phylogenetic tree.

Tree length = 1198 steps
 CI = 0.8
 RI = 0.9

