CERATOCYSTIS SPECIES ON TIMBER HARVESTED FOR EXPORT IN GHANA

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Ceratocystis species on timber harvested for export in Ghana

By

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DECLARATION

I, the undersigned, hereby declare that this thesis, submitted herewith for the degree *Magister Institutionis Agrariae* to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University.

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DEDICATION

I dedicate this thesis to my Late Father, Francis Fon Forcob and Mother, Elizabeth Forcob.

CONTENTS

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PREFACE

There has been an increase in research on the genus *Ceratocystis,* particularly in Africa, in the past ten years. This is due to the fact that these fungi represent economically important pathogens of agricultural and forestry crops, causing diseases of, for example, coffee, cacao, sweet potato, taro, oak and eucalypt trees globally. Despite this, little is known about the genus in Ghana. During studies of wood rot and other fungi in Ghana, the oak wilt pathogen, *C. fagacearum,* was reported from native Ghanaian trees. Previous to this report from Ghana, *C. fagacearum* was only known from oak trees and only from the United States of America, making this report from unrelated hosts in Ghana, highly significant. However, some doubt exists as to the accuracy of this identification since the fungus was identified based only on morphological observations. This study aims to try and confirm the occurrence of *C. fagacearum* in Ghana by identifying *Ceratocystis* isolates collected from the same hosts and from the same areas in the country, using modern molecular techniques.

Chapter one deals with an overview of the genus *Ceratocystis*, with a particular focus on its taxonomy and identification of species in the genus. The ecology and importance of *Ceratocystis* spp. is covered, including its movement and dispersal. A particular focus is also placed on information regarding the species that are known to occur in Africa, since this forms an important component of any quarantine system. The importance of *Ceratocystis* species as quarantine pests is, therefore, also discussed. This chapter concludes with a discussion of the features that enable *Ceratocystis* species to be listed as pests of quarantine interest.

Generally, the specific identity of a pathogen is a pre-requisite for proper classification and an understanding of its ecology and impact. Chapter two aims to identify isolates of *Ceratocystis* collected from logs of native trees in Ghana, using DNA sequence data and multiple-gene phylogenies. In using sequence data, three gene regions, the internal transcription spacer regions (ITS), including the 5.8S gene, the Beta-tubulin (BT) and transcription elongation factor-1α (TEF), are considered to compare isolates from Ghana with previously described *Ceratocystis* species to determine their identity.

This work was done over a period of five months using a collection of isolates from Ghana. Since the project was designed as a mini-thesis and was based only on a pilot study in Ghana,

more research is needed to clarify the morphology, ecology, distribution and impact of *Ceratocystis* spp. on both native and non-native plants in that country. This is especially important since vast quantities of native timber are exported to Europe and other regions, forming an important basis of the economy of Ghana.

CHAPTER 1

THE GENUS *CERATOCYSTIS*, WITH PARTICULAR REFERENCE TO ITS POTENTIAL AS QUARANTINE THREAT TO FORESTRY: A REVIEW

1.1 INTRODUCTION

The genus *Ceratocystis* Ellis & Halsted was established more than 100 years ago to accommodate the fungal pathogen of sweet potato (*Ipomoea batatas*), *Ceratocystis fimbriata* Ellis & Halst. *sensu stricto* (s.s) (Halsted & Fairchild 1891), and is today known as one of the most important groups of plant and tree pathogens of quarantine importance globally (Kile 1993, Baker & Harrington 2004, Roux & Wingfield 2009). *Ceratocystis* species have been implicated as the causes of important tree diseases such as oak wilt in the United States of America (USA) (Bretz 1952, Sinclair *et al*. 1987, Juzwik *et al*. 2008, Koch *et al.* 2010), wilt of *Acacia mearnsii* de Wild. trees in Eastern and Southern Africa (Morris *et al*. 1993, Roux *et al*. 1999b, Roux & Wingfield 2009), canker and wilt disease of coffee (Pontis 1951), cacao (Schieber 1969, Sanches *et al.* 2008) and mango (Al-subhi *et al.* 2006), to name but a few. Because of their economic impact, both in agriculture and forestry, *Ceratocystis* species are important quarantine threats. The following species have been listed to be of quarantine risk: *C. fimbriata* (South Africa, Turkey and USA), *C. fagacearum* (Bretz) J. Hunt and *C. platani* (J.M. Walter) Engelbr. & T.C. Harr. in the USA and Europe (ICPR 1999, Baker *et al.* 2004).

Ceratocystis spp. can be transmitted in several ways, including cuttings and propagating material (Baker *et al*. 2004), storage roots (Thorpe *et al.* 2005), soil (Hicks *et al*. 1980, Rossetto & Ribeiro 1990), vectored by nitidulid and some by bark-inhabiting insects (De Vay *et al*. 1963, Moller & De Vay 1968, Kile 1993, Heath *et al*. 2009a), silviculture equipment (Geldenhuis *et al.* 2004) as well as natural phenomena such as strong winds and hail (Walter *et al.* 1952, Vigouroux & Stojadinovic 1990). The movement of host material, including wood packing material (WPM) (Engelbrecht & Harrington 2005, Haack 2006, Brockerhoff *et al*. 2010), carries a high risk in *Ceratocystis* transmission if proper control measures are not in place.

Wood is a very important commodity, providing material for medicine, construction, furniture, fuel and pulp for paper (Carle *et al.* 2002). However, the natural forest supply base of current wood is falling short of demand in many countries (Fenning & Gershenzon 2002). The loss of 10 million hectares of rainforest in the tropics and subtropics, mostly through deforestation (Norris *et al.* 2010) has boosted extensive afforestation projects with exotic softwood species (Burgess & Wingfield 2002, Carle *et al*. 2002). The establishment of non-native forest plantations, co-existing with native forests, and an increase in the international

trade in plants is linked to an increase in incidences of pests and diseases which can be damaging in new ecosystems (Brasier 2008, Wingfield *et al*. 2008). The aim of this review is to provide a background for the research conducted in this mini-thesis, focusing on *Ceratocystis* species in Ghana. Particular emphasis is placed on the importance of this genus as quarantine pests, including difficulties in the taxonomy of *Ceratocystis* species, their ecology and pathways of spread. A focus is also placed on information regarding these fungi on particularly forest trees in Africa.

1.2 TAXONOMY OF THE GENUS *CERATOCYSTIS*

The genus *Ceratocystis* belongs to the ascomycota group of fungi and has traditionally been known as part of an aggregate group of morphologically similar fungi, the ophiostomatoid fungi. The ophiostomatoid fungi include fungi in at least two different orders, including well-known genera such as *Ophiostoma* H. Syd. & P. Syd., *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Ambrosiella* Arx & Hennebert, *Thielaviopsis* Went and *Gondwanamyces* G.J. Marais & M.J. Wingf. (Hunt 1956, Kile 1993, Wingfield *et al.* 1993, Marais *et al*. 1998, Viljoen *et al*. 1999, Réblová *et al*. 2011). *Ceratocystis* species and their anamorphs reside in the *Ceratocystidaceae* Locq. Ex Réblová, W. Gams & Seifert, fam. nov., one of four families currently recognized in the Microascales (Table 1) (Réblová *et al*. 2011).

Since the identification and description of *C. fimbriata* towards the end of the 19th century (Halsted & Fairchild 1891), there has been controversy surrounding the taxonomy of the genus *Ceratocystis* (Wingfield *et al.* 1993). Members, of what was known as the ophiostomatoid fungi, are characterized by the production of sexual states with globose ascomatal bases with long necks (Fig. 1a), at the tips of which sticky spore drops accumulate for easy insect disposal (Hunt 1956, Wingfield *et al.* 1993). This similarity in structure and the convergent evolution with insect dispersal has been the major reason for the confusion in the taxonomy of the ophiostomatoid fungi, as many of them are morphologically indistinguishable, except to experts in the field. This morphological similarity led to the situation where genera in two Orders, the Microascales and Ophiostomatales, and several families have for many years been confused (Réblová *et al*. 2011). This makes these fungi especially difficult to deal with in regards to accurate identification for quarantine purposes.

Several techniques have been used to classify *Ceratocystis* species and other fungi included in the ophiostomatoid fungi. These include ascospore characteristics (Hunt 1956, Upadhyay & Kendrick 1975), cell wall structure (Rosinski & Campana 1964, Smith *et al*. 1967, Spencer & Gorin 1971, Jewell 1974, Weijman & De Hoog 1975), biochemistry (Schneider 1956, Hicks *et al*. 1980, Harrington 1981), ultrastructure of centrum development (Van Wyk *et al.* 1991, Van Wyk *et al*. 1993), mating studies (Harrington & McNew 1997, Witthuhn *et al*. 2000, Engelbrecht & Harrington 2005), as well as DNA sequence data (Hausner *et al*. 1993, Spatafora & Blackwell 1993, 1994, Réblová *et al*. 2011). However, the most distinctive characteristic separating the genus *Ceratocystis* from other Ophiostomatoid fungi reside in their anamorph states. *Ceratocystis* species have their anamorph in *Thielaviopsis*, with enteroblastic conidiogenesis (Paulin-Mahady *et al.* 2002), while anamorph states of *Ophiostoma* reside in the genera *Pesotum* Crane (Upadhyay 1993) and *Sporothrix* Hektoen & Perkins (De Hoog & Scheffer 1984, Wingfield *et al*. 1993), with holoblastic conidial development. *Ceratocystiopsis* has its anamorph in *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. and *Grosmannia* Goid with a *Leptographium* Lagerb & Melin anamorph (Upadhyay 1981, Zipfel *et al*. 2006).

The advent of DNA sequencing and phylogenetic studies has made the identification of ophiostomatoid fungi much more reliable (Hausner *et al*. 1993, Witthuhn *et al*. 1999, Zipfel *et al*. 2006, Mullineux & Hausner 2009). The confusion over the taxonomic and phylogenetic placement of the genera within the ophiostomatoid fungi is no longer a matter of so much debate, and it is now clear that the genera, such as *Ceratocystis* and *Ophiostoma,* belong to different orders of the ascomycota (Spatafora & Blackwell 1994). The genus *Ceratocystis* resides in the family Ceratocystidaceae, order *Microascales*, while *Ophiostoma* resides in the family Ophiostomataceae, order *Ophiostomatales* (Fig. 2) (Spatafora & Blackwell 1994, Réblová *et al*. 2011). DNA sequence data comparisons have further been used to confirm the status of *Grosmannia* and *Ceratocystiopsis*, which represent distinct genera residing in the Ophiostomataceae, and different from *Ophiostoma* species (Zipfel *et al*. 2006). Species placed in the genus *Gondwanamyces* reside in the order *Microascales,* but are phylogenetically unrelated to *Ceratocystis* having their own family, the *Gondwanamycetaceae* Réblová, W. Gams & Seifert, fam. nov., although they share a number of morphological characteristics with species of *Ceratocystis* and *Ophiostoma* (Viljoen *et al*. 1999, Réblová *et al*. 2011).

The delineation of species within the genus *Ceratocystis* remains problematic, with several species complexes and cryptic species within the genus. At least four phylogenetic species complexes are recognized within the genus *Ceratocystis* (Marin & Wingfield 2006). These include the *C. fimbriata sensu lato* (s.l.) (Wingfield *et al.* 1996), *C. moniliformis* s.l. (Hedgc.) C. Moreau (Witthuhn *et al.* 1999, Van Wyk *et al*. 2006), *C. paradoxa* (Dade) C. Moreau (Moreau 1952) and *C. coerulescens* (Munch) B.K. Bakshi (Kile 1993, Harrington *et al*. 1996, Witthuhn *et al*. 1998) species complexes. It has been suggested that these species clades actually represent distinct genera (Wingfield *et al*. 2006, Harrington 2009).

Within the larger species clades in *Ceratocystis* s.l., significant problems in delineating species persist. Initially, disease of sweet potato (Halsted & Fairchild 1891), canker of stone fruits (De Vay *et al*. 1965) and wilt and death of eucalypts (Roux *et al*. 2004) were all ascribed to *C. fimbriata*. Already in the 1960s, Webster & Butler (1967), however, suggested that *C. fimbriata* may represent a complex of species. It is now recognized that *C. fimbriata sensu stricto* (s.s) should be reserved only for the pathogen of sweet potato (Engelbrecht & Harrington 2005), with other isolates being known as *C. fimbriata* s.l until such time as they can be clearly defined based on a combination of species concepts.

Current species concepts applied to species within *Ceratocystis* rely mostly on phylogenetics and to a lesser extent mating studies and morphology (Harrington & McNew 1997, Witthuhn *et al*. 2000, Roux *et al*. 2004, Kamgan Nkuekam *et al*. 2008, Heath *et al*. 2009b, Van Wyk *et al*. 2011). Each of these, however, has limitations in their application within the genus. For example, with *C. fimbriata* s.l., the ITS gene regions provide the best resolution between species (Barnes *et al*. 2003a), but problems are experienced in finding a second marker to support the groupings observed within this gene region (Van Wyk *et al*. 2006, Kamgan *et al*. 2008). Within *C. moniliformis* s.l., the ITS gene regions provide very limited distinction between species, with the BT and TEF gene regions being more informative (Kamgan Nkuekam *et al*. 2008, Van Wyk *et al*. 2011). Despite significant successes, the accurate identification of cryptic species within *Ceratocystis* remains a challenge. Advances in genomic studies will in future help in developing accurate markers for proper identification of organisms within this group.

1.3 ECONOMIC IMPORTANCE OF *CERATOCYSTIS* SPECIES

Species in the genus *Ceratocystis* have had a significant impact on agriculture and forestry since the first description of the genus in 1891, causing fruit and tuber rots of a number of important food crops as well as canker and wilt of forest trees globally (Kile 1993, Wingfield *et al*. 1993). Rot of sweet potato (*Ipomoea batatas*), taro (*Colocasia esculenta*), *Xanthosoma* spp. and *Syngonium* spp. has been attributed to species within *C. fimbriata* s.l. (Kile 1993, Thorpe *et al*. 2005), while *Thielaviopsis basicola* (Berk & Broome) Ferraris is responsible for black pod rot of groundnut and black root rot of chicory (Geldenhuis *et al*. 2004). *Ceratocystis cacaofunesta* Engelbrecht and T.C. Harrington causes a lethal wilt disease of cacao (*Theobroma cacao*) (Engelbrecht *et al*. 2007b) while *C. fimbriata* s.l. causes diseases of stone fruits in the USA (De Vay *et al*. 1963). Mango (*Mangifera indica*) sudden decline is a disease of economic importance in Oman and has been found to be associated with wilt caused by *C. manginecans* M. van Wyk, A. Al-Adawi, & M.J. Wingf. (Van Wyk *et. al*. 2007). In the forest, important tree diseases caused by *Ceratocystis* species and their anamorphs include oak wilt in the USA (Henry *et al*. 1944, Sinclair *et al*. 1987) and canker and death of *Platanus* species in Europe (Walter 1946, Walter *et al*. 1952), while in plantations of non-native trees, wattle wilt of *Acacia mearnsii* and *A. decurrens* Willd trees in South Africa, Uganda, Kenya and Brazil is caused by *C. albifundus* and *C. fimbriata* s.l. (Ribeiro *et al*. 1988, Roux *et al*. 2007). *Ceratocystis* spp. also cause diseases on fruit crops and vegetables of agronomic importance such as leaf and fruit root of pineapple (*Ananas comosus*), bitten leaf disease of cacao and soft rot of papaw (*Carica papaya* L.) caused by *C. paradoxa* (Kile 1993).

In Africa, *Ceratocystis* spp. have been reported causing diseases of agricultural crops, fruit trees, palms and plantation trees (Crous *et al*. 2000, Apetorgbor *et al*. 2004, Eziashi *et al*. 2006, Roux *et al*. 2007). The first known report of a disease in Africa caused by a species of *Ceratocystis* was a disease of *Hevea* sp. in the Congo and Uganda caused by *C. fimbriata* s.l. (Ringoet 1923, Snowden 1926). In the past two decades *Ceratocystis* spp. have been linked to wilting and death of *Eucalyptus* spp. in the Republic of Congo and Uganda (Roux *et al*. 1999a, Roux *et al*. 2001a) and diseases of *A. mearnsii* in Southern and Eastern Africa (Roux *et al*. 2007, Roux & Wingfield 2009). Although there is limited information on the genus *Ceratocystis* in Africa, it is surprising to note that about fifty-two species have been identified

on the continent, with a wide host range from native to non-native hosts, on plantation trees and crops of economic importance (Roux & Wingfield 2011).

Diseases caused by *Ceratocystis* spp. range from tuber and fruit rots, to sapstain, stem and branch cankers and vascular wilts of plant hosts (Fig. 3) and they affect both native and introduced hosts (Kile 1993, Roux & Wingfield 2009). Diseases of trees in their native environments, caused by species of *Ceratocystis*, include oak wilt (Henry *et al*. 1944), platanus canker (Walter 1946) and spruce wilting (Krokene *et al*. 2010). Probably the best known disease of native trees is oak wilt caused by *C. fagacearum* (Bretz) Hunt in North America (Bretz 1952, Juzwik *et al*. 2008). This disease threatens a significant percentage of oak forests in the United States of America where it has received a lot of attention from the forestry department, the public and conservationists (Billings 2000).

Apart from canker and wilt diseases of trees, some species of *Ceratocystis*, such as *C. coerulescens*, *C. adiposa* (Butl.) C. Moreau, *C. moniliformis* and *C. resinifera* Harrington & Wingfield are weaker pathogens and only result in the devaluation of timber. This happens as a result of sap stain caused by these fungi. *Ceratocystis moniliformis* s.s., for example, has been described as an agent of sapstain on trees in the U.S.A. (Grylls & Seifert 1993, Kile 1993). Similarly, *C. eucalypti* Z.Q. Yuan & Kile causes stain of eucalypt timber in Australia (Kile *et al*. 1996). The impact of these fungi, although only cosmetic, results in significant economic losses to timber companies.

1.4 ECOLOGY OF *CERATOCYSTIS* SPECIES

Dispersal within the genus *Ceratocystis* can be by air, water (Whitney & Blauel 1972), pruning equipment (Walter *et. al*. 1952), the movement of plant material such as timber (Panconesi 1999, Haack 2006), soil (Nag Raj & Kendrick 1975) and insects (Marin *et al.* 2005, Heath *et al*. 2009a). *Ceratocystis* species and their *Thielaviopsis* anamorphs have several morphological adaptations to facilitate their dispersal via these pathways. Some species, such as *Th. Basicola,* have thick walled conidia (aleuroconidia) and chlamydospores which can survive in the soil for long periods of time enabling dispersal via the movement of infected soil (Nag Raj & Kendrick 1975, Kile 1993). *Ceratocystis* species can also be dispersed through root grafts between uninfected and infected trees (Rexrode & Brown 1983,

Kile 1993). Sporulating *Ceratocystis* species are also disseminated with the help of water and wind (Vigouroux & Stojadinovic 1990, Grosclaude *et al.* 1991).

Ceratocystis species have evolved a number of morphological features to provide them with a competitive advantage and facilitate their insect associations (Kile 1993, Malloch & Blackwell 1993, Kirisits 2004, Marin & Wingfield 2006). The sexual state of these fungi, typically carry their ascospores at the tips of long ascomatal necks, lifting them above competing fungi and ensuring their preferential attachment to insect bodies. The ascospores of *Ceratocystis* species, furthermore, accumulate in a slimy matrix, facilitating adherence to insect bodies, equipment and other dispersal agents (Kile 1993, Upadhyay 1993). Some *Ceratocystis* species, particularly those in the *C. fimbriata* s.l. and *C. moniliformis* s.l. complexes, produce fruity aromas that are said to attract insects such as nitidulid (picnic) beetles and drosopholid flies, further enhancing their chances of dispersal (Moller & De Vay 1968, Kile 1993).

The association of *Ceratocystis* species with insects has been known for about 100 years (Upadhyay 1981, Wingfield *et al.* 1993). Their associations with insects, however, differ in their nature and it has generally been accepted that some species have more specific associations, while others have casual or loose insect associations. *Ceratocystis polonica* (Siemaszko) C. Moreau and *C. laricicola* Redfern & Minter have been shown to have very close vector associations, with specific bark beetles (Coleoptera, Scolytinae) (Kirisits 2004). *Ceratocystis polonica* known to cause blue stain on Norway spruce (*Picea abies*), has a mutual association with the bark beetles *Ips typographus* L., *I. typographus* f. *japonicas* Niijima*, I. amitinus* Eichh. and *I. duplicatus* Sahlb. (Mathiesen-Käärik 1960, Horntvedt *et al.* 1983, Krokene & Solheim 1998). However, *C. paradoxa, C. fagacearum* and *C. fimbriata* s.l. have apparently non-specific relationships with their insect vectors, such as flies (Diptera) and nitidulidae beetles (Coleoptera, Nitidulidae) (Kile 1993, Heath *et al.* 2009a). Nitidulid beetles that have been implicated in the dispersal of the wattle wilt pathogen, *C. albifundus,* include *Carpophilus bisignatus* Boheman. and *Brachypeplus depressus* Erichson (Heath *et al.* 2009a). Similarly, the major dessiminators of the oak wilt pathogen, *C. fagacearum* are recognized to include nitidulid beetles such as *Colopterus truncatus* (Juzwik & French 1983, Cease & Juzwik 2001). These vectors are attracted to sporulating mats growing on wounded or dead trees, and when they fly off, they are covered in fungal propagules which are then spread to wounds on other trees (Juzwik & French 1983).

Human activities play a major role in the dispersal of *Ceratocystis* species. Trading in germplasm within and between country borders (Old *et al.* 2003), the use of wood packaging material (WPM) in international trade (Grosclaude *et al*. 1988, Haack 2006) and silvicultural practices such as pruning (Kile 1993, Morris *et al*. 1993, Tarigan *et al.* 2010), all assist in transmitting *Ceratocystis* species. Cuttings used as propagative material have also been implicated as an agent of dispersal for fungi that cause Ceratocystis wilt in cacao farming (Engelbrecht *et al.* 2007a). The success of transmission is based on both the sexual and asexual spores of *Ceratocystis* species and their adaptation for survival under harsh conditions. As mentioned above, the slimy matrix in which ascospores accumulate, not only helps them to stick to insect bodies and equipment, but also protect the spores from desiccation and other environmental factors (Dowding 1970). Similarly, the thick-walled aleuroconidia and chlamydospores produced asexually by *Ceratocystis* species enable them to survive for long periods in the soil and in wood (Hunt 1956, Nag Raj & Kendrick 1975). *Ceratocystis* species have been shown to be able to survive on artificial wound surfaces of host plants causing significant lesions (Davidson 1935, 1944, Barnes *et al*. 2003a, Roux *et al.* 2004) and all these adaptations make them pathogens of significant importance.

1.5 *CERATOCYSTIS* SPECIES IN AFRICA

Studies of *Ceratocystis* species on the African continent have not in the past received the same attention as in the Northern Hemisphere. The majority of reports of *Ceratocystis* species from Africa (Table 2) is not backed up by living cultures and was based only on morphological identification (Roux & Wingfield 2011). The situation has changed in recent years, with an increase in information regarding these fungi, particularly on plantation grown tree species in Southern Africa (Wingfield *et al.* 1996, Roux 2002, Heath *et al.* 2009b, Roux & Wingfield 2009). However, significant research is still required to truly understand the diversity and impact of these fungi on the continent.

In Africa, various *Ceratocystis* species have been reported as serious pathogens of agronomic crops. *Ceratocystis fimbriata* s.l., for example has been reported as the cause of rot of sweet potato (Khiurani *et al*. 2000), while *Th. basicola* causes diseases of groundnuts and maize (Labuschagne *et al*. 1980, Crous *et al*. 2000). Another common food crop pathogen in Africa

is *C. paradoxa,* the cause of a range of diseases including pineapple disease and stem bleeding on banana, sugar cane and pineapple (Wingfield *et al*. 1993).

There has been a strong drive in recent years to increase knowledge on the impact of *Ceratocystis* species on mostly non-native, plantation grown eucalypts, *Acacia* species and forest trees in general (Roux *et al*. 1999b, Roux *et al*. 2001a, Roux *et al*. 2005) due to an increase in the importance of plantation forestry in Africa. More recently, these studies have been extended to include native African trees and together this has resulted in the description of 15 previously unknown *Ceratocystis* species from the continent (Wingfield *et al.* 1996, Kamgan Nkuekam *et al.* 2008, Heath *et al*. 2009b).

Diseases of important trees caused by *Ceratocystis* species common to Africa include wattle wilt of *Acacia mearnsii* caused by *C. albifundus* (Wingfield *et al*. 1996, Roux *et al*. 2005) and disease of eucalpyts caused by *C. eucalypticola* (*C. fimbriata* s.l.) (Roux *et al*. 1999a, Van Wyk *et al*. 2011). Both these diseases also illustrate the confusing taxonomy of *Ceratocystis* species and the difficulties of identifying these fungi based only on morphology. They were all initially reported as *C. fimbriata* and it was only later realized, using DNA sequence data, that both in fact represent distinct species (Wingfield *et al*. 1996, Van Wyk *et al*. 2011).

1.5.1 Ceratocystis wilt of Australian Black Wattle

The best studied tree disease caused by a *Ceratocystis* species on the African continent is that of wattle wilt (Ceratocystis wilt) of non-native, plantation grown *A. mearnsii* (black wattle) caused by *C. albifundus.* This pathogen easily infects wounds on *A. mearnsii* (Roux *et al*. 1999b) and is known to produce fruity aroma that attracts casual insect vectors which aids in its transmission (Heath *et al*. 2009a). Host infection by this pathogen commonly leads to rapid wilt and death of trees (Morris *et al*. 1993, Roux *et al*. 1999a). It is the cause of significant losses in the South African wattle growers industry and has attracted considerable research interest in recent years (Morris *et al*. 1993, Wingfield *et al*. 1996, Roux *et al*. 2007, Heath *et al*. 2009a). Most of the reports on *C. albifundus* on *A. mearnsii* are concentrated in Southern and Eastern Africa (Wingfield *et al*. 1996, Roux *et al*. 2001b, Roux *et al*. 2004).

A comparative analysis of the genetic diversity of *C. albifundus* with that of other *Ceratocystis* species, based on nuclear and mitochondrial DNA diversity, showed that *C.*

albifundus is most likely native to South Africa (Roux *et al*. 2001b). This study was later supported by a second (Barnes *et al*. 2003a) and a third study (Heath *et al*. 2009b) using microsatellite data and increased populations. Furthermore, its occurrence on more than eight families of native African trees, in the absence of disease on these trees, in numerous countries, further supports the hypothesis that it is native to Africa (Roux *et al*. 2001b).

1.5.2 Ceratocystis wilt of eucalypts

Diseases of eucalypts caused by *Ceratocystis* species in Africa first became known when *C. fimbriata* s.l., now *C. eucalypticola*, was reported as the cause of a wilt disease of eucalypts in the Republic of Congo in 1999 (Roux *et al*. 1999a). This disease was subsequently also reported from Uganda (Roux *et al*. 2001a). The origin of *C. eucalypticola* in Africa is not clear. Attempts have been made in recent years using DNA based microsatellite markers to understand the diversity of this fungus in South Africa (Barnes *et al*. 2001, Barnes 2002, Van Wyk *et al*. 2006a). The genetic diversity of *C. eucalypticola* in South Africa is low and the pathogen must have been accidentally introduced into Africa (Van Wyk *et al*. 2006a).

1.5.3 Other *Ceratocystis* species on eucalypts in Africa

Within *C. fimbriata* s.l., *C. pirilliformis* has been reported commonly from *Eucalyptus* species in South Africa (Roux *et al*. 2004, Kamgan Nkuekam *et al*. 2009). The occurrence of this pathogen on freshly harvested wounds of *E. grandis* was the first of this fungus in Africa and it was shown to have the potential to cause disease of eucalypts (Roux *et al*. 2004). This fungus is also known from Australia and population diversity studies suggest that it was introduced to South Africa, possibly from that country (Kamgan Nkuekam *et al*. 2009).

Other *Ceratocystis* species which have been associated with *Eucalyptus* species in Africa include *C. moniliformis* and *C. zombamontana* R.N. Heath & Jol. Roux. Neither of these fungi has, however, been associated with diseased or dying trees (Heath *et al*. 2009b).

1.5.4 *Ceratocystis* species associated with native African trees

It is only in the past ten years that any significant studies on *Ceratocystis* species associated with native trees on the African continent have been undertaken. This resulted in the description of many previously unknown *Ceratocystis* species.

The earliest reports of *Ceratocystis* species associated with native African plants on the continent were that of *C. paradoxa* from *E. guineensis* (Spaulding 1961) and *C. fimbriata* s.l. on *Crotalaria* sp. (Davet 1962). However, the following 40 years included only one report of a *Ceratocystis* sp., now known as *C. albifundus*, from a native African host, *Protea* sp. (Gorter 1977, Roux *et al*. 2001b). Recently, three previously unknown *Ceratocystis* species, *C. oblonga* R.N. Heath and Jolanda Roux, *C. savannae* Kamgan & Jol. Roux, and *C. tsitsikammensis* Kamgan & Jol. Roux were described from native trees in South Africa (Kamgan Nkuekam *et al.* 2008, Heath *et al*. 2009b).

An intriguing recent report of a *Ceratocystis* sp. from native African trees is that of the oak wilt pathogen, *C. fagacearum*, from harvested timber in Ghana (Apetorgbor *et al*. 2004). This identification was only based on morphological studies to determine biodeteriorating agents present on hardwood trees harvested from native forests in Ghana. Previously, *C. fagacearum* was only known to occur in North America (Bretz 1952) causing disease of native oak trees (Sinclair *et al*. 1987). It was however, recently suggested that it may be introduced into the USA, possibly from Central or South America (Juzwik *et al*. 2008). Its presence in Africa and on a native host could have far reaching consequences and needs to be confirmed.

1.6 QUARANTINE AND *CERATOCYSTIS* SPECIES

There is significant evidence to show that the number of non-native pests and pathogens entering countries globally have increased considerably over the past 100 years (Liebhold *et al*. 1995, Wingfield *et al*. 2001, Tatem *et al*. 2006, Brockerhoff *et al*. 2010). This has been attributed to an increase in transport, travel and trade in wood and forest products (Brasier 2008, Desprez-Loustau *et al*. 2010, Leal *et al.* 2010). Consequently, there has been an increase in the introduction of pests and diseases of crop plants, as well as trees posing a serious threat to plantations of non-native trees and equally native plants (Table 2) (Jones & Baker 2007, Waage *et al*. 2009).

The movement of plant material (seeds, seedlings, wood packaging material, dunnage etc.) carries a high risk of successfully introducing pests and pathogens into new areas (Haack 2006, Brasier 2008). During the years 1970-2004, the most common pathway for plant pathogens into Great Britain was through the importation of seedlings, tubers and scions

(Jones & Baker 2007). This is also true for seeds, where, for example, it is thought that the pine pitch canker pathogen, *Fusarium circinatum* Nirenberg & O'Donnell was introduced into South Africa on contaminated seeds imported from Mexico (Wikler & Gordon 2000). Similarly, the presence of *C. platani* in Europe has been attributed to the use of contaminated wooden packaging material during World War II, resulting in the movement of this pathogen from the USA to Europe (Panconesi 1999).

In order to deal with the increasing incidences of introduced plant pests and diseases, quarantine regulations have been introduced to prevent, or at least reduce, such incursions. Forest health is maintained internationally through the International Plant Protection Convention (IPPC). It is an international treaty on plant health that encourages harmonization of actions to prevent the introduction and spread of quarantine pests of plants and plant products and also to improve appropriate measures for their prevention, containment and eradication. The IPPC provides a framework for international cooperation between National Plant Protection Organisations (NPPO), which are official (government) bodies responsible for IPPC obligations (Anonymous 2002). The Commission on Phytosanitary Measures (CPM) governs the IPPC and each member country represented on the CPM to help direct its discussions and decisions. The activities of the IPPC are categorized into three main areas: Sharing Information on regulations through their website, providing Technical Assistance through training of less developed member countries on regulations, and Setting Standards. International Standards for Phytosanitary Measures (ISPM) are meant to reduce the spread of pests and facilitate trade through harmonization of phytosanitary measures applied in international trade. Amongst the different ISPMs, ISPM-15 adopted in 2002, is focused on minimizing the risk of quarantine pests associated with wood packaging material used in international trade (Anonymous 2002, Brockerhoff *et al*. 2010). As of December 2009, 172 member countries had signed on to the convention (MacLeod *et al*. 2010).

Entry of plant material and plant products recognised to carry pathogens is conditioned on treatment based on the ISPM-15 guidelines adopted in 2002 (Anonymous 2002, Haack 2006). These guidelines require two treatments: a temperature treatment at 56^{o}C for 30 minutes and fumigation with methyl bromide to kill insects (Haack & Petrice 2009). Examples of such treatments which have shown results include the use of temperatures of about 60^{o}C applied for 1 hour to kill *I. typograhus* (Annila 1969), a known vector of *Ceratocystis* species. In

another example, the oak wilt pathogen was shown to be eliminated from infected logs when treated for 6 hrs at temperatures greater than 54^{o}C (Jones 1973).

The significance of *Ceratocystis* species, including *C. fagacearum,* as pests of quarantine interest are due to several factors. These fungi have the ability to cause devastating losses to their host plants (Billing 2000) and they are well adapted to dispersal via wood and wood products, either on their own, or in combination with their insect vectors. *Ceratocystis* species can either be spread directly in infected wood, e.g. under the bark (Fig. 2), in soil associated with plant material, or with their insect vectors, also often under the bark of trees. Though debarking can be used to minimize the risk of insects and *Ceratocystis* survival, it exposes the nutrient-rich area of particularly sapwood to colonization, also by other fungal species. These effects eventually reduce the value of the wood or wood material, as such making *Ceratocystis* species significant quarantine pests.

An understanding of the pathway of pathogens is paramount to minimizing the risk of introduction into new environments. Implementing adequate and scientifically accepted measures, as prescribed under ISPM-15 (www.ippc.int) procedures for transporting wood material, will minimize the movement of insects and associated pathogens into new environments. The common practice which has often been used to mitigate the risk of pest and pathogen introduction is through publishing a pest and pathogen list of quarantine importance, such as that of the European Plant Protection Organization (http://www.eppo.org/QUARANTINE/quarantine.htm) and the IPPC (www.ippc.int). These lists, if not accurately and regularly updated, fail in their objectives. From the list of pests and pathogens of quarantine importance, which include three *Ceratocystis* species only two, *C. fagacearum* and *C. fimbriata* s.l., have been reported from Africa (www.ippc.int). As a quarantine pest, *C. fimbriata* has been reported on a wide range of host plants on every continent except for the Middle East and Eastern Europe and *C. fagacearum* on *Quercus* spp. across all the states in the United States (Table 3) (www.ippc.int). This list is, however, most likely incorrect as *C. fimbriata* may refer to several different species.

1.7 CONCLUSIONS

The genus *Ceratocystis* includes pathogens of numerous species of significant economic importance to agriculture and forestry, causing loss of quality and death of plants/trees. It has

a broad host and geographical range which includes angiosperms and gymnosperms from the temperate regions to the tropics. Generally, they are morphologically very similar, though phylogenetic analyses have greatly improved the accuracy of species delimitation in the genus. The association of *Ceratocystis* species with insects greatly assists them in their dissemination (Kirisits 2004, Heath *et al*. 2009a) over relatively long distances. This, together with the capacity for some species to switch between mating types (Witthuhn *et al.* 2000) makes them potentially highly successful invasive pathogens.

Information pertaining to *Ceratocystis* species on the African continent is relatively limited. Although a number of diseases of agricultural crops and forest plants have been attributed to *Ceratocystis* species, limited cultures are available to verify this (Roux & Wingfield 2011). However, studies of *Ceratocystis* species on native and plantation grown trees in Southern Africa over the past 12 years have identified numerous previously unknown species in this region, suggesting that the diversity of this genus on the continent is poorly understood.

In the following section of this mini-dissertation, the presence of a *Ceratocystis* sp., previously only known from the USA, in Ghana, will be investigated. This fungus was identified based on morphology only and it is highly unlikely that it was correctly identified. The correct identification of this fungus is of importance since it is a registered quarantine tree pathogen in the USA. Its introduction into Africa and its impact on related trees is of concern in Ghana. Therefore, the aim of this investigation is to identify and correctly characterize the *Ceratocystis* species present in Ghana using PCR, sequencing and phylogenetic techniques.

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Table 1: Taxonomic classification of the genus *Ceratocystis*.

Ceratocystis sensu stricto		
Characters	***Ceratocystis***	***Ophiostoma***
Kingdom	Fungi	Fungi
Division	Eumycota	Eumycota
Phylum	Dikaryomycota (Ascomycota)	Dikaryomycota (Ascomycota)
Order	Microascales	Ophiostomatales
Families	*Ceratocystidaceae*	Ophiostomataceae
Genus	*Ceratocystis* sp.	*Ophiostoma* sp.
Anamorph	*Thielaviopsis*	*Pesotum, Sporothrix*

Table 2: List of *Ceratocystis* spp. and their hosts reported from the African continent. Anamorph states are listed as reported in literature (e.g. *Chalara* spp.) and some may not actually represent *Ceratocystis* spp., since the accepted anamorphs for the genus is restricted to *Thielaviopsis* spp. The table was undated and adjusted from the one published by Roux & Wingfield 2011.

SPECIES	HOST	COUNTRY	REFERENCE
C. albifundus	*Acacia mearnsii*	Kenya	Roux *et al*. 2004a
		South Africa	Morris *et al*. 1993; Wingfield *et al*. 1996
		Uganda	Roux *et al*. 2001c
	A. decurrens	South Africa	Morris *et al*. 1993
	A. caffra	South Africa	Roux *et al*. 2004b
	Burkea africana	South Africa	Roux *et al*. 2004b
	Combretum molle	South Africa	Roux *et al*. 2004b
	C. zeyheri	South Africa	Roux *et al*. 2004b
	Faurea saligna	South Africa	Roux *et al*. 2004b
	Ochna pulchra	South Africa	Roux *et al*. 2004b
	Ozoroa paniculosa	South Africa	Roux *et al*. 2004b
	Protea acaulos	South Africa	Crous *et al*. 2000
	P. cynaroides	South Africa	Morris *et al*. 1993; Crous *et al*. 2000
	P. eximia	South Africa	Crous *et al*. 2000
	P. gaguedi	South Africa	Roux *et al*. 2004b

	P. gigantea	South Africa	Gorter 1977
	P. grandiceps	South Africa	Crous *et al*. 2000
	P. magnifica	South Africa	Crous *et al*. 2000
	P. neriifolia	South Africa	Crous *et al*. 2000
	P. repens	South Africa	Crous *et al*. 2000
	Terminalia sericea	South Africa	Roux *et al*. 2004b
C. fagacearum	*Ceiba pentandra*	Ghana	Apetorgbor *et al*. 2004
	Celtis mildbraedii	Ghana	Apetorgbor *et al*. 2004
	Pterygota macrocarpa	Ghana	Apetorgbor *et al*. 2004
C. fimbriata	*Acacia mearnsii*	South Africa	Roux 1998; Roux *et al*. 2004
	Crotalaria sp.	Ivory Coast	Davet 1962
	Eucalytpus grandis X tereticornis	Republic of Congo	Roux *et al*. 1999b
	E. pellita X urophylla	Republic of Congo	Roux *et al*. 1999b
	E. grandis	South Africa	Roux *et al*. 2004a
		Uganda	Roux *et al*. 2001a
	Hevea sp.	Uganda	Ringoet 1923; Snowden 1925
	Ipomoea batatas	Kenya	Khiurani *et al*. 2000
C. moniliformis	*Erythrina* sp.	South Africa	Witthuhn *et al*. 1999

	E. grandis	South Africa	Roux *et al*. 2004a
	Macaranga capensis	South Africa	Van Wyk *et al*. 1991
C. moniliformis f. theobromae	*Theobromae cacao*	Madagascar	Luc 1952
C. moniliformis f. pycnanthi	*Pycnanthus cambo*	Cameroon	Luc 1952
C. paradoxa	*Ananas comosus*	Kenya	Upadhyay 1981
	A. comosus	South Africa	Malan 1954; Crous *et al*. 2000
	Cocus nucifera	Ivory Coast	Spauling 1961
	C. nucifera	Nigeria	Spaulding 1961; Upadhyay 1981
	Coffea abeokutae	Ivory Coast	Spaulding 1961
	C. canephora	Ivory Coast	Spaulding 1961
	C. liberica	Ivory Coast	Spaulding 1961
	Cupressus macrocarpa	Kenya	Nattrass 1961
	Elaeis guineenses	Ghana	Spaulding 1961
	E. guineenses	Ivory Coast	Spaulding 1961
	E. guineenses	Nigeria	Upadhyay 1981
	E. guineenses	Republic of Congo	Spaulding 1961
	E. guineenses	Sierra Leone	Spaulding 1961
	Ipomoea batatas	Nigeria	Upadhyay 1981

	Musa sp.	South Africa	Roth & Loest 1965; Crous *et al*. 2000
	Musa sp.	Tanzania (1953)	Upadhyay 1981
	Saccharum officinarum	South Africa	Crous *et al*. 2000
	Theobromae cacao	Ghana	Spaulding 1961
C. pirilliformis	*E. grandis*	South Africa	Roux *et al*. 2004a
C. radicicola	*Phoenix dactylifera*	South Africa	Linde & Smit 1999
C. tsitsikammensis	*Rapanea melanophloeos*	South Africa	Kamgan Nkuekam *et al*. 2006
C. savanna	*Acacia nigrescens*	South Africa	Kamgan Nkuekam *et al*. 2006
	Sclerocarya birrea		
C. oblonga	*A. mearnsii*	South Africa	Heath *et al*. 2009
C. polyconidia	*A. mearnsii*	South Africa	Heath *et al*. 2009
C. tanganyicensis	*A. mearnsii*	Tanzania	Heath *et al*. 2009
Chalara alabamensis	*Unknown*	Ivory Coast	Heredia-Abarca 1994
Ch. austriaca	*Pinus austriaca*	Ivory Coast	Nag Raj & Kendrick 1975
Ch. elegans	*Crotalaria junca*	South Africa	Nag Raj & Kendrick 1975
	C. junca	Zimbabwe	Nag Raj & Kendrick 1975
Ch. hughesii	*Elegia capensis*	South Africa	Lee *et al*. 2004
Ch. kendrickii	*Brassica* sp.	Sierra Leone	Nag Raj & Kendrick 1975
Ch. paradoxa	*Ananas comosus*	Kenya	Nag Raj & Kendrick 1975

	Cocus nucefera	Nigeria	Nag Raj & Kendrick 1975
	Elaeis guineensis	Nigeria	Nag Raj & Kendrick 1975
	Ipomoea batatas	Nigeria	Nag Raj & Kendrick 1975
	Musa sp.	Tanzania	Nag Raj & Kendrick 1975
	Theobromae cacao	Ghana	Nag Raj & Kendrick 1975
Ch. phaeospora	*Cyathea* sp.	Kenya	Kirk 1985
Ch. transkeiensis	Dead wood	South Africa	Morgan-Jones *et al*. 1992
Chaetochalara africana	*Brachystegia spiciformis*	Zambia	Ellis 1971; Nag Raj & Kendrick 1975
Chaetochalara ramosa	*Dalbergia lacteal*	Tanzania	Nag Raj & Kendrick 1975
Thielaviopsis basicola	*Arachis hypogaea*	South Africa	Labuschagne *et al*. 1980; Prinsloo 1980; Crous *et al*. 2000

Avena sativa	South Africa	De Villiers 1987; Crous *et al*. 2000
Capsicum annuum var. longum	South Africa	Crous *et al*. 2000
C. frutescens	South Africa	Gorter 1977; Crous *et al*. 2000
Cenchrus ciliaris	South Africa	De Villiers 1987; Crous *et al*. 2000
Cichorium intybus	South Africa	Doidge 1950; Prinsloo 1986
Citrus sp.	South Africa	Wehner *et al*. 1986; Crous *et al*. 2000
Crotalaria juncea	South Africa	De Villiers 1987; Crous *et al*. 2000
Gossypium hirsutum	South Africa	De Villiers 1987; Crous *et al*. 2000
Lycopersicum esculentum	South Africa	De Villiers 1987; Crous *et al*. 2000
Nicotiana tabacum	South Africa	Doidge & Bottomley 1931; Gorter, 1977; Crous *et al*. 2000
Pennisetum clandestinum		De Villiers 1987
P. glaucum	South Africa	Crous *et al*. 2000
Pisum sativum	South Africa	De Villiers 1987; Crous *et al*. 2000
Triticum aestivum	South Africa	De Villliers 1987; Crous *et al*. 2000
Vigna unguiculata	Zimbabwe	Whiteside 1966

	Zea mays	South Africa	Crous *et al*. 2000
Th. paradoxa (syn. Th. ethacetica)	*Ananas comosus*	South Africa	Doidge 1950; Gorter 1977
	A. sativus	Africa	Wallace 1930; Moreau 1948
	Cocus nucifera	Somalia	Castellani & Ciferri 1937
	Musa sp.	South Africa	Doidge 1950; Gorter 1977
	Saccharum officinarum	South Africa	Doidge 1950; Gorter 1977

Table 3: *Ceratocystis* species of quarantine interest and some of their known hosts and distribution.

PATHOGEN[A]	HOST	LOCATION	REFERENCES
Ceratocystis fimbriata s.l.	*Acacia decurrens*	Brazil	Mendes *et al*. 1998
	A. mearnsii	South Africa	Morris *et al*. 1993[B]
	Alocasia sp.	Florida	Miller 1992
	Annona muricata	Colombia	Van Wyk *et al*. 2009
	Betula papyrifera	Canada	Mendes *et al*. 1998
	Cajanus cajan	Brazil	Mendes *et al*. 1998
	Cassia renigera	Brazil	Mendes *et al*. 1998
	Cinchona pubescens	Guatemala	Spaulding 1961
	Citrus limon	Colombia	Van Wyk *et al*. 2009
	Citrus sp.	Colombia	Benjamen 1969
	Cocos nucifera	Japan	Kobayashi 2007
	Cocos sp.	Haiti	Benjamin & Slot 1969
	Coffea arabica	Guatemala,	Mendes *et al*. 1998
	Colocasia esculenta	China	Huang *et al.* 2008
	Crotalaria juncea	Brazil	Mendes *et al*. 1998
	Crotalaria sp.	Colombia	Mendes *et al*. 1998
	Crotalaria spectabilis	Brazil	Mendes *et al*. 1998
	Eucalyptus grandis	Brazil, Congo Rep., South Africa Uganda, Uruguay	Roux *et al*. 2004, Barnes *et al*. 2003a

	Eucalyptus sp.	Rupublic of Congo,	Roux *et al*. 2000a
	Eucalyptus x grandis-tereticornis	Republic of Congo	Roux *et al*. 2000
	Eucalyptus x pellita-urophylla	Republic of Congo	Roux *et al*. 2000
	Fagus crenata	Japan	Matsushima 1975
	Gmelina arborea	Brazil	Mendes *et al*. 1998
	Havea brasiliensis	Brazil	Spaulding 1961
	Ipomoea batatas	Brazil	"
	Mangifera indica	Brazil	Mendes *et al*. 1998
	Platanus sp.	France	Witthuhn *et al*. 1999
	Populus balsamifera	Canada	Vujanovic *et al*. 1999
	Protea cynaroides	South Africa	Gorter 1977
	Prunus amygdalus	USA	De Vay *et al*. 1968
	Punica granatum	China	Huang *et al*. 2008
	Quercus ellipsoidalis	USA	Campbell 1960
	Syngonium podophyllum	USA	French 1989
	Theobroma cacao	Brazil	Mendes *et al*. 1998
C. fimbriata f.platani	*Platanus occidentalis*	USA	McCracken & Burkhardt 1977
C. fagacearum	*Quercus* sp.	USA	Peacher *et al*. 1975

[A] Reports are as they appear in quarantine lists and do not necessarily represent the latest, updated taxonomy of the pathogens.

[B]This report of *C. fimbriata* is incorrect, as this fungus was later described as the new species, *C. albifundus* (Wingfield *et al*. 1996).

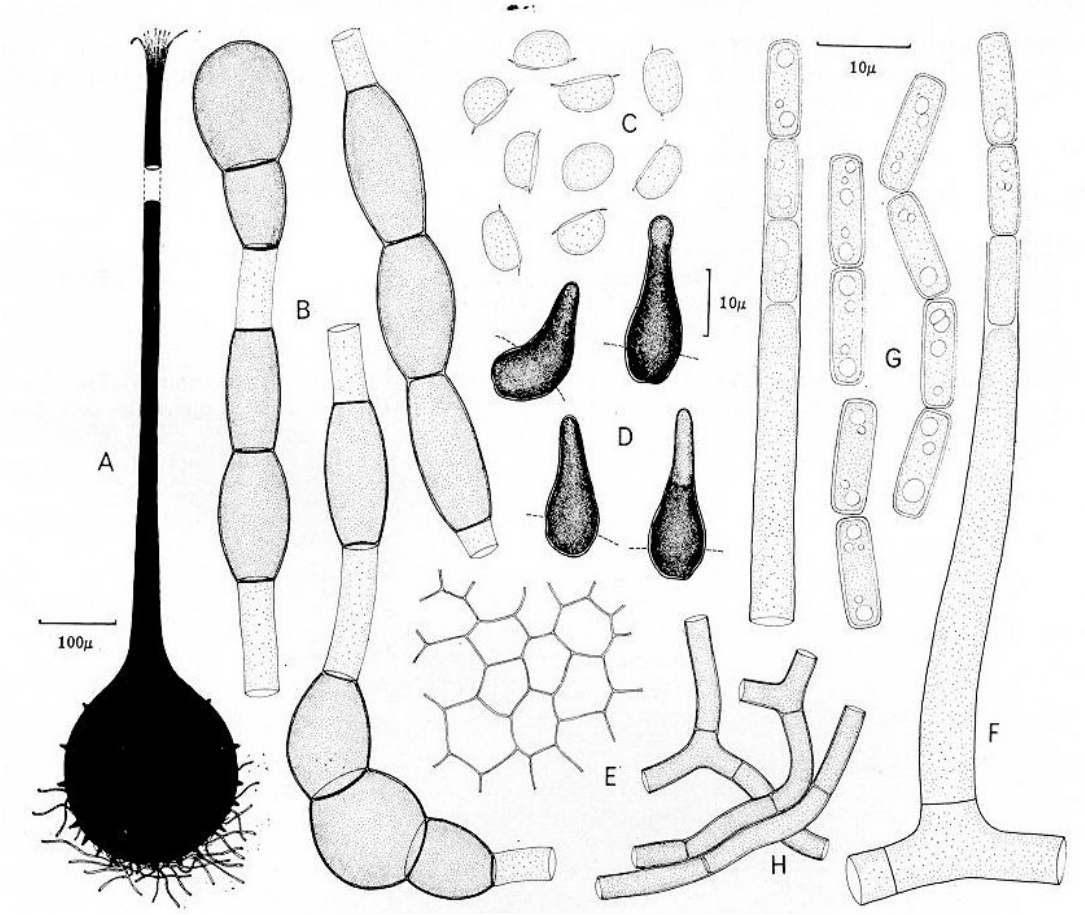

Figure 1: Characteristic morphological structures of *Ceratocystis moniliformis*. (A) Perithecium (B) chlamydospores (C) ascospores (D) perithecial appendages (E) surface of perithecium wall (F) conidiophores (G) conidia (H) mycelium (Moreau 1952).

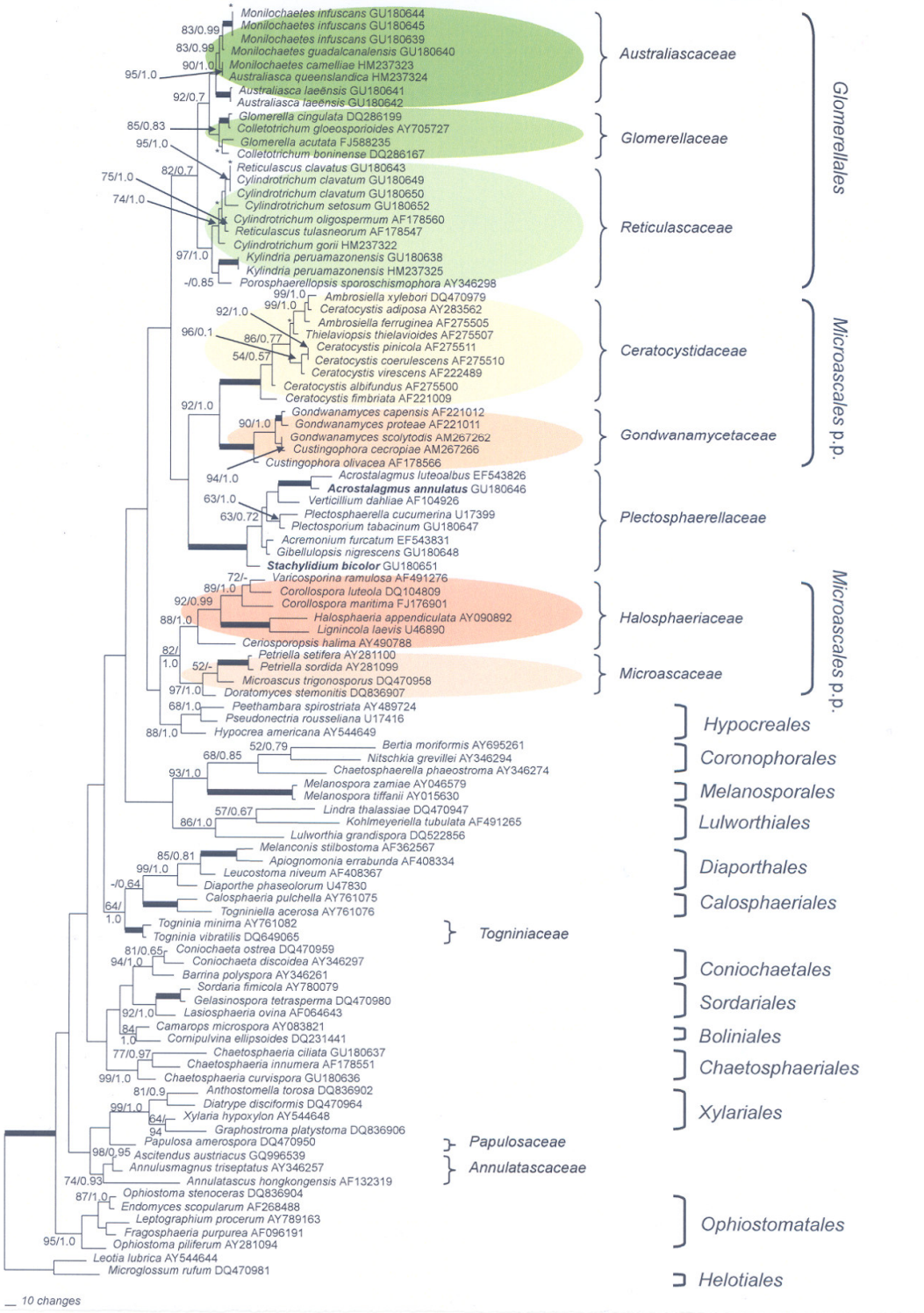

Figure 2: One of 16 most parsimonious trees from a heuristic analysis of ncLSU rDNA sequences. Thickened branches indicate posterior probability values = 1.0 PP and 100% bootstrap support. Bootstrap support values ≥ 50% and Posterior probability values ≥ 0.5 are included at the notes. Branch lengths are drawn to scale. An asterics above or below a branch marks branches that collapse in the strict consensus tree (Réblová *et al*. 2011).

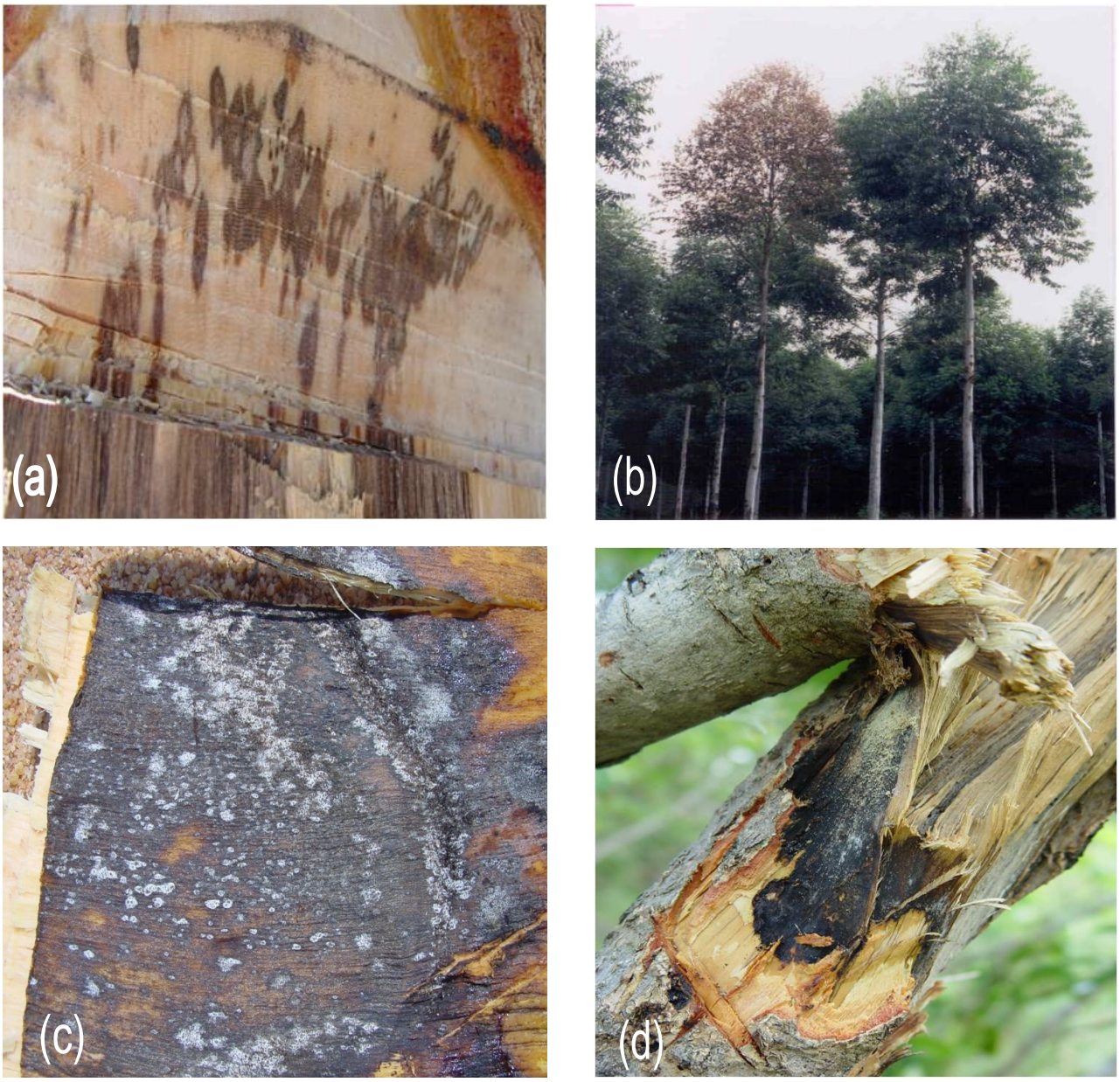

Figure 3: Symptoms of infection and disease of trees caused by *Ceratocystis* spp. (a) Xylem discolouration of a native African tree caused by a *Ceratocystis* spp. (b) Wilt and die-back caused by *C. eucalypticola* in Uruguay (c) Grey mycelium and fruiting bodies of *Ceratocystis* spp. under the bark of a wounded native South African tree (d) Natural infection of wounds caused by *Ceratocystis* spp. on an indigenous African *Combretum molle* tree.

Photos (a, c, d) were taken by Prof. Jolanda Roux and (b) by Prof. Micheal J. Wingfield, of FABI, University of Pretoria, Pretoria, South Africa.

CHAPTER 2

IDENTIFICATION OF *CERATOCYSTIS* SPECIES ON TIMBER HARVESTED FOR EXPORT IN GHANA

ABSTRACT

Ceratocystis species are important pathogens of tree species worldwide. They are responsible for sap stain and loss of the aesthetic value of wood and wood products, as well as the death of trees. In 2004, *C. fagacearum*, the cause of oak wilt of native oak trees in the USA, was reported from timber harvested in native forests in Ghana. This was the first report of this pathogen in a country other than the USA. The aim of the current study was to follow up on this report to confirm the presence of *C. fagacearum* in Ghana using DNA sequence data. Surveys were conducted of harvested trees in the same areas from which the report in 2004 obtained their isolates, since no isolates from 2004 remained for analyses. The isolates obtained in this study were identified using DNA sequence data for the ITS, β-tubulin, and transcription elongation factor 1-α gene regions. Isolates were all delineated into three clades belonging to the *C. moniliformis sensu lato* species complex, closely related to *C. savannae* and *C. oblonga* and no *C. fagacearum* isolates were obtained. Reports of the oak wilt pathogen from Ghana, therefore, remain unconfirmed.

2.1 INTRODUCTION

Globally, the demand for wood and wood products is continuing to increase (Leal *et al*. 2010, Anonymous 2011). This phenomenon is not going unnoticed in Ghana. Ghana is a major exporter of timber and wood products to Europe and other countries, contributing approximately 7.2% to the country's gross domestic product (GDP) (Anonymous 2011). Major tree species harvested in Ghana include Odum [*Melicia excelsa* (Welw.) C.C. Berg. and *M. regia* (A. Chev.) C.C. Berg.], African Mahogamy (*Khaya ivorensis* A. Chev., *K. anthotheca* C.DC.), *Celtis mildbraedii* Engl., *Ceiba pentandra* (Linn.) Gaertn, *Pterygota macrocarpa* K. Schum. and Kokrodua [*Pericopsis elata* (Harms) Meeuwen], amongst others (Apetorgbor *et al.* 2004, Baatuuwie *et al*. 2011).

Despite the importance of timber and other forest products in Ghana, and other African countries, limited to no information is known regarding microbial pathogens of these trees. The situation is only slightly better with regards to pest (invertebrates) problems on timber trees on the continent (Beaver 1989, Wagner *et al*. 1991, Rao *et al*. 2000, Bosu & Apetorgbor 2009). Some attention has been given to those native tree species of economic importance, and which have been planted in plantations (Bosu & Apetorgbor 2009). The extent of the damage caused by fungal biodeterioting agents in Ghana has been estimated to be as much as one-third of the annual crop production (Liese 1975). For example, several fungal species were reported to cause sap stain and rot of *C. mildbraedii, C. pentandra* and *P. macrocarpa.* in the country (Apetorgbor *et al*. 2004). The most common fungi associated with the stain and rot was *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Ofosu-Asiedu 1973) and *Ceratocystis fagacearum* (Bretz) Hunt (Apetorgbor *et al*. 2004). Apart from the report of *C. fagacearum* from native timber trees in Ghana, previous reports of *Ceratocystis* species are limited. This includes report of *C. paradoxa* (Dade) Moreau and its anamorph *Thielaviopsis paradoxa* (de Seynes) Hōhn on *Elaesis guineense* Jacq. and *Theobroma cacao* L. respectively (Spaulding 1961, Nag Rag & Kendrick 1975).

Ceratocystis fagacearum is the cause of the devastating disease of native oak trees (*Quercus* species) in the United States of America (Henry *et al*. 1944). This disease results in the death of trees and has been responsible for devastating losses in 860 counties of 23 eastern and Midwestern states of the USA (USDA 2006). It was originally suspected that it may be native to the USA, however, recent reports suggest that it is possibly of South American origin

(Juzwit *et al*. 2008). It has an opportunistic relationship with nitidulid beetles and many species of flies which are attracted to it by the fruity odour it produces, and the insects acting as vectors in its transmission (Cease & Juzwik 2001). The fungus has never been reported from any country other than the USA and its report from Ghana (Apetorgbor *et al*. 2004) is of great importance.

The accurate identification of *Ceratocystis* Ellis & Halsted has been problematic since the first description of this pathogen in 1891 and remains so, even with the help of DNA sequence data. *Ceratocystis* species have a high degree of morphological similarity, including with fungi in other genera such as *Ophiostoma* H. Syd. & P. Syd. and *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. (Wingfield *et al*. 1993). Where in the past, use was made mostly of morphological identification (Upadhyay 1993), it is today standard practice to include multiple species concepts in attempts to characterize these fungi. The most common of these include a combination of two or more biological concepts (Wingfield *et al*. 1996, Harrington & Wingfield 1998, Witthuhn *et al*. 1999, Barnes *et al*. 2003, Van Wyk *et al*. 2006). Previously, use was made of a single gene region for the phylogenetic species concept in *Ceratocystis* (Barnes *et al*. 2003a), but it is today recognized that this species concept should include differences in at least two, unlinked, gene regions (Marin *et al*. 2005, Kamgan Nkuekam 2011). The most common gene regions to delineate species of *Ceratocystis* are the Internally Transcribed Spacer Regions (ITS1, ITS2) and the 5.8s gene of the ribosomal RNA operon (commonly designed together as ITS), the β-tubulin (BT) and Translation Elongation Factor-1α (TEF) gene regions. These gene regions, however, are not equally informative for the four main phylogenetic lineages within the genus *Ceratocystis*. For example, where the ITS region is highly informative for species in the *C. fimbriata sensu lato* (s.l.) species group (Barnes *et al*. 2003), it is virtually useless for the *C. moniliformis* s.l. (Hedgc.) C. Moreau species group (Van Wyk *et al*. 2006). The similarity in morphology between different *Ceratocystis* species makes it highly unlikely that the species identification based only on morphology of the Ghana isolates are accurate.

Therefore, the aim of this study is to identify or confirm the identity of *Ceratocystis* spp. present on native trees in Ghana, using multi-gene phylogenetic analyses. With ITS, BT and TEF gene regions, we hope to properly place these isolates into their correct genetic lineage.

2.2 MATERIALS AND METHODS

2.2.1 Fungal Isolates

Isolates (Table 1) of *Ceratocystis* used in this study were obtained from the culture collection (CMW) of FABI (Forestry and Agricultural Biotechnology Institute), University of Pretoria, South Africa. These isolates originated from Ghana where they were isolated from native and non-native trees during a survey of *Ceratocystis* species in that country. The survey was aimed specifically at confirming the presence of *C. fagacearum* in Ghana and was done in collaboration with Dr. Mary Apetorgbor of the Forestry Research Institute in Kumasi, Ghana. Samples were obtained from the forest (logs in loading bays and recently felled trees), as well as from logs awaiting processing in saw mills, and originated from the same saw mills and forest areas where *C. fagacearum* was reported from 2004. In all cases, samples were obtained from beneath bark still attached to the logs (Fig. 1). Isolates were grown on 2% malt extract agar (MEA, 20g malt extract, 15g agar, Biolab, Midrand, South Africa) supplemented with the antibiotic streptomycin sulfate (Sigma, Steinheim, Germany). Culture purification was done by transferring single ascospore masses from the apices of ascomatal necks or conidiophores to 2% MEA and incubated for 7 days at 25^{o}C. Three replicate plates of each isolate were considered for culture growth.

2.2.2 DNA Extraction and Polymerase Chain Reaction (PCR)

Mycelium was scraped from the surfaces of pure cultures using a sterile hypodermic needle and transferred to 1.5 ml Eppendorf tubes. DNA was extracted from all isolates using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The presence of DNA was verified by checking the concentration of 1µl supernatant using a Nanodrop ND-1000 and accompanying software (Nanodrop Technologies, Rockland, USA).

Three gene regions, the ITS, BT and TEF, were amplified using the Polymerase Chain Reaction (PCR) in an Eppendorf Mastercycler (Merck, Hamburg, Germany). The primer pairs ITS1 and ITS4 (White *et al.* 1990) for the ITS region, βt1a and βt1b (Glass & Donaldson 1995) for the BT gene region and EF1F and EF2R (Jacobs *et al.* 2004) for the TEF.

A 25µl PCR reaction mixture was prepared using 60 ng of DNA template. The mixture also contained 2.5 µl of 10x reaction buffer with $MgCl_2$ (25 mM) (Roche), 2.5 µl $MgCl_2$ (25 mM) (Roche), 1U of Taq polymerase, 2.5 µl of deoxynucleotide triphosphate mix (dNTP) (10 mM), 1 µl of each primer (10 mM) and enough sabax water. The thermal cycling conditions were as follows: an initial denaturation step of the DNA at 95°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 35 s, annealing at 50°C for 30 s for the ITS and β-tubulin genes and 55°C for the elongation factor-1α gene, primer extension at 72°C for 1 min and a final extension step at 72°C for 10 min. An aliquot of 4 µl of the PCR products were pre-stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA) and separated on a 1% agarose gel and visualized under UV light. Product sizes were estimated using a Lambda DNA/*Eco*RI+*Hin*dIII marker 3 (Fermentas Life Sciences, USA).

PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich) following the manufacturer's instructions. The concentration of the purified PCR products were then measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

2.2.3 DNA Sequencing and Phylogenetic analyses

DNA sequencing was carried out using the same primers as those for PCR amplification, except that only one primer was used for each sequencing PCR reaction. The PCR amplicons were sequenced in both directions and sequencing reactions were performed using the Big Dye Cycle Sequencing Kit with Amplitaq® DNA polymerase, FS (Perkin-Elmer, Warrington, UK), following the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Between 40-150 ng of purified PCR product was used to prepare 10 µl of sequencing PCR that contained 2 µl of ready reaction mixture (Big dye), 1.5 µl of 5x reaction buffer, 1 µl of primer (10 mM) and enough sabax water to bring the volume to 10 µl. Both DNA strands were sequenced for each gene region.

Sequences of both strands of each isolate were examined visually and consensus sequences were combined using Sequence Navigator version 1.0.1.™ (Applied Biosystems Division, Perkin-Elmer, Foster City, CA, USA). A preliminary identity of each isolate was obtained by performing a standard nucleotide BLAST (using blastn) against the GenBank database (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Sequences of

Ceratocystis species, including those generated in the current study as well as those of related species obtained from GenBank were aligned automatically using the online version of Mafft ver. 5.851 (Katoh *et al.* 2002).

After manual confirmation of the aligned sequences in sequence navigator, analyses of sequence data was done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Sinauer Associates, Sunderland, MA, USA) (Swofford 2002). Data for the three gene regions sequenced were analysed separately from each other, then combined and the same type of analyses was carried out for each dataset. A partition Homogeneity Test (PHT) was run to determine whether the sequences of the three gene regions could be combined (Swofford 2002). Phylogenetic trees were constructed with PAUP based on best tree only. Trees were obtained using a stepwise random addition of 1000 replicates. Tree searches were done using Tree Bisection Reconnection (TBR) branch swapping algorithms with the MULPARS option based on the maximum parsimony (MP) method. Confidence levels of the phylogenies were estimated with 1000 bootstrap replicates. Gaps were treated as fifth character and confidence intervals of branch nodes, with the full heuristic search option, were calculated. *Ceratocystis moniliformopsis* Yuan & Mohammed was used as an out-group and treated as a monophyletic sister group to the in-group. All sequences derived from this study have been deposited in GenBank (Table 1).

Bayesian analyses for each gene region as well as the combined dataset were performed using MrBayes 3.1 (Ronquist & Huelsenbeck 2003), based on the Markov Chain Monte Carlo (MCMC) method. The most appropriate substitution models were determined using the Akaike information criterion (AIC) in MrModeltest 2.2 (Nylander 2004) for analyses of each dataset. The models applied to the combined, TEF and BT datasets were HKY+I, K80+G and HKY+1 respectively. The suggested partition-specific models were then included in the Bayesian analyses. Phylogenetic trees based on Bayesian inference were generated using MrBayes. One million generations were run during which random trees were generated using the MCMC procedure. Four chains were applied and sampled every 100^{th} generation. To discard trees that had been sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before stabilization. This was done by means of the burn-in procedure in MrBayes. The posterior probabilities in the majority-rule consensus trees were calculated by MCMC sampling in MrBayes 3.1, using the best-fit model of evolution mentioned above.

2.3 RESULTS

2.3.1 Fungal Isolates

Ceratocystis isolates used in this study were mostly from *Ceiba pentandra* and one from *Pycnanthus macrocarpus* (Table 1), since these were the most commonly harvested species at the time of fungal collection. Fungal mats, consisting of pure *Ceratocystis* cultures were commonly found beneath the bark of these trees (Fig. 1). Isolations were made directly from these mats by transferring single ascospore drops to MEA. Ascomatal structures were common within 5 days of culturing. Ascospores were then easily transferred from perithecial tips to MEA plates to obtain pure cultures of each of the isolates. Although a total of 14 isolates were initially deposited in the culture collection, only 9 survived and managed to grow at the time of this study.

Isolates of the obtained *Ceratocystis* species in culture on MEA were whitish in colour when young but darkened as they grew older. Submerged mycelium had a honey-like colour with cream-buff aerial mycelium. Generally, the cultures had a banana like odour, after growing for 7 days. Ascomata had black elongated necks with black globose bases. Ostiolar hyphae were divergent and ascomatal bases had spines and disc-shaped attachment points where the necks and the bases converge. Isolates were fast growing, covering the surface of 65 mm Petri plates within one to two weeks.

2.3.2 DNA Extraction and Polymerase Chain Reaction (PCR)

Amplification of the β-tubulin and the ITS gene regions resulted in PCR products of about 500 base pairs (bp) and 300 bp for the TEF region. Successful amplification were achieved for all the isolates considered for this study.

2.3.3 DNA Sequencing and Phylogenetic Analyses

From the sequence results obtained for *Ceratocystis* isolates from Ghana (CMW24924, CMW24925, CMW24927, CMW24928, CMW24929, CMW24950), blast results showed

that all isolates collected in this study represent species within the *C. moniliformis* s.l. species complex. Phylogenetic analyses of the ITS gene region (32 taxa, 396 characters) showed little separation between species, with isolates from Ghana grouping into two larger clades, each including several other species (Fig. 3). It resulted in 383 constant, 13 variable (parsimony-uninformative) and 13 parsimony informative characters, with a consistency index (CI) and retention index (RI) of 1.000 and 1.000 respectively. Five of the isolates CMW24924, CMW24950, CMW24928, CMW24925 and CMW24927 were most closely grouped to *C. savannae* Kamgan & Jol. Roux and *C. oblonga* R N. Heath and Jolanda Roux, while one isolate from Ghana grouped closest to a recently described species from South Africa, *C. decipiens* Kamgan & Jol. Roux.

Analyses of the BT gene region (32 taxa, 442 characters) grouped the six isolates from Ghana into two distinct phylogenetic clades (Fig. 4). The data set had a CI and RI of 0.848 and 0.944 respectively with 399 constant characters, 43 variable and 43 parsimony informative characters. This gene region grouped four isolates (CMW24924, CMW24950, CMW24928, CMW24925) close to *C. omanensis* Al-Subhi, M. J. Wingf., M. Van Wyk & Deadman.

The TEF gene region (32 taxa, 197 characters) analyses indicated a CI and RI value of 0.814 and 0.928 respectively with 187 constant, 10 variable and 100 parsimony informative characters. The isolates from Ghana grouped into three clades (Fig. 5), two of which were close to *C. oblonga* and *C. savannae*. One isolates (CMW24927) grouped with isolates of *C. decipiens*.

A P-value of 0.001 was obtained from the PHT for the combined dataset of the three gene regions. Despite having a low value, it has been argued that this type of data can still be combined (Sullivan 1996, Cunningham 1997). Phylogenetic analyses of the combined dataset using maximum parsimony (MP) consisted of 1135 characters including gaps. It showed 964 constant and 13 variable or parsimony uninformative characters, with 158 parsimony informative characters. Eight most parsimonious trees were obtained after the analyses with 1 selected for representation (Fig. 6) with tree length of 275, consistency index (CI) of 0.775 and retention index (RI) of 0.952.

The combined dataset of the BT, TEF and ITS gene regions grouped the isolates from Ghana into at least three different clades. The larger group (CMW24924, CMW24950, CMW24928,

CMW24925) are most closely related to, but separate from *C. savannae*. Isolate CMW24927 grouped on its own, adjacent to the *C. savannae* and *C. oblonga* clade, while isolate CMW24929 grouped closest to *C. decipiens*.

2.4 DISCUSSION

No previous studies using DNA sequence data had been conducted on *Ceratocystis* species associated with forestry trees in Ghana. In this study we aimed to confirm the presence of *C. fagacearum* in the country. This pathogen of oak trees in the USA (Koch *et al.* 2010), was previously reported from Ghana, based only on studies of the morphology of isolates (Apertorgbor *et al*. 2004). Despite sampling similar tree species, in the same regions of Ghana, we did not obtain any isolates of *C. fagacearum* in the current study. All isolates sequenced in this study belonged to the *C. moniliformis* s.l. species complex, most probably representing at least two different and undescribed species.

In the study by Apetorgbor *et al*. (2004), *C. fagacearum* was reported from the native trees, *Pterygota macrocarpa*, *Ceiba pentandra* and *Celtis mildbraedi* in Nyamibe Bepo (6°10'N, 1°22'N) and Numia (6°02'N, 1°24'W) which are adjacent to each other. Isolates for the current study were obtained in the same forest regions as those studied by Apertorgbor *et al*. (2004) and included both field samples from harvested trees and logs in loading bays, as well as from logs in sawmills. Of the isolates originally collected in Ghana, only 9 remained alive in the culture collection. Of these, sequence results for all three gene regions could only be obtained for 6 isolates. The colony morphology of the 3 for which sequence results could not be obtained, resembled that of species in the *C. moniliformis* s.l. species clade.

Phylogenetically, *Ceratocystis* species from this study are most closely related to *C. savannae*, *C. oblonga* and *C. decipiens*, all species in the *C. moniliformis* s.l. species complex described previously from numerous hosts in South Africa (Kamgan Nkuekam *et al*. 2008, Heath *et al*. 2009, Kamgan Nkuekam 2011). Fungi in the *C. moniliformis* s.l. species complex can be readily distinguished from *C. fagacearum*. The most outstanding differences being in the shape of the ascospores, which are hat-shaped for species in *C. moniliformis* s.l. (Van Wyk *et al*. 1991) and flask-shaped, elliptical or slightly curved for species of *C. fagacearum* (Bretz 1952). The ascomata of *C. moniliformis* s.l. species have typical disk shaped attachment points at the bases of the ascomatal necks, short conical spines on the ascomatal

bases and both cylindrical and barrel shaped conidia (Hedgcock 1906, Yuan & Mohammed 2002, Al-Subhi *et al*. 2006, Van Wyk *et al*. 2006). On the other hand, *C. fagacearum* lacks the thickened neck based attachment to the ascomatal base as well as spines (Bretz 1952, Moreau 1952).

In this study we made use of three gene regions, the ITS, BT and TEF, which have most commonly been used to identify species in the *C. moniliformis* s.l. species complex (Van Wyk *et al*. 2004, Heath *et al*. 2009, Kamgan Nkuekam *et al*. 2008, Tarigan *et al*. 2010). Similar to the previous studies, we found very limited resolution between species using the ITS sequence data. Additionally, arrangements of clades for the BT and TEF gene regions did not match completely, with some isolates grouping in different positions for these two gene regions. A combined gene phylogenetic tree of all three gene regions, however, provided good resolution of the species, and confirmed the arrangements of clades observed with the BT gene region. Because of the clear lack of resolution between isolates based on the three regions studied, we chose not to currently describe isolates from Ghana as new species. Additional gene regions are clearly needed to fully understand species delineation within this group of fungi.

At least two possibly undescribed species of *Ceratocystis* were identified from Ghana during this study. This includes one group, most closely related to *C. oblonga* and *C. savannae* and one group possibly related to *C. decipiens*. All three of these species are currently only known from South Africa, originating from dry savanna regions, totally unrelated to the tropical forest area from which Ghanaian isolates were obtained. We have refrained from attempting to describe the isolates from Ghana at this stage. This is partly because of lack of sufficient isolates for all the clades and partly because of the above mentioned problems in delineating species within the complex. These isolates will be incorporated into a larger study of *Ceratocystis* species from Cameroon, an area with similar climatic conditions and tree species.

Results from the study, although based on a very limited number of isolates, confirms the skepticism regarding the report of *C. fagacearum* from Ghana. *Ceratocystis fagacearum* has mostly been reported on *Quercus* species from the United States of America (Bretz 1955, Tainter *et al*. 2007, Juzwik *et al*. 2008) with geographic conditions totally different from the tropics where isolates for this study were obtained. The native host plants reported in Ghana

are totally different to its known host (Apetorgbor *et al*. 2004), making it highly unlikely that the pathogen had been correctly identified.

The lack of information on *Ceratocystis* species in Ghana, and the identification of at least two undescribed species on only a small number of hosts and limited geographic area in Ghana is an indication of how poorly these fungi have been studied in this region. If one considers studies of *Ceratocystis* species in other regions, such as South Africa, where at least 10 *Ceratocystis* species have been recorded (Kamgan Nkuekam *et al*. 2009, Roux & Wingfield 2011, Kamgan Nkuekam 2011), it is highly likely that numerous additional *Ceratocystis* species await discovery in Ghana. Considering that some of these isolates were collected beneath tree bark that were not completely removed from logs, proper identification and implementing ISPM 15 is necessary for increasing Ghana's wood market share in Europe and America where standards are rigorously implemented. More investigations are needed to establish the presence and origin of *Ceratocystis* species on both native and exotic trees to more accurately quantify their potential threats as quarantine pests.

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Table 1: *Ceratocystis* isolates and those selected with accession numbers used for sequence comparisons in this study.

ISOLATE	COUNTRY	GENBANK ACCESSION NOS.				CULTURE NO.	HOST	COLLECTOR
		TEF	BT	ITS	OTHER NUMBERS			
C. bhutanensis	Bhutan	AY528954	AY5289564	AY528959	CBS115772, BH 8/8	CMW8399	*Picea spinulosa*	T. Kirisitis & DB. Chhetri
C. bhutanensis	Bhutan	AY528953	AY528963	AY528958	CBS114290 PREM57805	CMW8215	*P. spinulosa*	T. Kirisits & DB. Chhetri
C. decipiens	South Africa	HQ236437	HQ203236	HQ203218	n/a	CMW25918	*Eucalyptus cloeziana*	NG. Kamgan & J. Roux
C. decipiens	South Africa	n/a	n/a	n/a	n/a	CMW30829	*E. cloeziana*	NG. Kamgan & J. Roux
C. decipiens	South Africa	n/a	n/a	n/a	n/a	CMW30701	*Brachypeplus depressus*	NG. Kamgan & J. Roux
C. decipiens	South Africa	HQ236437	HQ203236	HQ203236	n/a	CMW30830	*B. depressus*	NG. Kamgan & J. Roux
C. inquinans	Indonesia	EU588674	EU588666	EU588587	n/a	CMW21106	*Acacia mangium*	M. Tarigan

C. inquinans	Indonesia	EU588675	EU588667	EU588588	n/a	CMW21107	*A. mangium*	M. Tarigan
C. microbasis	Indonesia	EU588679	EU588671	EU588592	n/a	CMW21115	*A. mangium*	M. Tarigan
C. microbasis	Indonesia	EU588680	EU588672	EU588593	n/a	CMW21117	*A. mangium*	M. Tarigan
C. moniliformis	South Africa	AY529006	AY528985	AY431101	CBS116452	CMW9590	*Eucalyptus grandis*	J. Roux
C. moniliformis	Bhutan	AY529016	AY529005	AY528995	n/a	CMW8379	*Cassia fistula*	M.J. Wingfield
C. moniliformopsis	Australia	AY529008	AY528987	AY528998	CBS109441	CMW9986	*E. obliqua*	Z.Q. Yuan
C. moniliformopsis	Australia	AY529009	AY528988	AY528999	CBS115792, ORB 33	CMW10214	*E. sieberi*	M.J. Dudzinski
C. oblonga	South Africa	EU244951	EU244991	EU245019	CBS122291	CMW23803	*A. mearnsii*	R.N. Heath
C. oblonga	South Africa	EU244951	EU244992	EU245020	CBS122820	CMW23802	*A. mearnsii*	R.N. Heath
C. omanensis	Oman	DQ074737	DQ074732	DQ074742	CBS115780, PREM57815	CMW11048	*Mangifera indica*	AO.Al-Adawi
C. omanensis	Oman	DQ074734	DQ074729	DQ074739	CBS118112 PREM57814	CMW11046	*M. indica*	AO.Al-Adawi

C. salinaria	South Africa	n/a	n/a	n/a	n/a	CMW30704	*E. maculata*	NG Kamgan & J. Roux
C. salinaria	South Africa	HQ236432	HQ203230	HQ203213	n/a	CMW25911	*E. maculata*	NG Kamgan & J. Roux
C. savannae	South Africa	EF408572	EF408566	EF408551	PREM59423	CMW17300	*A. nigrescens*	NG. Kamgan & J. Roux
C. savannae	South Africa	EF408573	EF408566	EF408552	n/a	CMW17297	*Combretum zeyheri*	NG. Kamgan & J. Roux
C. sumatrana	Indonesia	EU588676	EU588668	EU588589	n/a	CMW21109	*A. mangium*	M. Tarigan
C. sumatrana	Indonesia	EU588677	EU588669	EU588590	n/a	CMW21111	*A. mangium*	M. Tarigan
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24924	*Ceiba pentandra*	J. Roux & M. Apetorgbor
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24925	*C. pentandra*	J. Roux & M. Apetorgbor
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24927	*C. pentandra*	J. Roux & M. Apetorgbor
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24928	*C. pentandra*	J. Roux & M. Apetorgbor
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24929	*Pycnanthus macrocarpus*	J. Roux & M. Apetorgbor
***Ceratocystis* sp.**	Ghana	n/a	n/a	n/a	n/a	CMW24950	*C. pentandra*	J. Roux & M. Apetorgbor

C. tribiliformis	Indonesia	AY529015	AY528994	AY529004	CBS115949	CMW13015	*Pinus merkusii*	M.J. Wingfield
C. tribiliformis	Indonesia	AY529014	AY528993	AY529003	CBS115949	CMW13013	*P. merkusii*	M.J. Wingfield

Figure 1: White and grey mats of sporulating *Ceratocystis* sp. on the cambium of a *C. pentandra* tree after removal of the bark (Photo by Jolanda Roux of FABI, University of Pretoria, Pretoria South Africa).

Figure 2: Mycelium and sporulating ascocarps of *Ceratocystis* sp. growing under the bark of a *C. pentandra* tree in Ghana (Photo by Jolanda Roux of FABI, University of Pretoria, Pretoria South Africa).

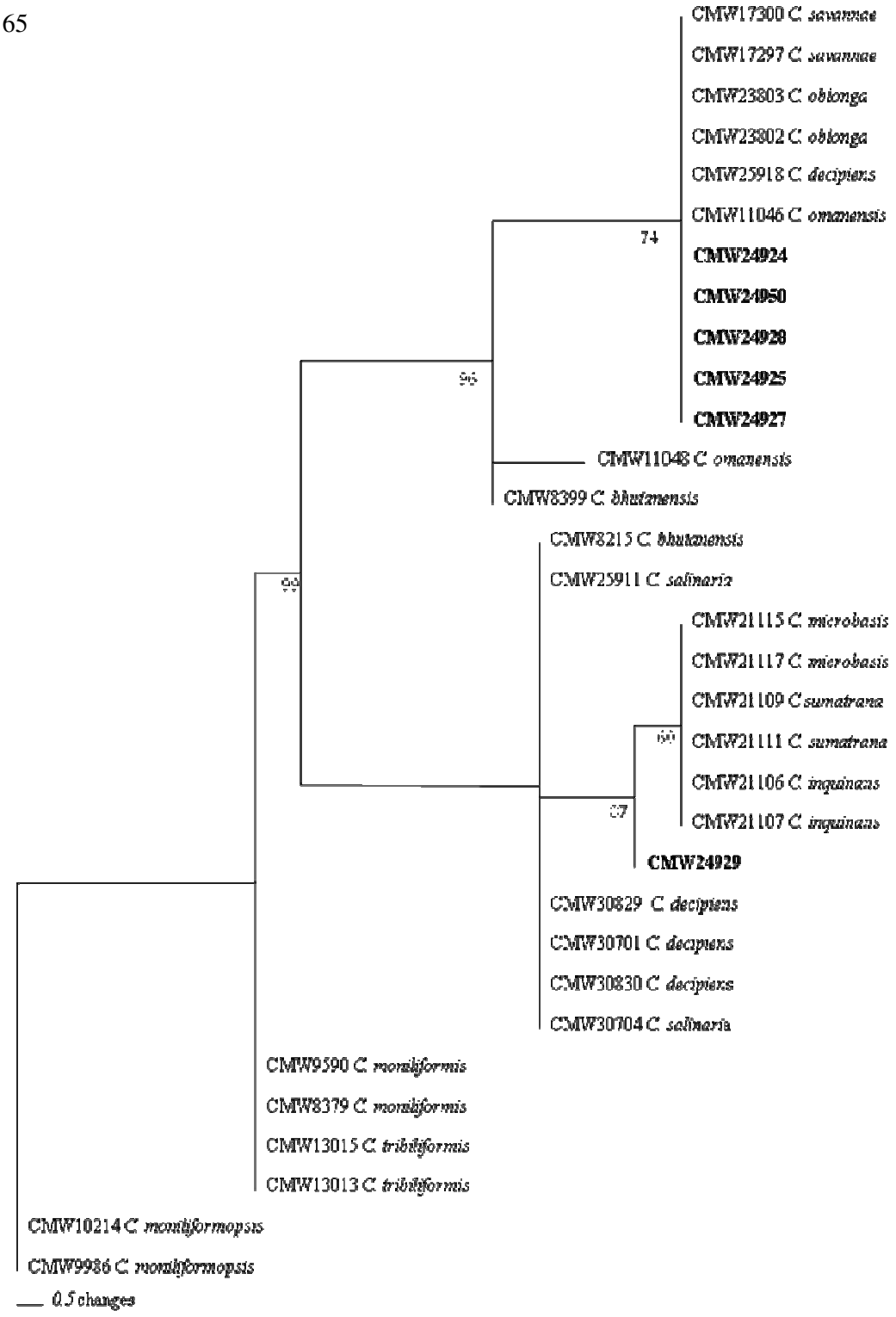

Figure 3: Phylogenetic tree based on the sequence data set of ITS gene region showing relationships between *Ceratocystis* species in the *C. moniliformis* s.l. complex and isolates sequenced in this study represented in bold. Bootstrap values are indicated at the branch nodes and Bayesian posterior probabilities are excluded because the ITS region has a poor resolution with this group of fungi. *Ceratocystis moniliformopsis* were selected as the outgroup taxon.

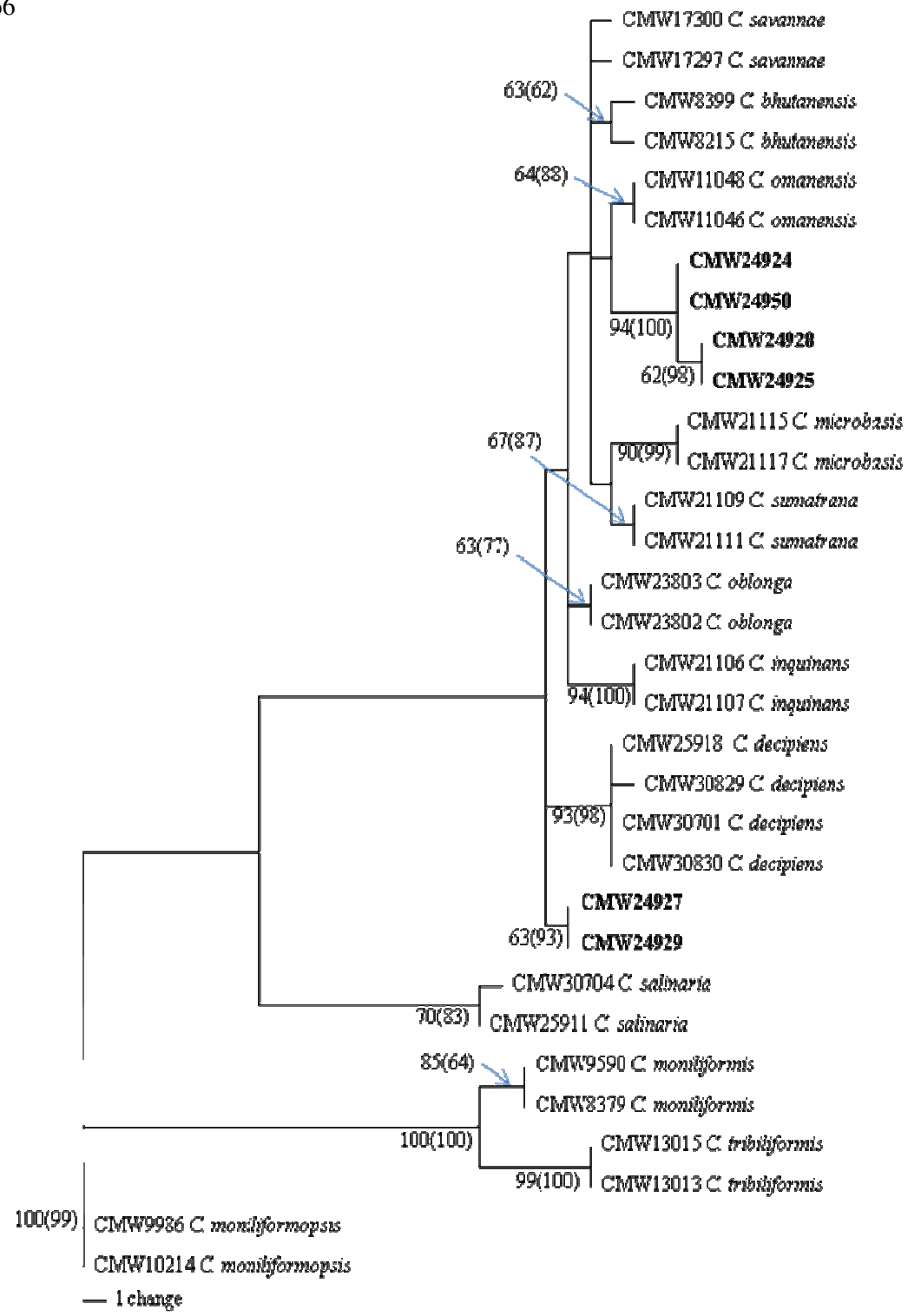

Figure 4: Phylogenetic tree based on the sequence data set of β-tubulin gene region showing relationships between *Ceratocystis* species in the *Ceratocystis moniliformis* s.l. complex and isolates sequenced in this study represented in bold. Bootstrap values and Bayesian posterior probabilities (*in brackets*) are indicated at the branch nodes. *Ceratocystis moniliformopsis* were selected as the outgroup taxon.

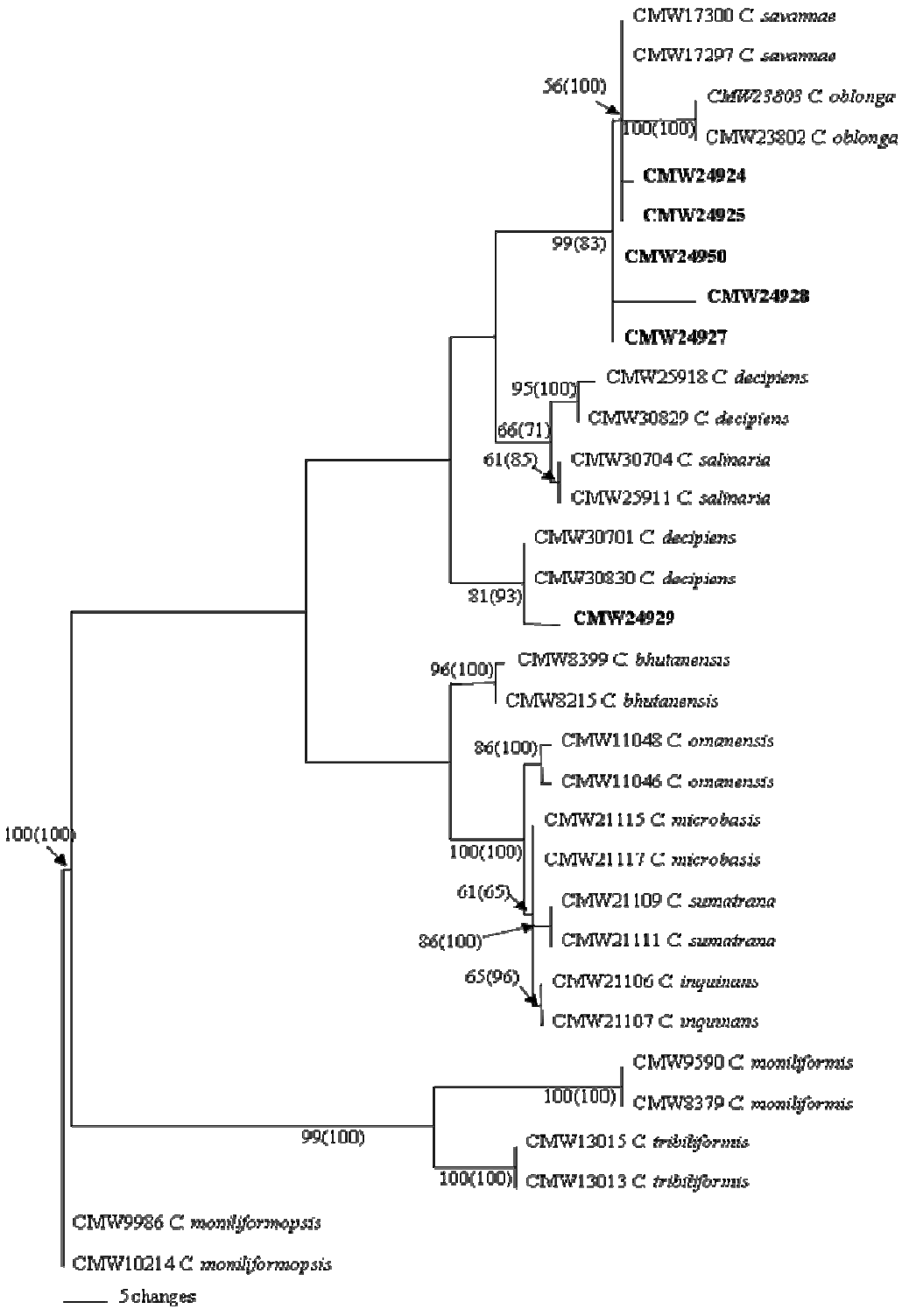

Figure 5: Phylogenetic tree based on the sequence data set of TEF gene region showing relationships between *Ceratocystis* species in the *C. moniliformis* s.l. complex and isolates sequenced in this study represented in bold. Bootstrap values and Bayesian posterior probabilities (*in brackets*) are indicated at the branch nodes. *Ceratocystis moniliformopsis* were selected as the outgroup taxon.

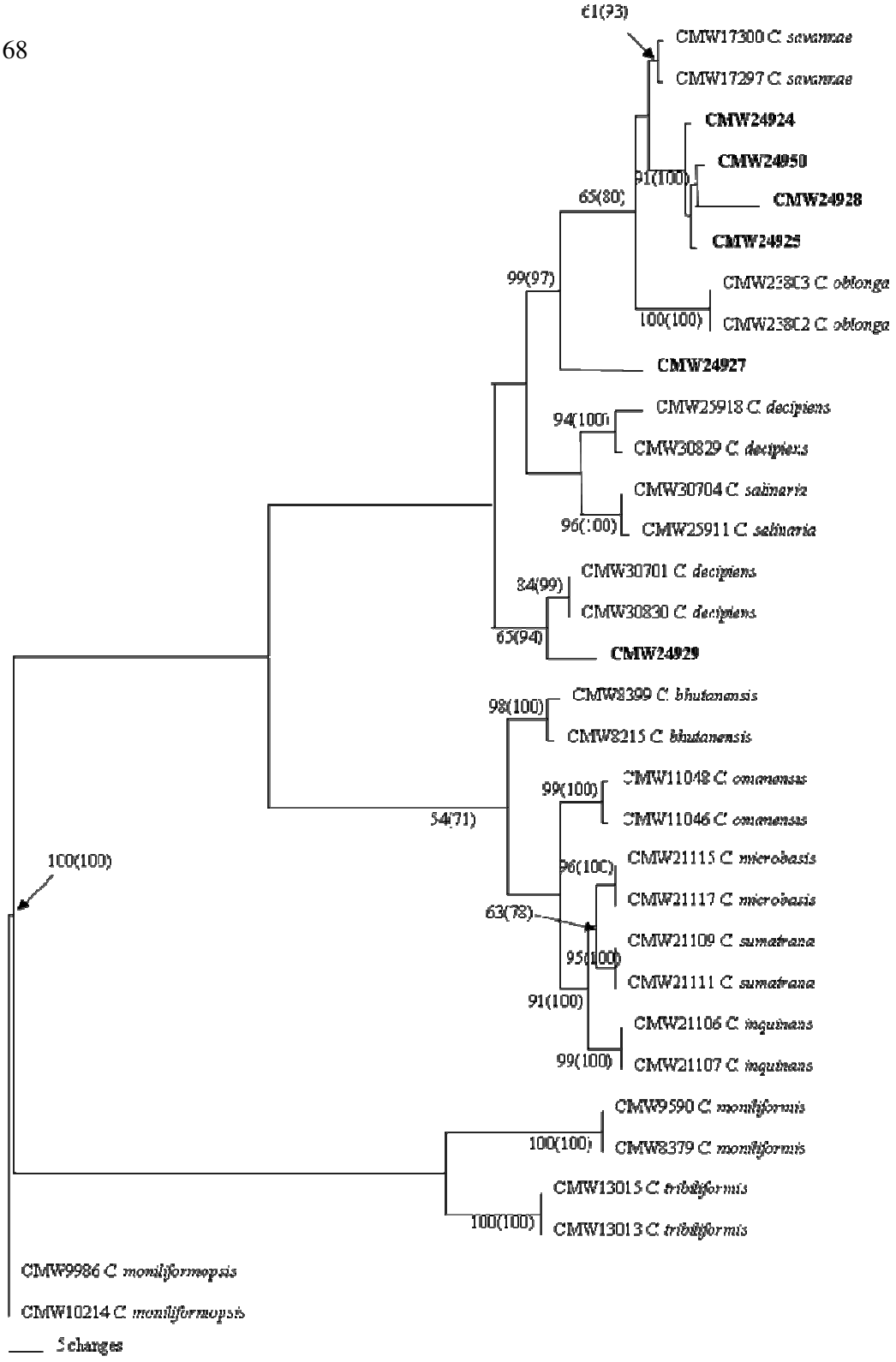

Figure 6: Phylogenetic tree based on the combined sequence data of three gene regions (ITS, BT1 and TEF) showing relationships between *Ceratocystis* species in the *C. moniliformis* s.l. complex and isolates sequenced in this study represented in bold. Bootstrap values and Bayesian posterior probabilities (*in brackets*) are indicated at the branch nodes. *Ceratocystis moniliformopsis* were selected as the outgroup taxon.