Ophiostomatoid fungi and their insect associates on *Eucalyptus* trees in Australia and South Africa

By

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DECLARATION

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

Kamgan Nkuekam Gilbert

July 2011

I dedicate this thesis to my mother Ngangnou Helene and my late father Nkuekam Rene

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PREFACE

The so-called Ophiostomatoid fungi include species in at least five genera of the Ascomycota. These include *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, *Ophiostoma* and *Gondwanamyces*. Although morphologically similar, these fungi reside in different orders that are phylogenetically unrelated. *Ceratocystis*, for example, resides in the Microascales, while *Ophiostoma* belong in the Ophiostomatales. These fungi are typically adapted to insect dispersal and share similar ecological niches. *Ceratocystis* and *Ophiostoma*, the two nominal genera in the Ophiostomatoid fungi were erected in the late 19th century. The type species of *Ceratocystis*, *C. fimbriata*, was first described in 1890 and recognized as the causal agent of sweet potato root rot in the USA. Thereafter, the taxonomic placement of *Ceratocystis* and *Ophiostom*a, as well as the other genera in this grouping, became the source of extended controversy. However, it is now well accepted that species of *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, *Ophiostoma* and *Gondwanamyces* belong in different orders of the Ascomycota. To date more than 150 Ophiostomatoid species have been described worldwide. The Ophiostomatoid fungi include species of economic importance that cause diseases of trees and crop plants globally. However, the wide majority cause sap stain of timber, downgrading its commercial value substantially. The ability of these fungi to cause plant, human and animal diseases and the ease with which they spread with insects and timber over long distances, have made them one of the most important groups of fungi today.

The Ophiostomatoid fungi require wounds for infection and rely on a wide variety of insects to reach infection sites on trees. Some species have highly specific associations with insects, while others, such as species of *Ceratocystis fimbriata sensu lato* and *C. fagacearum* have what has commonly been described as loose associations with insects. Amongst the insects associated with Ophiostomatoid fungi, those in the Nitidulidae, especially, have less specific associations with these fungi. Increasing evidence is, however, appearing to suggest that the association between the Nitidulidae and the Ophiostomatoid fungi is more complex and specific than previously thought. This association clearly needs further study, especially in Africa, where only two studies considering this association have been undertaken.

The majority of eucalypts are native to Australia, where they grow in a diverse range of conditions. Today, these trees represent the most widely grown non-native tree species internationally, with more than 20 million hectares of forestry plantations having been established globally. Numerous disease and insect problems have emerged in the countries where eucalypts are grown as non-natives, necessitating intensive breeding and management programmes to grow these trees

commercially. Limited information exists regarding the fungi occurring on these trees in their native habitats. These fungi and insects represent a huge threat to eucalypt trees in non-native plantation situations. Eucalypts can be regarded as sources of intercontinental movement of fungi and insects pests. Studying the fungal associates of eucalypts, both in native and non-native situations will provide valuable information regarding the possible movement of fungal pathogens between Australia and other countries.

Chapter one of this dissertation presents a review of literature pertaining to the taxonomy, biology and ecology of Nitidulidae and their association with fungi. Particular focus is placed on Ophiostomatoid fungi isolated from these insects. The role of the Nitidulidae in transmitting *C. fagacearum* is illustrated in a disease cycle represented schematically to summarize this association. Furthermore, the nature of the factors that mediate attraction of sap beetles to each other, to fungi and to wounds on trees is discussed.

The remaining chapters of this dissertation aim to increase our knowledge regarding the Ophiostomatoid fungi, and their nitidulid vectors, on eucalypts in Australia and in Southern Africa. Chapter two concerns *Ophiostoma* spp., and their *Pesotum* and *Sporothrix* anamorphs that infect wounds of eucalypt trees in Australia. The Ophiostomatales include important pathogens of trees. However, limited reports of fungi in this order are available from Australia. The study focuses on the Eastern and Southern parts of Australia and targets naturally occuring wounds on eucalypt trees. Use was made of both morphological studies and multigene sequence phylogenies for identification and taxonomic purposes. Furthermore, the pathogenicity of the isolated species was determined on *Eucalyptus* trees grown in a quarantine greenhouse.

Chapter three of the dissertation addresses the biodiversity and phylogeny of *Ceratocystis* spp. and their nitidulid vectors on eucalypt trees in Eastern and Southern Australia. Prior to this study, only five *Ceratocystis* spp. had been reported from eucalypts in Australia. Similar to studies in Chapter two, fungi and their insect vectors were collected from naturally occurring and harvesting wounds on eucalypts in several regions of Australia, and these were studied using morphology and multi-gene DNA sequence data.

Chapters four and five deal with Ophiostomatoid fungi and their associated insect vectors on eucalypt trees grown as non-natives in commercial plantations in South Africa. Only three *Ophiostoma* spp. and four *Ceratocystis* spp. have previously been reported from eucalypts trees in South Africa. For both genera, the insects that might carry them in the country have not been

considered. Studies presented in these two chapters, therefore, addresses this lack of knowledge. Morphological identification coupled to multigene phylogenies were used to explore various aspects of the diversity of *Ceratocystis* spp. and *Ophiostoma* spp. collected from wounds on *Eucalyptus* spp. in the major plantation areas of South Africa.

CHAPTER I

The Nitidulidae: Taxonomy, biology and ecology with special reference to their associations with fungi

1.1. INTRODUCTION

The Nitidulidae encompass insects in the order Coleoptera, commonly known as sap beetles or picnic beetles. The family comprises beetles generally of small size, less than 10 mm long, having a variety of colors, clubbed antennae and visible segments on the abdomen (Dowd 1991, Borror *et al.* 1976). These insects have a worldwide distribution and many are important pests of plant crops and stored products (Hinton 1945). They infest a wide variety of materials such as sap flows on plants and trees, flowers, decaying plant material, fresh fruits and vegetables, maize (corn), dried fruits, stored grain, peanuts, cottonseed, spices, sugar and many more (Hinton 1945).

The Nitidulidae include several species of economic importance. Species in the genus *Carpophilus* Stephens, for example are well known cosmopolitan pests with broad host ranges. They cause damage to a large variety of agricultural products including fruit and grains, both before and after harvest. This is either by direct infestation or by transmission of pathogenic fungi (Hinton 1945, Bartelt *et al.* 1992a).

Examples of important pests include the dried fruit beetle, *Carpophilus hemipterus* (L.), a cosmopolitan pest of fresh and dried fruit, as well as many fresh and stored grains, spices, drugs, and seeds (Dowd & Bartelt 1991, Hinton 1945). Similarly, *C. davidsoni* Dobson, a small reddish-brown sap beetle occurring in a number of countries including Australia, New Zealand, and Micronesia, is a pest of Australian sweet corn, figs and peaches (Dobson 1952, Gillogly 1962, Bartelt & James 1994). *C. obsoletus* Erichson, a small dark-brown sap beetle that occurs throughout the tropical, subtropical, and milder temperate regions of the world is a serious pest of dried fruit commodities, but also infests maize (Petroski *et al.* 1994). The corn sap beetle, *C. dimidiatus* Fabricius, is a pest of maize but has also been reported from about 89 different plant species (Connell 1975).

Many nitidulid beetles are adapted to woody environments, confined to forests and trees where they feed on fungi, pollen and most importantly on sap flowing from wounds on trees (Kirejtshuk 1998a). These insects are not recognized as primary pests, due to the fact they do not create wounds on trees themselves, but rather rely on wounds arising from other factors. However, their associations with important pathogens of trees, such as those in the Ophiostomatoid group of fungi, have made them some of the most important threats especially to hardwood plantation trees, worldwide. Important examples of pathogens illustrating this relatively recently discovered role of the Nitidulidae include *Ceratocystis fagacearum* (Bretz) Hunt, the cause of oak wilt diseases in the

United States of America (USA) (Jewell 1956, Juzwik & French 1983), *Ceratocystis albifundus* De Beer, Wingfield & Morris, the cause of wattle wilt disease of *Acacia mearnsii* trees in Southern Africa (Roux & Wingfield 2009) and *C. fimbriata sensu lato*, that cause canker on aspen, and is the so-called "mallet wound pathogen" of almond trees (Hinds 1972, Moller & Devay 1968).

The aim of this review is to provide a summary of the biology and ecology of nitidulid beetles. Additional focus is placed on their associations with fungi, especially members of the Ophiostomatoid fungi, and the impact they have on hardwood plantation forestry. Other important issues considered include the chemical communication, both between nitidulid beetles and between these insects and their hosts, especially the chemical basis mediating host attraction and host detection. The type of chemical communication reported in the literature and the impact this might have on mating, ecology and population dynamics of nitidulids is also considered.

1.2. THE NITIDULIDAE

1.2.1. Evolutionary history and phylogeny

Nitidulid beetles reside in the order Coleoptera, suborder Polyphaga, series Cucujiformia and superfamily Cucujoidae (Parsons 1943, Kirejtshuk 1998b). The Coleoptera appeared on earth more than 285 millions years ago (Crowson 1981, Hunt *et al*. 2007), followed by radiations of the sub-orders Archostemata, Adephaga and Polyphaga, which have been found in fossils from the middle Triassic onwards (Crowson 1981, Hunt *et al*. 2007). Members of the Nitidulidae as well as other Cucujoid beetles have been recorded exclusively from different layers of the early Cretaceous, increasing in number to the end of this period (Kirejtshuk 1998b). This early period, according to Hunt *et al.* (2007), corresponds approximately to 127.7 million years ago (Figure 1).

Nitidulid beetles are thought to have originated from aquatic environment. This view emerged from the fact that these insects bear a number of features and traces of ancient behaviors typical of aquatic insects. Almost all nitidulid species pupate in moist soil despite the diverse ecological living and feeding habits of active immature and mature stages. This may be regarded as an example of a trace of ancient behavior, which raises the supposition that their ancestral larvae inhabited more or less wet, or liquid substrates and left these for pupation in comparatively drier conditions such as in soil (Kirejtshuk 1991). The moist environments probably included tree sap and soft fruits fermented with yeast and other microorganisms. Many adults of modern nitidulid beetles belonging to different sub-families are still attracted to such substrata and some of them use these niches as breeding sites for their larvae (Kirejtshuk 1991).

Hunt *et al.* (2007), published results of an extensive molecular genetic investigation of beetle phylogeny. They constructed multi-gene phylogenies of Coleopteran genera, inferred from three gene regions and for nearly 1900 beetle species, representing more than 80% of the world's recognized beetle families. In the resulting phylogenetic tree, the series Cucujiformia was strongly supported as monophyletic (Hunt *et al.* 2007). However, within this hyperdiverse series, the superfamily Cucujoidae was polyphyletic. The family Nitidulidae forms a monophyletic group within the Cucujoidae in subclades including fungivorous species (Figure 2), and most closely related to the Erotylid series and the Cucujid series, respectively (Hunt *et al*. 2007).

1.2.2. Taxonomy and classification of the Nitidulidae

The Nitidulidae represent the second largest family (after the Coccinellidae that includes more than 6000 species) of the superfamily Cucujoidea in the Coleoptera (Lawrence 1982). The family comprises more than 4000 species in more than 200 genera (Lawrence 1982, Kirejtshuk 2008). This number is highly underestimated and will likely increase several times, when more comprehensive work dealing with the family at species level is undertaken. In a treatise of the Coleoptera at family and sub-family levels, seven sub-families were recognized in the Nitidulidae, but nothing was mentioned regarding the number of species in the family (Lawrence & Newton 1995). More recently, Kirejtshuk (2008) provided a list of genera in the Nitidulidae, organized into sub-families, but once more nothing was mentioned regarding the number of species for each sub-family and genus.

According to Kirejtshuk (2008), the Nitidulidae include more than 206 genera, subdivided into ten sub-families. The sub-families and the number of genera they might contain (in brackets) are as follows: Nitidulinae (110), Cillaeinae (29), Cryptarchinae (15), Meligethinae (14), Carpophilinae (7), Amphicrossinae (2), Epuraeinae (21), Calonecrinae (1), Maynipeplinae (1) and Cybocephalinae (8). There are three additional genera in the Cybocephalinae, for which the generic status still needs to be elucidated (Kirejtshuk 2008). Characteristics and keys that separate members of the sub-families from each other are summarized in Table 1.

The family Nitidulidae, as it currently stands, resides in three main lineages (Table 2). These are the Nitiduline lineage, comprising the sub-families, Nitidulinae, Cillaeinae, Cryptarchinae, Cybocephalinae and Meligethinae; the Carpophyline lineage that comprises the sub-families Carpophilinae, Amphicrossinae and Epuraeinae and the Calocrine lineage that comprises the sub-families Calonecrinae and Maynipeplinae (Kirejtshuk 1994, 1998a, 2008).

The three lineages of the Nitidulidae are defined based on their morphology and geographic distribution. Thus the Nitiduline and the Carpophiline can be distinguished based on the structure and function of the male genitalia (Kirejthuk 1994, 1998a). The tegmen in the Carpophiline is more frequently well sclerotized, deeply excised, and divided into two lobes, between which a membranous penis protracts (Figure 3). The aedeagus of the Nitiduline is formed by two plates, often equally strongly sclerotized, protracting in the same translational motion outwards, and opening as a knife leaves before the inner sac of the penis is ejected (Kirejtshuk 1998a). Members of the Nitiduline occur worldwide and exhibit higher levels of abundance and diversity in the western hemisphere. In contrast the Carpophiline occurs mainly in the eastern hemisphere (Kirejtshuk 1994, 1998a). Members of the Calocrine share a number of structural characteristics with both the Nitiduline and the Carpophiline (Kirejtshuk 1998b).

1.2.3. Macro-morphology

Nitidulids are typically small black or brown beetles, in some cases with red, yellow or orange spots on their elytra, which are broad and flattened (Kistner 1982). Body shapes are often oval, some elongate and depressed, with sizes ranging from 1.5-12.0 mm in length (Habeck 2002). Their antennae are 11 segmented with an apical club made-up of three antenomeres and a tarsal formula of 5-5-5 or 4-4-4 (Habeck 2002). Larvae are elongate, 2-20 mm, most less that 12 mm long, parallel sided or fusiform, straight or curved ventrally, occasionally curved dorsally and subcylindrical to strongly flattened (Habeck 2002). Almost all larvae have a ventral head structure and ventral mouthparts (Lawrence 1991).

1.3. LIFE HISTORY

The life history, differs between nitidulid species with regards to mating, oviposition, longevity and length of the immature stage. Nitidulids may overwinter as adults or as larvae and pupate in the soil or in detritus (Dorsey & Leach 1956, Schmidt 1935, Balzer 1942). In the following section the life histories of a few species in three genera of the Nitidulidae are discussed as examples to illustrate the varying life histories of beetles in this family.

Connell (1956), studied the life histories of eight *Carpophilus* spp. from field observations and laboratory-reared populations and observed very minor differences in the generation time among *C. freemani* Dobson, *C. hemipterus*, *C. humeralis* Fabricius, *C. lugubris* Murray and *C. melanopterus* Erichson. These beetles incubate as eggs for 1-2 days and 3 instars develop in 15-18 days. They spend 5-8 days as prepupae and 6-9 days as pupae (Connell 1956, Skalbeck 1976). The life history

is relatively short for *C. semitectus* Say, which lasts from 21-25 days (Dorsey & Leach 1956). In contrast *C. dimidiatus*, under optimum conditions, can complete its life cycle in 18 days in summer, whereas in winter it may extend over 150-200 days (Balzer 1942). Furthermore, the adults of *C. dimidiatus* crawl during winter but do not attempt to fly, they resume flying activities during warm days. Adults live about 90-117 days in summer while overwintering specimens live as long as 200 days (Balzer 1942).

Dickason (1954), studied the life history and biology of *Meligethes seminulum* Lec. (Coleoptera: Meligethinae) in Western Oregon (USA). There is one generation of this beetle per year. The adults overwinter in plant debris and in protected areas near the soil surface, and emerge in Spring, more precisely late in March during the flowering period of the yellow mustard plant *Brassica campestris* L. where feeding occurs on the pollen prior to migration of the beetles to other leguminous plants. Mating occurs late in April to early June on the flowers of Fabaceae. Oviposition is coincidental with the development of flowers, starting in the basal florets and progressing outwards as the flower develops. The majority of eggs are laid in May and June. Eggs hatch in three to four days under laboratory conditions. Larvae may be found in the field within flowers from early May until July where they feed primarily on pollen and casually on plant tissue in the undeveloped flowers. After up to three weeks spent in this niche, the mature larvae leave the florets and drop to the ground where they penetrate the loose soil and pupate. Under laboratory conditions the pupal stage requires eight to nine days. The majority of adults emerge by mid-July, where they are found on the flowers of numerous plants, particularly leguminous plants.

McCoy & Brindley (1961), first described the biology and life history of *Glischrochilus quadrisignatus* (Say) (Coleoptera: Nitidulinae). They suggested that the beetle has two generations a year in Illinois, United States of America (USA), but Luckamann (1963) later reported a single generation of the beetle per year. The beetle overwinters as an adult, most commonly beneath the bark of logs, in tree wounds, in clumps of grass, in the soil, and in or beneath the residue of decomposing vegetables, fruits, or grain. Adults emerge in late April, mostly on warm days and become active and are soon attracted to tree wounds, decaying vegetable matter, either in direct contact with the soil or totally buried. Mating occurs late in April or early in May. Oviposition begins in April, but most eggs are deposited during May and can continue into early July (Luckmann 1963). Larvae can often be in this niche late in May. Adult emergence of the first generation occurs in mid-July and this marks the start of the second generation, which would have emerged in early September (McCoy & Brindley 1961).

1.3.1. Reproduction

Some Nitidulids species have a high potential of reproduction. For example, it has been found that each female of *Colopterus semitectus* Say lays an average of 72 eggs, and eggs begin to hatch three days after oviposition. Mature larvae enter the pupal stage within 2 days and emerge as adults within 3-4 days (Dorsey & Leach 1956). The females of *C. dimidiatus* lay about 175-225 eggs that start to hatch within 24 hours after oviposition (Balzer 1942), while some females of *C. humeralis* produce on average 14.66 eggs per day (Schmidt 1935).

1.4. BIOLOGY AND ECOLOGY OF NITIDULIDAE

1.4.1. Habitats

Nitidulid beetles live in a wide variety of environments world-wide. However, woody ecosystems consisting of trees and brushes are preferred habitats (Kirejtshuk 1998b). For example, members of the sub-families Epuraenae, Amphicrossinae, Calonecrinae and Cryptarchinae are mostly confined to forests, while most members of the Carpophilinae, Nitidulinae and Cillaienae are associated with fungi growing on the wood or sap of tree wounds (Kirejtshuk 1998b). Numerous species in many genera of the Nitidulinae, such as *Thalycra*, *Pocadius*, *Quadrifons* and *Thalycrodes* are mostly associated with sub-terranian fungi in forests. Others, with close connections to woody environments, include members of the parasitoid genera *Cybocephalinae*, and *Cychramptodini*, which prey on white flies and coccids living with trees and shrubs (Kirejtshuk 1998b). Only one species has an aquatic habitat. *Amphicrossus japonicus* Reitter breeds in bamboo sap and subsequently enters water filled-bamboo calus where it breathes via a ventral air sheath (Kovac *et al.* 2007).

Nitidulid beetles are designated as sap and fungus beetles due to their feeding habits and the type of environments with which they are associated. These insects and their larvae are mostly found in moist tree wounds and sapwood under loosened bark of living or dying trees (Dorsey & Leach 1956), where they feed on sap and fungi associated with this type of environment. The adult beetles have flattened bodies adapted for inhabiting small crevices between the bark and sapwood of wounded trees. When disturbed in their natural habitat, they rapidly disappear by hiding in cracks, by feigning death and dropping to the ground, or by flying away (Dorsey & Leach 1956).

Wounds on trees, and especially sap-flows from these wounds, are important ecological niches for Nitidulidae. The quality of the sap produced by trees seems to be an important factor on which the beetles rely to select their host trees. For example, it has been found that certain tree species, such

as members of the white oak group (*Quercus alba* L, *Q. stellata* Wang, *Q. muhlenbergii* Engelm., etc.) provide sap-flows attractive to nitidulid beetles, mostly because of the high sugar content, or at least the fragrance of their fermenting sap (Vogt 1950). Saps produced by some tree species of the black oak group are entirely unattractive to these insects (Vogt 1950). Included in this last category are conifers, with their repellent resins and some well known hardwood trees such as American elm (*Ulmus americana* L.) and cherry trees that exude watery and gummy sap respectively, none of which are attractive to nitidulid beetles (Vogt 1950).

1.4.2. Feeding habits and social life

Members of the Nitidulidae have a large feeding repertoire and habitats. Most nitidulids feed on flowers, fruit and fungi. A few are carnivorous (Kistner 1982). Some feeding habits and social life styles known to nitidulid beetles include: mycophagy, anthophily, phytophagy, necrophagy, predation and inquilinism. In the following sections these feeding and social habits are discussed in detail.

1.4.2.1. Inquilinism

Inquilinism refers to organisms that are associates or symbionts of social insects, thus inquiline Nitidulidae are those species in the family that share the nests of their social hosts. There are two types of inquilines amongst Nitidulidae. The first includes species that are associates, or symbionts, of bees. Species involved in this association are referred to as melittophiles. The second type includes species that are associates, or symbionts, of ants and species involved in this type of association are referred to as myrmecophiles (Kistner 1979).

An example of a melittophile is the hive nitidulid, *Aethina tumida* Murray (Coleoptera, Nitidulidae). This nitidulid is native to sub-saharan Africa, but has recently also been reported from the southern USA and Eastern Australia (Ellis & Hepburn 2006). In its native range, *A. tumida* is both a scavenger and a symbiont of *Apis mellifera* L., an African sub-species of the western honey bee. The nitidulid beetles share the nests of their bee hosts, feeding on pollen, honey and bee brood, and damaging the hive in the process (Kistner 1982, Ellis & Hepburn 2006). The bee may benefit from this association by being relieved of weakened, diseased or abandoned hives, which are potential sources of pathogens (Ellis & Hepburn 2006). The nitidulid beetles spend their egg, larval and adult stages in the hive, but pupate in soil, and their reproduction is often maximized in bee colonies that abscond from the hives (Kistner 1982, Ellis & Hepburn 2006). Within its non-native range, *A. tumida* has been reported as a serious pest, causing devastation to colonies of European

sub-species of the honey bee in the USA and Australia (Ellis & Hepburn 2006, Hoffmann *et al.* 2008).

The nitidulid beetle, *Amphotis marginata* Fabr., is an example of a myrmecophile. These beetles obtain their food from the ant *Lasius fuliginosus* Latreille (Kistner 1979). Unlike melittophiles, *A. marginata* do not share the nests of their ant hosts, but live near the nest where most of the trails of the ants converge. When a fully loaded ant returns from her foraging expedition, the beetle induces the ant to regurgitate food by palpating the labrum. The ant then releases some food which the beetle eats (Kistner 1979). These beetles are heavily armored to enable them to withstand attacks from the ants (Kistner 1979).

1.4.2.2. Mycophagy

Mycophagy, also known as fungivory or mycetophagy, refers to a habit of some members of the Nitidulidae that are more closely associated with fungi. These insects feed on fungi and are also involved in transmission of fungal pathogens (Lawrence 1991). Nitidulidae feed on a wide variety of fungi including ascomycetes and basidiomycetes (Lawrence 1977, 1991, Lawrence & Milner 1996). Some *Epuraea* spp. for example, feed on conidia of molds (Eurotiales), *Prometopia sexmaculata* Say has been associated with *Hypoxylon* spp. (Xylariales) and *Soronia hystrix* Broun feeds on sooty molds (Dothideales: Capnodiaceae) in New Zealand (Lawrence 1977, 1991, Lawrence & Milner 1996).

Nitidulidae associated with basidiomycetes are numerous and most inhabit the sporocarps of higher basidiomycetes. These species were reviewed by Lawrence (1991), who lists, amongst others, nitidulid species in the genera *Pallodes*, *Neopallodes*, *Cychramus* and *Cyllodes* associated with cap fungi such as those in the genera *Armillaria*, *Lampteromyces*, *Pleurotus*, *Pholiota* and *Russula*. *Epuraea monogama* Crotch, on the other hand, feeds on spores of the polypore *Cryptoporus volvatus* (Peck) Shear (Gillogly & Gillogly 1954). Gasteromycetes have also been reported as hosts for some Nitidulidae. *Pocadius* spp. for example, are known to feed on puff-ball spores (Lawrence 1991, Leschen & Carlton 1994).

Examples of Nitidulidae vectoring fungal pathogens include species of *Colopterus*, *Carpophilus*, *Haptoncus* and *Glischrochilus* that vector *C. fagacearum* between oak trees (Norris 1953, Dorsey & Leach 1956, Dorsey *et al.* 1953, Jewell 1956, Juzwik & French 1983) and aspen (Hinds 1972) and *Urophorus humeralis* (F), *C. hemipterus* and *Haptoncus ocularis* (Fairmaire) which vector *C. paradoxa* (Dade) Moreau in sugarcane (Chang & Jensen 1974). *Glischrochilus* spp. are known to

transmit species of *Fusarium* in maize (Windels *et al.* 1976), while some *Epureae* spp. may transmit rust diseases in pine trees as they feed on the nectar exuded by the pycnidial stages of rust fungi such as *Cronartium* and *Peridermium* (Basidiomycetes, Uredinales) (Powell 1971). *Brachypeplus depressus* Erichson, *C. bisignatus* Boheman and *C. hemipterus* have been shown to be associated with the wilt pathogen *C. albifundus* on *Acacia mearnsii* trees in plantations in South Africa (Heath *et al.* 2009).

1.4.2.3. Predation

Predation is known in members of the Cybocephalinae that prey and feed on a wide variety of insects including armored scales insect (Diaspididae), whiteflies (Aleyrodidae), mealybugs (Pseudococcidae), and the citrus red mite, *Panonychus citri* (McGregor) (Kirejtshuk *et al.* 1997, Smith & Cave 2006, Lawrence 1991, Smith & Cave 2006). Other nitidulid beetles known as predators belong to the sub-family Nitidulinae. A well known example is *Glischrochilus quadripustulatus* L. that preys on the eggs and larvae of several bark beetles. *G. fasciatus* is reported to be a predator of *Trypenodendron lineatum* Olivier and *G. quadrisignatus* has been shown to prey on the larvae on the European corn borer (McCoy & Brindley 1961).

1.4.2.4. Necrophagy

Feeding on dead plant or animal tissue that has been partially broken down by bacteria, fungi, or necrosis may be referred to as saprophagy, detritivory or necrophagy (Lawrence & Milner 1996). Necrophagy is common in the Nitidulidae. Although the habitat and feeding habits of nitidulid beetles are variable and cosmopolitan, the majority of species feed on decaying and fermenting plant tissues (Parsons 1943). The dried-fruit beetle, *C. hemipterus*, and the pineapple beetle *U. humeralis* for example, are well known scavengers on all types of decaying plant material. They have been reported to feed on fermented stalks of sugar cane (Schmidt 1935), and decaying tree fruits both in commercial plantations and in natural forests (Schmidt 1935). In pineapple fields, they are always found associated with decaying plant parts or in places where fermentations or necrosis have started (Schmidt 1935).

Some nitidulids occur on animal cadavers and can be of use in forensic entomology (Smith 1986). For example, Payne & King (1970) found nine species of Nitidulidae on pig carcases during the dry stages, including species of *Carpophilus* (*C. pilosellus* Mots., *C. mutilatus* Er., *C. lugubris* Murray), *Nitidula* (*N. rufipes* L.), Omosita (*O. colon* L.), *Stelidota* (*S. geminata* Say, *S. strigosa* Gyll.) and *Glischrochilus* (*G. fasciatus* Olivier, *G. quadrisignatus* Say). However, nothing was mentioned as to whether these beetles feed on either the meat, or on the body fluids.

1.4.2.5. Anthophily

Feeding or living on regenerative organs (flowers) of plants is referred to as anthophily or anthophagy. Probable visitors of flowers occur among almost all nitidulid sub-families except the Calonecrinae, Amphicrossinae and Cybocephalinae (Kirejtshuk 1997). Adults and larvae of all species in the sub-family Meligethinae, with known biologies live and feed in flowers of angiosperm plants, mainly the dicotyledons (Kirejtshuk 1997). Many Carpophilinae adults visit flowers and the participation of some species in the pollination of plants has been recorded (Kirejtshuk 1997). Complete anthophagy is recorded for some representatives of at least three genera of the sub-family Epuraeinae (Epuraea, Propetes and Grouvellia) and this feature can be expected among many forms with unstudied life cycles (Kirejtshuk 1997).

1.4.3. Flight habit

Most Nitidulid beetles are able to fly. Morris *et al.* (1955), demonstrated that two tagged nitidulids could fly for 1 mile (about 1.6 km) from the point of release within a 24-hour periods. Forest inhabiting species fly both at night and during the day (Dorsey & Leach 1956). A few species including *C. hemipterus* have, however, been observed flying mainly during daylight hours (Barnes & Kaloostian 1940).

1.4.4. Slime production in mycophagous Nitidulidae

Some nitidulid beetles have been reported to produce slime. These reside in the sub-family Nitidulinae and include *Pallodes* spp. that feed exclusively on a variety of basidiomycetes, *Pocadius* spp. that feed exclusively on puffballs, *Psilopyga* that feed on Phallales (Lawrence 1991, Leschen & Carlton 1996) and *Eusphaerius lubricus* Leschen & Carlton associated with sporocarps of coral fungi (Leschen & Carlton 1996). Larvae of these nitidulid beetles produce slime that is thought to be correlated with feeding substrates (Leschen & Carlton 1996). Larvae removed from their fungal substrates cannot secrete slime (Leschen & Carlton 1996). In addition, larvae of the beetles living within the fungal tissues will produce less slime that those occurring on the surface of the fungi (Leschen & Carlton 1996). The functions of the slime are numerous and include the protection of the larvae against desiccation, assisting with locomotion, serving as antibiotic to protect larvae from fungal and microbial infections, or to deter attacks by predators and parasitoids (Leschen & Carlton 1996).

1.5. NITIDULIDAE AND THEIR ASSOCIATED FUNGAL PATHOGENS

The Nitidulidae include numerous species of economic importance. These are mostly species transmitting fungal pathogens of agronomic crops and forest trees. On forest trees, members of the Nitidulidae have been reported as vectors of the oak wilt pathogen, *C. fagacearum* (Jewell 1956, Juzwik & French 1983), the Aspen canker pathogens in the genus *Ceratocystis* (Hinds 1972), the mallet wound pathogen of almond, *C. fimbriata* Ellis & Halsted (Moller & Devay 1968) and *C. albifundus*, the cause of wattle wilt disease of *Acacia mearnsii* (Heath *et al.* 2009). The remainder of this review will focus on those Nitidulidae that have been found associated with fungal pathogens of trees.

1.5.1. Nitidulids associated with *Ceratocystis fagacearum*

Oak wilt, caused by *C. fagacearum* is an important disease of oak trees (*Quercus* spp.) in the USA (Juzwik *et al.* 2008, USDA Forest Service 2006, Sinclair & Lyon 2005). The fungus is thought to have been accidentally introduced into the USA from Mexico, where it is thought to be native (Juzwik *et al.* 2008). It has caused devastation of *Quercus* trees in 23 Eastern and Midwestern states in the USA (USDA Forest Service 2006), especially where oak trees are planted as ornamentals along roadsides (French 1995, Tainter & Baker 1996, Billing 2000). Red oak is highly susceptible to the pathogen, with infection leading to tree death in as little as three weeks (Gibbs & French 1980). Although white oak is also susceptible to the pathogen, disease development on this host is slower and often does not lead to tree death (Gibbs & French 1980). *C. fagacearum* is transmitted from tree to tree below ground via root grafts, while above ground it is vectored by insects, especially nitidulid beetles (Gibbs & French 1980).

Leach *et al*. (1952), first reported the association of nitidulid beetles with the sporulating mats of *C. fagacearum*. They predicted that these insects would be found as vectors of the oak wilt pathogen and this hypothesis has since been confirmed in numerous studies (Dorsey *et al*. 1953, Norris 1953, Himelick *et al*. 1954, Juzwik & French 1983). Nitidulid beetles mostly occur on sap associated with fresh wounds on the trees, but they are also attracted by aromas emanating from fungi, such as *C. fagacearum* mats growing on moist wounds on living or dying trees. Once the beetles have arrived at the site, they soon begin to feed and mate (Dorsey & Leach 1956). As they feed on the fungi, fungal spores commonly adhere to their bodies or are ingested and thus carried in their intestinal tracts.

Conidia and ascospores of *C. fagacearum* can remain viable for a number of weeks, in association with nitidulid beetles (Jewell 1954). It has been demonstrated that conidia of *C. fagacearum* ingested by nitidulids can pass through the intestinal tracts of the insects, remaining viable until excretion (Jewell 1954). Likewise, *C. fagacearum* has been isolated from the external surfaces of adult nitidulids (Yount *et al*. 1955). Isolation of the fungus from the insects is often made in the spring and late autumn when new mats are being formed (Yount *et al.* 1955). Furthermore, it has been shown that *C. fagacearum* can live for more than three months on the bodies of insects kept in the refrigerator at 9˚C (Yount *et al.* 1955), adding credence to the view that nitidulid beetles are effective vectors of *C. fagacearum*.

Insect dispersal of *Ceratocystis* spp., including *C. fagacearum*, between trees is by ascospores or conidia ingested or attached to the insect bodies. However, the transport of *C. fagacearum* over long distances between trees appears to be mainly by ascospores attached to the insect body surfaces (Gibbs 1980). This is due to the fact that ascospores in most *Ceratocystis* spp. posses a numbers of characteristics that make them suitable for attachment and transport. Ascospores are produced in slimy droplets at the apices of the ascomatal necks (Ingold 1961), which prepare them for acquisition by the passing nitidulid beetles. The slimy matrix in which the spores accumulate is an adhesive and is believed to aid in attaching the spores to the surface of the insect (Malloch & Blackwell 1993). In addition, ascospores of most *Ceratocystis* spp. have a concave surfaces, which further facilitates insect transport by enabling them to come into contact with the insect body surfaces at more than one point (Malloch & Blackwell 1993). A large area of contact combined with adhesive substances ensures that ascospores of *Ceratocystis* are not easily dislodged from insect body surfaces (Malloch & Blackwell 1993). In addition, the ascospores of *Ceratocystis* spp. are enveloped in complex sheaths that would act to preserve them for long distance spread (Van Wyk *et al.* 1993).

Although all Nitidulidae were considered vectors of *C. fagacearum* in early studies (Dorsey & Leach 1956), only certain species seem to be implicated in the direct transmission of the pathogen to healthy trees. Skalbeck (1976), summarized early reports of 27 nitidulids species visiting oak wilt mats in Illinois (Curl 1955), Iowa (Norris 1956), West Virginia (Dorsey & Leach 1956, Jewell 1956), and Wisconsin (McMullen & Shenefelt 1961). Numerous studies followed dealing with the epidemiology of *C. fagacearum* in Texas (Appel *et al.* 1986, 1990) and Minnesota (Juzwik & French 1983, 1986, Cease & Juzwik 2001, Juzwik *et al.* 2004). Studies conducted in Minnesota provided evidence that the association between some nitidulid beetles and *C. fagacearum* is not as casual as originally believed (Cease & Juzwik 2001, Juzwik *et al*. 2004). Certain species in the

family such as *Coleopterus truncatus* Randall and *Carpophilus sayi* Parsons are more closely associated with the fungus and are likely the principal vector species (Juzwik *et al*. 2004). This view, however, requires further study.

1.5.2. Nitidulidae associated with *Ceratocystis albifundus*

Ceratocystis albifundus is the most important pathogen of non-native *Acacia mearnsii* trees in South Africa (Wingfield *et al*. 1996, Roux & Wingfield 1997). It causes a disease known as wattle wilt that is characterized by the formation of stem cankers, wilting and death of trees (Morris *et al.* 1993, Roux & Wingfield 1997, Roux *et al*. 1999). *C. albifundus* is known only from Africa where it is thought to have originated (Roux *et al*. 2001, Nakabonge 2002, Barnes *et al.* 2005). First isolated from native *Protea* trees in South Africa, *C. albifundus* is now known to have a broad host range including non-native trees in plantations (Roux *et al.* 2005) and many other trees in their native range (Roux *et al.* 2007). In a recent study considering the epidemiology of *C. albifundus* in South Africa, *C. albifundus*, along with a previously unknown taxon, *Ceratocystis oblonga* R. N. Heath & Jolanda Roux, were isolated from three nitidulids; *Brachypeplus depressus* Erichson, *C. bisignatus* Boheman and *C. hemipterus* (Heath *et al.* 2009). These insects originated from both native forests and a plantation of non-native *A. mearnsii* trees, where they were commonly collected from either traps or from under bark flaps on cut stumps (Heath *et al.* 2009). A relatively low percentage of insects yielded cultures of *C. albifundus*. Yet their presence on flying nitidulids collected in traps as well as from insects occurring on fungal mats growing under bark flaps suggests strongly that nitidulid beetles are vectors of *C. albifundus* and *C. oblonga* on both native trees and *A. mearnsii* trees in South Africa (Heath *et al.* 2009).

1.5.3. Nitidulidae associated with *Ceratocystis fimbriata*

The *Ceratocystis fimbriata sensu lato* complex includes important and cosmopolitan fungal pathogens of trees and crop plants (Kile 1993). They occur globally on a wide range of hosts including woody and herbaceous plants (Upadhyay 1981, Kile 1993). The fungi are currently referred to as belonging to the *C. fimbriata s.l.* complex because they include morphologically similar and sometimes host specialized cryptic species or strains commonly referred to as ‘’types’’, ‘’races’’ or ‘’forms’’ (Baker *et al.* 2003, Harrington & Baker 2002, Van Wyk *et al.* 2007).

The association of *C. fimbriata s.l.* with insects, especially nitidulid beetles, has been demonstrated in a number of studies (Hinds 1972, Crone & Bachelder 1961, Moller & Devay 1968). Species in the *C. fimbriata s.l.* species complex produce strong fruity odors that attract numerous insects including nitidulid beetles (Hunt 1956, Moller & Devay 1968). The fungi serve as food for the

beetles, and as they feed on them, spores and fungal propagules commonly adhere to their body surfaces or are transported with them in their intestinal tracts. These serve as an innoculum when the beetles visit fresh wounds on healthy trees, thus resulting in new infection courts for the fungi (Hinds 1972, Crone & Bachelder 1961).

Crone & Bachelder (1961), first demonstrated the transmission of *C. fimbriata f.sp. platani* (now *C. platani* (Walter) Engelbrecht & Harrington) by nitidulid beetles. They obtained the infection on London plane tree by placing beetles on cultures of the fungus prior to using them as inoculating agents. In addition, they also showed that nitidulid beetles collected from diseased plane trees can transmit *C. platani* to healthy trees (Crone & Bachelder 1961). Later, Moller & Devay (1968) demonstrated that *Carpophilus* spp. are the main vectors of *C. fimbriata s.l.* in stone fruits trees. They also provided evidence for transmission of *C. fimbriata s.l.* by these insects to almond trees. Later, Hinds (1972) found that numerous insects that visit cankers on trembling aspen in Colorado can transmit numerous *Ceratocystis* spp., especially *C. fimbriata s.l.* to wounds on healthy trees. However, nitidulid beetles were the most common of these insects and included species such as *Epureae avara* (Randall), *E. erichsoni* Reit, *E. terminalis* Pars., *Colopterus truncatus*, *Glischrochilus moratus* Br., and *G. vittatus* (Say) (Hinds 1972).

1.5.4. Nitidulidae associated with *Ceratocystis paradoxa*

Ceratocystis paradoxa (Dade) Moreau causes disease of sugar cane worldwide and can results in huge economic losses through rotting and souring of mature cane stalks (Wismer 1961). It has been shown that three nitidulid beetles, *U. humeralis*, *C. hemipterus* and *Haptoncus ocularis* (Fairmaire) vector the pathogen in sugar cane plantations (Chang & Jensen 1974). The nitidulid beetles are attracted to diseased cane, which provides a good source of sap and fungi on which they feed. In turn, the nitidulid beetles spread the pathogen by carrying spores and mycelia of *C. paradoxa* on their bodies (Chang & Jensen 1974).

1.5.5. Nitidulidae as vectors of the entomopathogen *Beauveria bassiana*

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a fungus that occurs naturally in soil throughout the world (Meyling & Eilenberg 2007). It causes a disease known as white muscadine of insects. When spores of the fungus come into contact with the exoskeleton of susceptible insects, it germinates and grows through the skin into the insect's body, producing toxins and draining the insect of nutrients, eventually killing it (Meyling & Eilenberg 2007). Due to its entomopathogenic characteristics, *B. bassiana* has been registered as biological control for many insects, applied in agriculture and forestry (Dowd & Vega 2003, Meyling & Eilenberg 2007).

Sap beetles are known to carry a variety of disease-causing microorganisms to crops (Dowd 1995, Jewell 1956, Moller & deVay 1968). Nitidulid beetles feed on *B. bassiana* and have been demonstrated to be capable of vectoring the fungus both via their fecal material and externally, by transporting them on their body surfaces (Bruck & Lewis 2002, Meyling & Eilenberg 2007). The fungus was first reported in limited quantity from the body surfaces of *Glischrochilus quadrissignatus* Say in a number of studies (Foott & Timmins 1979, Peng & Williams 1990). In another study, *B. bassiana* was isolated from various *Carpophylus* species, but very low level of mortality of the beetles were recorded after allowing them to walk through sporulating cultures of *B. bassiana* for two weeks (Dowd & Vega 2003). Although nitidulid beetles are susceptible to infection by *B. bassiana*, it was demonstrated that passage of the fungus through the digestive tract or phoretically on their exoskeleton occurs before the beetles become incapacitated (Bruck & Lewis 2002).

Entomopathogenic fungi are generally dispersed by living hosts which migrate and die in another place than where they became infected (Hajek 1997). Because sap beetles can vector pathogenic fungi, they have been tested and used as potential vectors of biocompetitors, transporting biological control agents as a mean of controlling populations of sap beetles. Vega *et al.* (1995), first designed laboratory assays with inoculated sap beetles. Sap beetles placed into the autoinoculative devices containing *B. bassiana* were shown to become contaminated with the fungus and to carry it to other uncontaminated sap beetles (Vega *et al.* 1995). Sap beetles treated in the same manner were shown to carry the fungus to entomopathogen-free aggregating beetles in the field, thus increasing infection rates and mortality (Vega *et al*. 1995, Dowd & Vega 2003). In this regard, the dispersing ability of fungal pathogens by sap beetles make them a strong candidates for use as natural vectors of *B. bassiana* in controlling populations of sap beetles and other insect pests susceptible to the pathogen.

1.6. NITIDULIDAE ASSOCIATED WITH MYCOTOXIN PRODUCING FUNGI RELEVANT TO HUMAN AND ANIMAL FOOD CONSUMPTION

Insects are one of the best known means by which mycotoxin producing fungi are introduced to commodity crops (Widstrom 1979, Dowd 1995). However, sap beetles have been found to be the most important group of insects that introduce *Aspergillus* spp. and *Fusarium* spp. to maize in numerous countries world-wide (Dowd 1991, 1995, Windels *et al.* 1976). There are three types of mycotoxin producing fungi associated with nitidulid beetles. These fungi reside predominantly in

the genera *Aspergillus*, *Fusarium* and *Penicillium* (Cardwell *et al.* 2000, Marasas 1988). Fungi in the genus *Fusarium* produce toxins known as fumonisins (Cardwell *et al.* 2000, Marasas 1988), those in *Aspergillus* produce aflatoxins (Cardwell *et al*. 2000), while ochratoxin is a mycotoxin produced by different species of *Aspergillus* and *Penicillium* (Van der Merve *et al*. 1965, Bankole & Adebanjo 2003).

Contamination of foodstuffs and many other commodities by mycotoxins has for many years been a serious problem in developed countries as they cause diseases that result in reduced life expectancy (Bankole & Adebanjo 2003). Fumonisins was first discovered from *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushina) Nirenberg cause animal diseases, such as leucoencephalomalacia in equines, porcine pulmonary oedema, rat liver cancer and haemorrhage in the brain of rabbits (Marasas 1995). Most importantly, this toxin has been suggested as part of the cause of human oesophageal carcinoma in South Africa and China (Yoshizawa *et al.* 1994, Marasas *et al.* 1988). Aflatoxins produced by *Aspergillus flavus* Link has been suggested to be the cause of liver cancer in many parts of Africa (Oettle 1964, Bankole & Adebanjo 2003), while ochratoxin, first isolated from cultures of *A. ochraceus* G. Wilh. have immunosuppressive, teratogenic, mutagenic and genotoxic properties (Bankole & Adebanjo 2003)

Fusarium spp. associated with nitidulid beetles are mostly pathogens of crop plants such as maize. Maize is one of the world's most important cereals and represents an important staple food for many African countries. However, diseases of maize caused by *Fusarium* spp. are recurrent problem in many countries where maize is planted. Several pathogenic species of *Fusarium* have been reported from maize and these include *F. verticillioides*, *F. proliferatum*, *F. graminearum* Schwabe and *F. anthophilum* (A. Braun) Wollenweber (Fandohan *et al.* 2003), of which *F. verticillioides* is the most frequently associated with diseased maize, worldwide (Munkvold & Desjardins 1997, Fandohan *et al.* 2003).

Nitidulid beetles are the most important group of insects that introduce *Fusarium* spp. to maize (Dowd 1991). Nitidulid beetles are attracted to the plant volatiles alone or in synergistic combination with their pheromone, including the common maize volatile, and are also attracted to maize damaged by other insects or any other factors (Dowd 1991, Dowd & Bartelt 1991). Nitidulid beetles become contaminated with *Fusarium* or other mycotoxin producing fungi while feeding on maize ear residues. They then carry the fungi to pollen, leaf axels and ultimately to milk stage kernels of healthy maize plants resulting in infection and increased disease incidence (Attwater & Busch 1983, Dowd 1995). Monroe *et al.* (1947), first reported the association of sap beetles with

Fusarium moniliforme Scheld. in maize. This was followed by Windels *et al*. (1976) who reported that *G. quadrissignatus* vectors *Fusarium* spp. to maize. They isolated many *Fusarium* spp. including *F. solani f. sp. pisi* (F.R. Jones) W.C. Snyder & H.N. Hansen, (1941), *F. moniliforme*, *F. roseum* Gibbosum, *F. oxysporum* and *F. tricinctum* (Corda) Sacc. from both the outside and inside of various insect development stages, including larvae, pupae or adults of the beetles collected from infested ears of standing field corn. Attwater & Busch (1983), demonstrated the role of *G. quadrissignatus* in the epidemiology of *F. graminearum* maize ear rot. Numerous other studies have referred to Nitidulidae favouring maize infection by *Fusarium* spp. (Munkvold & Desjardins 1997, Cardwell *et al.* 2000, Ako *et al.* 2003).

The role of sap beetles in vectoring aflatoxin producing fungi was demonstrated only two decades ago. Lussenhop & Wicklow (1990), identified *C. lugubris*, *C. hemipterus* and *C. freemani* from infested maize and established their role in vectoring *A. flavus*. This was followed by a number of studies establishing the role of sap beetles in vectoring aflatoxin producing fungi. Wicklow *et al.* (1998), isolated *A. flavus* from *C. lugubris* trapped in maize fields. Rodriguez-Del-Bosque *et al.* (1998), identified coleopteran beetles belonging to seven different families, from maize ears damaged by either birds or lepidopteran larvae in Northeastern Mexico. Of these, sap beetles were by far the most common insects, and infection of maize by *A. flavus* and aflatoxin contamination was correlated with ear wounding and incidence of sap beetles.

Sap beetles appear to be well-adapted for vectoring mycotoxin producing fungi (Dowd 1991, 1995). Firstly, sap beetles reside in environments where mycotoxin producing fungi occur naturally, and these include soil, stored products, decaying materials, fresh and dried fruit, as well as woody environments. They are attracted to fungal and host volatiles alone and in synergistic combination with their own aggregation pheromone (Dowd 1991, 1995). Nitidulid beetles are resistant to mycotoxins as they can metabolize these compounds up to ten times more effectively than any other insects, and they can carry these fungi both internally and externally (Dowd 1995). Furthermore, the presence of various pits and hairs that cover the sap beetles provide important collection sites for the spores of the fungi (Dowd 1995, Juzwik & French 1986).

1.7. OTHER FUNGI ASSOCIATED WITH NITIDULIDAE

Nitidulid beetles have been found feeding and ovipositing on wounds infested by numerous fungi including *Ophiostoma pluriannulatum* (Hedgc.) H. & P. Sydow, *Graphium rigidum* (Pers.) Sacc. and several species of yeast (Dorsey & Leach 1956). Spores of these fungi are not adapted to wind

dispersal by virtue of the sticky matrix in which they are embedded, therefore they probably rely on the insects for dissemination to fresh wounds (Dorsey & Leach 1956). Nitidulid beetles have also been reported in association with *Monilinia fructicola* (Wint.) Honey, the cause of brown rot in stone fruit in California (Tate & Ogawa 1975), with yeasts that cause diseases of fig trees (Miller & Mrak 1953) and with the oyster mushroom *Pleurotus ostreatus* Fries (Cline & Leschen 2005). Some *Epuraean* spp. are casually associated with fungi in the Boletales. Adults of these beetles have been found occasionally with bolete basidiocarps and were regarded as secondary fungivores of these fungi (Bruns 1984).

1.8. NATURE OF THE INTERACTION BETWEEN FUNGI AND NITIDULIDAE

The association between fungi and insects is generally regarded as mutualistic, as it has been reported for many bark and ambrosia beetles (Beaver 1989, Grosmann 1967) living in symbioses with fungi. However, this is not the case for nitidulid beetles. The association between nitidulid beetles and the fungi they carry, and on which they feed, is not obligatory for the beetles (Lin & Phelan 1992). Nitidulid beetles do not rely exclusively on the fungi for their diet as they can feed on a variety of ripening or decaying fruits, sap oozing from tree wounds and plant tissues such as flowers and pollen (Lin & Phelan 1992). However, fungal infection does increase the attractiveness and possibly even the nutritional value of food substrates for nitidulids. For example, *C. paradoxa*-infected sugarcane stalks were more attractive to nitidulid beetles than healthy stalks in the field. In the same manner, larvae of *U. humeralis* reared on a diet of mixed sugarcane juice and *C. paradoxa* developed faster, gained more weight and had a higher percentage of pupation than those on sugarcane juice only (Chang & Jansen 1974).

In the case of oak wilt, it has been assumed that there is not specificity or co-evolution between *C. fagacearum* and nitidulid beetles. This view is changing. There is now growing evidence to suggest that the association between some nitidulid beetles and *C. fagacearum* is not as casual (Juzwik *et al.* 2004, Hayslett *et al.* 2008). Further studies are clearly needed to better understand this question.

1.9. ATTRACTION OF NITIDULID BEETLES TO PHEROMONE AND HOST VOLATILES

Chemical communication is widespread among insects, including nitidulid beetles. It occurs in a diversity of forms and functions. Chemical communication among insects is a mechanism by which nest mates recognize one another (Gamboa *et al.* 1987, Ali & Morgan 1990). In nitidulid beetles, chemical communication is common and has been considered in numerous studies. This mostly includes chemicals such as pheromones or kairomones, which mediate attraction and mass aggregation of nitidulid beetles for mating and feeding. Likewise, volatiles released from host trees and food sources, such as fungi, crops, and sap oozing from tree wounds, mediate attraction of nitidulid beetles to these sources. The following paragraphs focus on the factors that either mediate mass aggregation of nitidulid beetles or host location by these insects. Most importantly, the importance these might have in the development of control strategies for nitidulid beetles is considered.

1.9.1. Attraction to pheromones

Female insects often release chemicals (pheromones) into the air to make their presence known, while males release similar chemicals (sex pheromones) to induce the female into mating (Ali & Morgan 1990). Numerous insects are also known to rely on chemicals to induce mass aggregation (aggregation pheromones) in the vicinity of the source of the chemical, either for feeding, mating or hibernation (Ali & Morgan 1990). Pheromones are defined as substances that are secreted to the outside by an individual and received by a second individual of the same species, in which they induce a specific reaction (Karlson & Butenandt 1959, Karlson & Lüscher 1959, Ali & Morgan 1990). In other terms, these are chemicals that are released by an organism for intraspecific communication. Nitidulid beetles respond to a wide range of host-related volatiles as well as aggregation pheromones (Bartelt *et al.* 1992a, b, 1993a, b). In most cases, pheromones produced by one species can have kairomonal activity where they induce interspecific communication among insects or mass aggregation of insects of different species in the vicinity of the source where the chemical is released (Bartelt *et al*. 1993b).

Mating associated pheromones or aggregation pheromones have been identified for a number of nitidulid beetles, including *Carpophilus antiquus* Melsheimer (Bartelt *et al.* 1993b), *C. freemani* (Bartelt *et al.* 1990a), *C. hemipterus* (Bartelt *et al.* 1990b, 1992a), *C. lugubris* (Bartelt *et al.* 1991), *C. mutilatus* Erichson (Bartelt *et al.* 1993a) and *C. obsoletus* Erichson (Petroski *et al.* 1994). These pheromones are mostly blends of triene or tetraene hydrocarbons and they share components

between species (Bartelt *et al.* 1990a, b, 1992a, Petroski *et al*. 1994). Therefore, pheromones of some nitidulid species have been found to have kairomonal activities attracting nitidulid beetles of different species. For example, cross-attraction of various *Carpophilus* spp. to *C. hemipterus* and *C. mutilatus* pheromones has been reported in a number of studies (Bartelt *et al.* 1992b, Blumberg *et al*. 1993, James *et al*. 1993). *C. obsoletus* also responds significantly to the pheromone blend of *C. hemipterus* in field tests (Bartelt *et al.* 1992b).

1.9.2. Attraction to host volatiles

Selection of appropriate hosts by sap beetles is based on many chemical components and their interactions. Numerous studies have shown that sap beetles respond to a wide range of host-related volatiles (Smilanick *et al.* 1978, Dowd & Bartelt 1991, Bartelt *et al.* 1992a, 1993a). Control of nitidulid beetles by mass trapping using fermenting fruit baits can be economically feasible (Bartelt *et al.* 1992b, Warner 1961). Smilanick *et al.* (1978), first developed a synthetic version of fermenting fruit volatiles and demonstrated that it can be used as a bait to trap nitidulid beetles. This was followed by Alm *et al.* (1986) who showed that a mixture of propyl propionate or butyl acetate can be used to selectively trap *G. quadrisignatus*. However, in both these cases the rate of release of the lures turned out to be much higher than that expected from a natural host plant, thus could not reveal the role these chemicals play in the mediation of host-selection for nitidulid beetles.

The chemical basis mediating the orientation of nitidulid beetles to fruit and fungal odours was considered for *C. hemipterus* in wind tunnel bioassays to agar medium, fruits, fungi or fruits inoculated with fungi. The beetles were attracted to odours of the yeast *Saccharomyces cerevisiae* Meyen: E.C. Hansen, aseptic banana, or banana inoculated with *S. cerevisiae*, although both banana substrates elicited greater responses than the yeast alone (Phelan & Lin 1991). When presented with a two-choice bioassay, the yeast inoculated banana attracted twice as many beetles as the aseptic banana (Phelan & Lin 1991). Analysis of head-space volatiles above these odour sources, using gas chromatography, revealed eighteen compounds (eight esters, six alcohols, three ketones and one aldehyde) identified from the four substrates (Phelan & Lin 1991). Agar medium and *S. cerevisiae* on yeast medium were qualitatively and quantitatively poor, with only two and seven compounds detected respectively, and these were present in low concentrations. In contrast, the aseptic banana possessed 15 of the compounds identified while yeast-inoculated banana possessed all 18 components. However, the two substrates were quantitatively distinct, with the inoculated banana producing twice the concentration of volatiles overall, and with the largest increase occurring in the production of alcohols (Phelin & Lin 1991). A blend of the eighteen compounds identified also mimicked the natural substrate in behavioural activity, eliciting upwing attraction in *C. hemipterus*

at levels comparable to the inoculated banana (Phelan & Lin 1991). This was the first occasion where chemical compound mediating attraction or host finding by nitidulid beetles was discovered and clearly emphasized that similar compounds are also released by other plants and fungi, which are natural hosts for nitidulid beetles.

1.9.2.1. Fruit odours and the aromas produced by *Ceratocystis* spp.

Many *Ceratocystis* spp. produce compounds that are thought to attract insects that feed on them therefore, contributing to the spread of the fungi. Over the years, research has been undertaken exploring the essence of "fruity" aromas from *Ceratocystis* sp., and literatures pertaining to these studies has been summarised by Hanssen (1993). Davidson (1944) first described odor produced by *C. coerulescens* as similar to "banana-oil" (amyl acetate), and those of *C. virescens* (Davidson) Moreau as "musty and penetrating". This was followed by Hunt (1956) who referred odor of *C. moniliformis* (Hedgcock) Moreau as similar to "banana-oil, or "banana-like" aroma. Lanza *et al.* (1976) reported that *C. moniliformis* produces volatiles with "fruit-banana", "peach-pear" and "citrus" aroma.

The chemical constituents of aromatic flavor produced by some *Ceratocystis* spp. has been considered in analyses using gas-chromatography. Odors emanating from *Ceratocystis* cultures are due mainly to a mixture of short-chain alcohols and esters (Lanza *et al.* 1976, Hanssen 1993). Theses compounds are similar to those mediating orientation of nitidulid beetles to fruit and fungal odors as described by Phelin & Lin (1991). It is likely that these compounds play similar role in attracting these insects to the niche where *Ceratocystis* spp. occur.

1.9.3. Pheromones synergized by host volatiles

Most of the pheromones identified from nitidulid beetles, to which both sexes respond, have proved very active when combined with food volatiles under field conditions in numerous studies conducted in the USA (Bartelt *et al.* 1990a, b, 1992a, b, 1993a), Israel (Blumberg *et al.* 1993), and Australia (James *et al.* 1993). Bartelt *et al.* (1990b), reported the first example of an aggregation pheromone from *C. hemipterus*, which interacts synergistically with some host volatiles. Subsequently, they identified an aggregation pheromone from males of *C. lugubris* and also demonstrated its synergistic effect with bread dough in attracting this insect (Bartelt *et al.* 1991). This was followed by a number of studies testing the behavioural activity of either synthetic or natural host odour blends when presented alone and in combination with aggregation pheromone (Lin *et al.* 1992), or synthetic aggregation pheromone when presented alone and combined with natural host odour (James *et al.* 1993). All these studies showed that either host odours or

aggregation pheromones are often attractive to nitidulid beetles on their own, but host odours act as effective synergists for the pheromone as aggregation pheromones attraction to these insects are always enhanced when they are combined with the host volatiles.

1.9.4. Attraction to wounds

Wounds, either on trees or crop plants, are important in the biology of nitidulid beetles. These insects are often attracted to sweet smells, mostly sap exuding from decayed or fresh wounds, which constitute important components of their diet (Vogt 1950, Tate & Ogawa 1975). However, the stimuli that may induce nitidulid beetles to seek out injuries are being investigated, and it has been found that moisture is an important factor influencing the attraction of these insects to injured fruits (Tate & Ogawa 1975). It is, therefore, believed that moisture was probably a stimulus for nitidulid beetles involved in the results of Moller *et al.* (1969), who found that bark injuries made on trees when the soil is moist not only provide better infection courts for *C. fimbriata s.l.*, but appeared to be more attractive to *C. freemani* (Moller *et al.* 1969). Thus, considerable water loss to the atmosphere from bark injuries is necessary to provide the stimulus to attract nitidulid beetles (Tate & Ogawa 1975).

1.10. CONCLUSIONS

The beetle family, Nitidulidae (Coleoptera) comprises ten sub-families spanning more than 8000 species. Nitidulidae include species with a wide variety of body shapes, color and length. Although sap oozing from fresh wounds on trees is considered as a food source for nitidulid beetles, these insects live and feed in a wide variety of habitats including stored products, soil, pollen, fungi and under decaying bark. Some nitidulid beetles are predators feeding on scale insects, while others occur on cadavers and have been used for forensic entomology.

Numerous nitidulid beetles are species of economic importance as they are pests of fruits and vegetables as well as stored products. They are also implicated in the fertilization and dissemination of important microorganism such as fungi, many of which are important pathogens of trees and crop plants, or are mycotoxin producing fungi that may affect human and animal health. Of the pathogenic fungi known to be vectored by nitidulid beetles, species of *Ceratocystis* are probably the best known.

Monitoring populations of sap beetles has involved either trapping or the use of various chemical compounds for mass killing of these insects. However, with the knowledge of various elements

involved in attracting nitidulid beetles, such as pheromones and numerous host volatiles, alternative control strategies have been developed using these attractants, and these have been very successful in controlling these insects.

Despite the numerous records of nitidulid beetles worldwide, there is still limited knowledge pertaining to the diversity of fungi associated with them, many of which are likely important plant pathogens. Of the tree hosts on which these insects occur, eucalypts have been overlooked for many decades. Similarly, very little is known regarding nitidulid beetles on the African continent, especially regarding their fungal associates. This lack of information has prompted the studies presented in this dissertation. The aims are particularly to identify the Ophiostomatoid fungi associated with harvesting wounds on eucalypt trees in Australia and South Africa. An important component of this dissertation is the identification of nitidulid beetles infesting wounds on *Eucalyptus* trees and to identify the Ophiostomatoid fungi that they carry.

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Table 1. Sub-families of Nitidulidae and characteristics that separate them.

Sub-families	Number. of genera	Morphological characteristics	References
Calonecrinae	1	❖ 10 segmented antennae ❖ 1 segmented and undepressed club ❖ Elitra simple at outer apical corners ❖ Maxillae with a single lobe ❖ Aedeagus symmetrical, with fused parameres and phallobase ❖ Ovipositor with sclerites well defined	Kirejtshuk 1982, 1998b, 2008
Amphicrossinae	2	❖ Bodies widely oval and usually larger, flattened ventrally, evenly and moderately convex dorsaly ❖ Pronotal and elytral with long dense ciliae ❖ Male anal sclerite not exposed from under pygidial apex ❖ Hypopygidium with a large movable apex	Kirejtshuk 1998b, 2008
Cryptarchinae	15	❖ Labrum connate with the clypeus, the suture more or less distinct	Kirejtshuk 1998b, 2008 Parsons 1943
Meligethinae	14	❖ Mid and hind tibiae strongly depressed dorsoventrally and with one outer border bearing setae or marked hair different from those on remainder of these structures ❖ Pygidial base with a pair of very wide arc-like depressions, usually partly covered by preceding tergite	Kirejtshuk 1998b, 2008
Epuraeinae	21	❖ Male anal sclerite projects distally, with hypopygidium not strongly excised while	Kirejtshuk 1986, 1998b, 2008
Maynipeplinae	1	❖ Possesses 9-segmented antennae ❖ One-segmented antennal club	Kirejtshuk 1998a, 2008
Nitidulinae	110	❖ Mesosternum not carinated ❖ Prosternum depressed behind the coxae and not prolonged ❖ Pronotum margined at base	Parsons 1943 Kirejtshuk 2008
Carpophilinae	7	❖ Strongly curved, bilobed tegmen with an extremely deep excision between lobes and a nearly membranous penis	Parsons 1943, Kirejtshuk 1986, 2008
Cillaeinae	29	❖ flattened tegument, fused lobes and a more or less flattened and sclerotized penis	Kirejtshuk 1986, 2008
Cybocephalinae	8	❖ Predators feeding on scale insects ❖ Cybocephalids adults have a 4-4-4 tarsal formula ❖ five visible ventral plates and five abdominal spiracles ❖ Body retractile, allowing the mandibles in repose to rest against the metasternum ❖ Larvae's head without dorsal sutures, lack pregomphi and urogomphi on abdominal tergite XI, and have hypostomal rods with divergent hypostomal ridges present posteriorly, hypopharynx without a sclerome and bracons, maxillae without mola, and annular spiracles with two lateral air tubes	Kirejtshuk 1998b, 2008, Smith & Cave 2006

Table 2. Diagram illustrating the current taxonomic status of the family Nitidulidae within the kingdom animalia. The numbers of genera within each sub-family are indicated in brackets. (Sources: Parsons 1943, Kirejtshuk 2008).

Kingdom	Animalia		
Phylum	Arthropoda		
Superclass	Hexapoda		
Class	Insecta		
Order	Coleoptera		
Suborder	Polyphaga		
Series	Cucujiformia		
Superfamily	Cucujoidea		
Family	Nitidulidae		
Lineages	**Nitiduline**	**Carpophyline**	**Calocrine**
Sub-families	Nitidulinae (110), Cillaeinae (29), Cryptarchinae (15), Cybocephalinae (8), Meligethinae (14)	Carpophilinae (7), Amphicrossinae (2), Epuraeinae (21)	Calonecrinae (1), Maynipeplinae (1)

Table 3. Nitidulid beetles and their fungal associates grouped in distinct sub-families.

Nitidulid beetles	Sub-family	Host trees	Fungal associate	References
Brachypeplus depressus	Cillaeinae	*A. mearnsii*	*C. albifundus, C. oblonga*	Heath *et al.* 2009
Carpophilus bisignatus	Carpophilinae	*A. mearnsii*	*C. albifundus, C. oblonga*	Heath *et al.* 2009
C. brachyopterus	Carpophilinae	Oak tree	*C. fagacearum*	Curl 1955, Skalbeck 1976
C. corticinus	Carpophilinae	Oak tree	*C. fagacearum*	Skalbeck 1976
C. hemipterus	Carpophilinae	Sugar cane, *Acacia mearnsii*, Stone fruit tree, Oak tree	*C. fagacearum, C. albifundus, C. oblonga, C. fimbriata, C. fagacearum*	Chang & Jensen 1974, Heath *et al.* 2009, Moller & Devay 1968, Skalbeck 1976
C. freemani	Carpophilinae	Stone fruit tree	*C. fimbriata*	Moller & Devay 1968
C. freemani, C. hemipterus, C. mutilatus	Carpophilinae	Stone fruit trees	*Scherotinia* (*Monilinia*) *fructicola*	Tate & Ogawa 1975
C. lugubris	Carpophilinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976
C. lugubris	Carpophilinae	*Platanus acerifolia*	*C. platani*	Crone & Bachelder 1961
C. sayi	Carpophilinae	Oak tree	*C. fagacearum*	Curl 1955, Norris 1956, Skalbeck 1976, Cease & Juzwik 2001, Juzwik *et al.* 2004
Carpophilus spp.	Carpophilinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Juzwik & French 1983
Colopterus maculatus	Cillaeinae	Oak tree	*C. fagacearum*	McMullen & Shenefelt 1960, Skalbeck 1976, Appel *et al.* 1990
Colopterus maculatus	Cillaeinae	*Q. fusiformis*	*C. fagacearum*	Appel *et al.* 1986
Co. morio	Cillaeinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956
Co. niger	Cillaeinae	Oak tree	*C. fagacearum*	Jewell 1956, Skalbeck 1976, Hayslett *et al.* 2008
Co. semitectus	Cillaeinae	Oak tree	*C. fagacearum*	Juzwik *et al.* 2004, Hayslett *et al.* 2008, Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976
Co. truncates	Cillaeinae	Trembling aspen, Oak tree	*C. fimbriata, C. moniliformis, C. populina, C. crassivaginata, C. fagacearum*	Hinds 1972, Juzwik & French 1983, Cease & Juzwik 2001, Skalbeck 1976, Juzwik *et al.* 2004, Hayslett *et al.* 2008
Co. truncates	Cillaeinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
Cryptarcha ampla	Cryptarchinae	*Platanus acerifolia*, Oak tree	*C. platani, C. fagacearum*	Crone & Bachelder 1961, Dorsey & Leach 1956, Skalbeck 1976
Cr. concinna	Cryptarchinae	Oak tree	*C. fagacearum*	Appel *et al.* 1990
Cr. concinna,	Cryptarchinae	*Quercus fusiformis*	*C. fagacearum*	Appel *et al.* 1986
Cyllodes biplagiatus	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005

Epuraea avara	Epuraeinae	Oak tree, Trembling aspen	*C. fagacearum, C. fimbriata, C. moniliformis, C. populina, C. crassivaginata*	McMullen & Shenefelt 1960, Skalbeck 1976, Hinds 1972
E. boraeades	Epuraeinae	Oak tree	*C. fagacearum*	McMullen & Shenefelt 1960, Skalbeck 1976, McMullen & Shenefelt 1960, Skalbeck 1976, McMullen & Shenefelt 1960, Skalbeck 1976
E. corticina	Epuraeinae	Oak tree	*C. fagacearum*	Juzwik *et al.* 2004, Norris 1956, McMullen & Shenefelt 1960, Skalbeck 1976, Cease & Juzwik 2001
E. erichsoni	Epuraeinae	Trembling aspen, Oak tree	*C. fimbriata, C. moniliformis, C. populina, C. crassivaginata, C. fagacearum*	Hinds 1972, Skalbeck 1976
E. terminalis	Epuraeinae	Trembling aspen, Oak tree	*C. fimbriata, C. moniliformis, C. populina, C. crassivaginata, C. fagacearum*	Hinds 1972, Skalbeck 1976
E. umbrosa	Epuraeinae	Oak tree	*C. fagacearum*	Skalbeck 1976
E. umbrosa	Epuraeinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
Epureae spp.	Epuraeinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976, Juzwik & French 1983
Epuraea spp.	Epuraeinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
Glischrochilus confluentus	Cryptarchinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976
G. fasciatus	Cryptarchinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976, Juzwik & French 1983, Cease & Juzwik 2001
G. fasciatus	Cryptarchinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
G. fasciatus	Cryptarchinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
G. moratus	Cryptarchinae	Trembling aspen	*C. fimbriata, C. populina, C. crassivaginata*	Hinds 1972
G. obtusus	Cryptarchinae	Oak tree	*C. fagacearum*	Curl 1955, Skalbeck 1976
G. obtusus	Cryptarchinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
G. quadrisignatus	Cryptarchinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
G. quadrisignatus	Cryptarchinae	Oak tree	*C. fagacearum*	Juzwik & French 1983, Dorsey & Leach 1956, Skalbeck 1976, Cease & Juzwik 2001
G. sanguinolentus	Cryptarchinae	*Quercus* tree	*O.piceae*	Juzwik & French 1983
G. sanguinolentus	Cryptarchinae	Oak tree	*C. fagacearum*	Juzwik & French 1983, Dorsey & Leach 1956, Jewell 1956, Skalbeck 1976, Cease & Juzwik 2001
G. vittatus	Cryptarchinae	Trembling aspen	*C. fimbriata, C. moniliformis, C. populina, C. crassivaginata*	Hinds 1972
Haptoncus luteolus	Epuraeinae	Stone sruit trees	*Scherotinia* (Monilinia) *fructicola*	Tate & Ogawa 1975
H. ocularis	Epuraeinae	Sugar cane	*C. fagacearum*	Chang & Jensen 1974
Lobiopa undulate	Nitidulinae	Oak tree	*C. fagacearum*	Appel *et al.* 1990

L. undulate	Nitidulinae	Oak tree	*C. fagacearum*	Skalbeck 1976
Nitidula bipunctata	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
Oxycnemus histrinus	Nitidulinae	Oak tree	*C. fagacearum*	Norris 1956, Skalbeck 1976
Pallodes austrinus	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
P. pallidus	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
P. pallidus	Nitidulinae	*Carya* spp., Hickory spp.	*Amanita onusta, Laetiporus sulphureus, Megacollybia platyphylla,Tylopilus felleus, Strobilomyces sp., Hygrocybe sp., Russula sp.*	Epps & Arnold 2010
Phenolia grossa	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
Ph. grossa	Nitidulinae	*Carya* spp., Hickory spp.	*Laetiporus sulphureus, Mycena leaiana*	Epps & Arnold 2010
Prometopia sexmaculata	Nitidulinae	Oak tree	*C. fagacearum*	Dorsey & Leach 1956, Norris 1956, Skalbeck 1976
Stelidota geminata	Nitidulinae	Oak tree	*C. fagacearum*	Norris 1956, Skalbeck 1976
S. geminata	Nitidulinae	NA	*Pleurotus ostreatus*	Cline & Leschen 2005
S. octomaculata	Nitidulinae	*Carya* spp., Hickory spp.	*Paxillus atrotomentosus*	Epps & Arnold 2010
Urophorus humeralis	Carpophilinae	Sugar cane	*C. fagacearum*	Chang & Jensen 1974

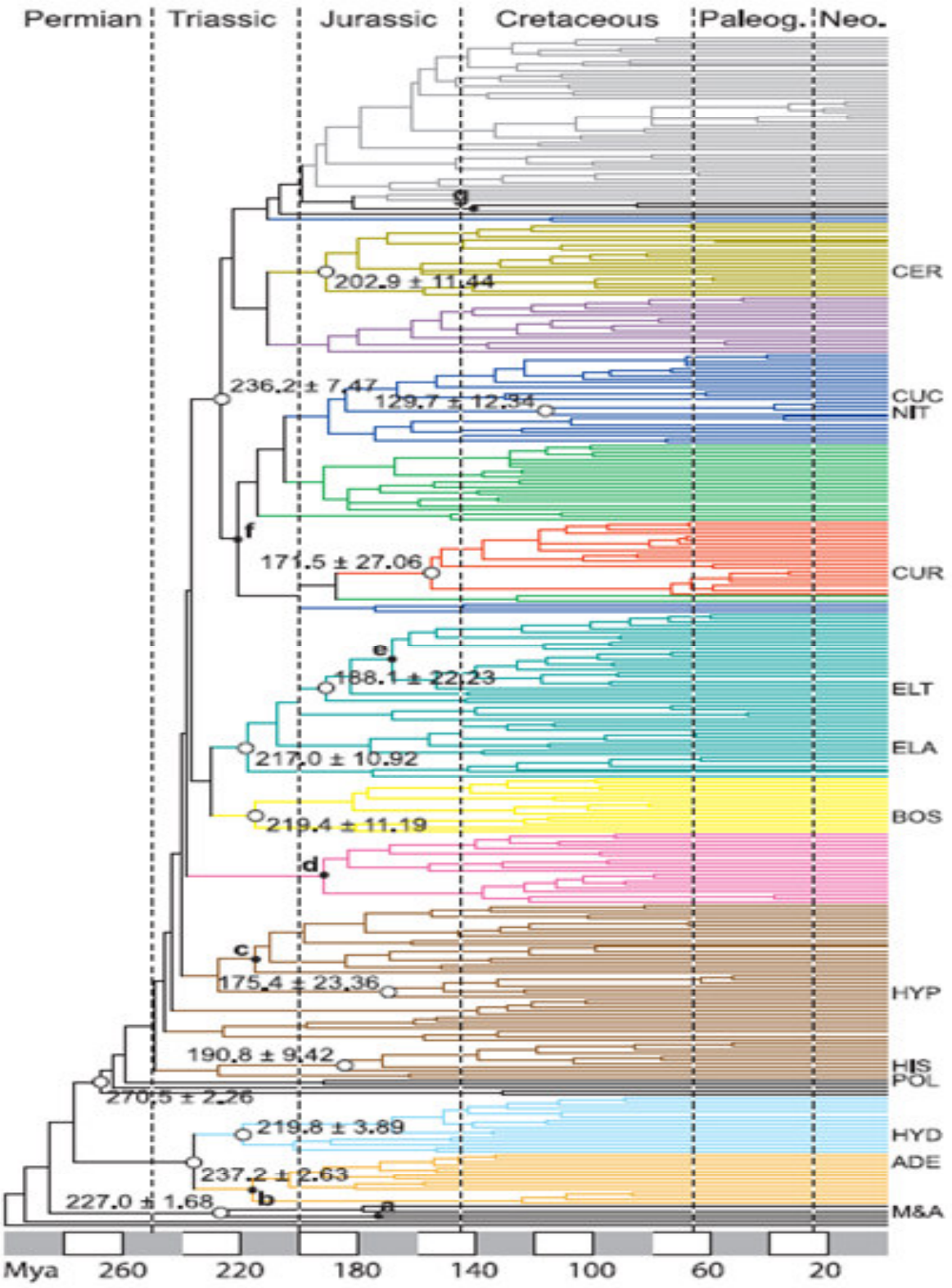

Figure 1: A dated 340-taxon concensus tree of Coleoptera from Bayesian analysis indicating the Coleopteran beetles: CER, Cerylonid series; CUC, Cucujiformia; NIT, Nitidulidae; CUR, Curculionoidae; ELT, Elateroidea; ELA, Elateriformia; BOS, Bostrichiformia; HYP, Hydrophiloidea; HIS, Histeroidea; POL, Polyphaga; HYD' Hydradephaga; ADE, Adephaga; M & A, Myxophaga and Archostemata. (Source: Hunt *et al.* 2007).

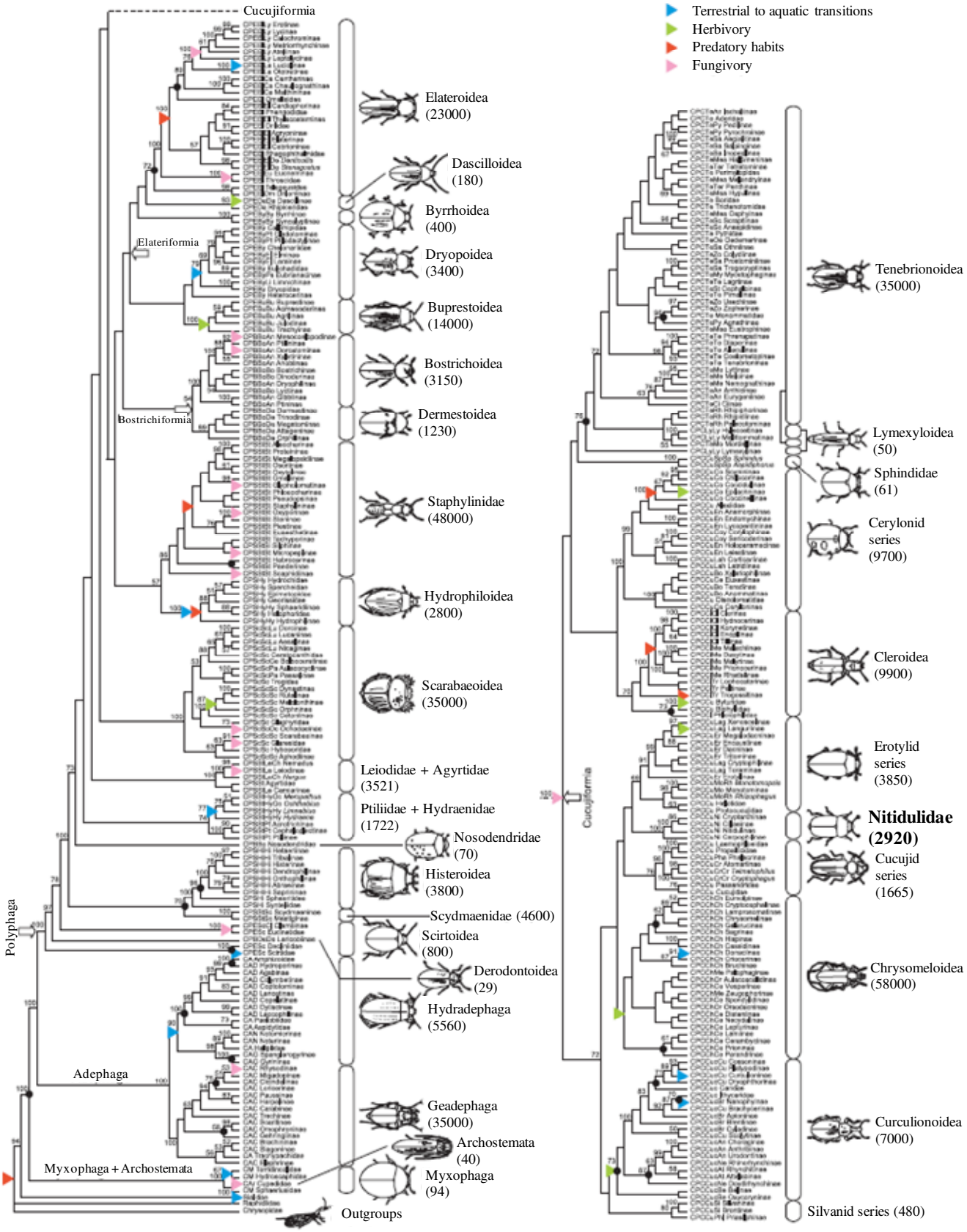

Figure 2: The phylogeny of Coleoptera at the family level, showing the position of the Nitidulidae among the beetles (Source: Hunt *et al.* 2007).

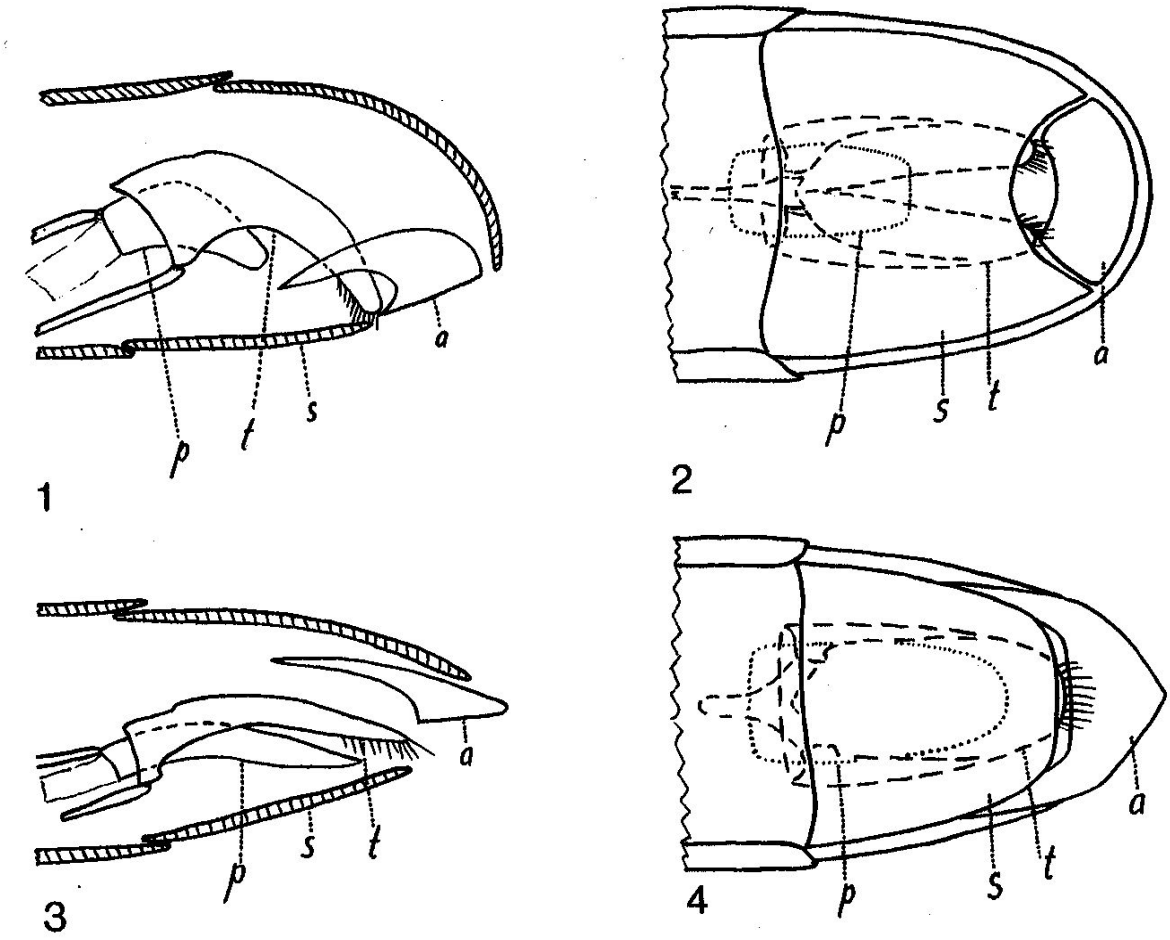

Figure 3: Generalized structures of male terminal abdominal segments of some Nitidulidae. 1, 2, Carpophilinae (Carpophiline lineage). 3, 4, Cillaeinae (Nitiduline lineage). [1, 3, cross section; 2, 4, ventral view; a = anal sclerite, p = penis, s = last sternite, t = tegmen]. (Source: Kirejtshuk 1986).

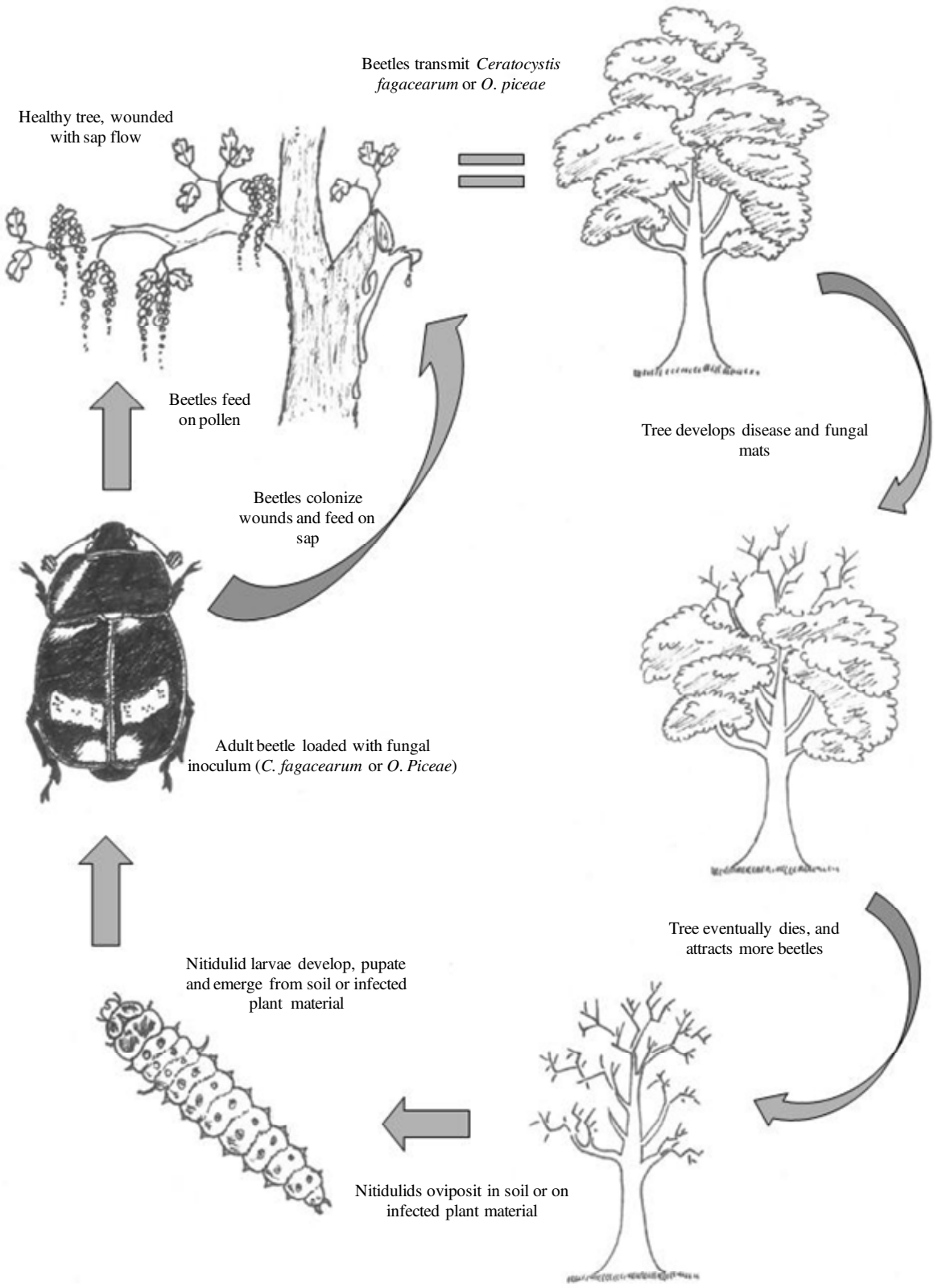

Figure 4: Diagrammatic representation of the role of nitidulids in the spread of *C. fagacearum*.

CHAPTER II

Ceratocystis species, including two new species associated with nitidulid beetles on *Eucalyptus* in Australia

ABSTRACT

The genus *Ceratocystis* includes important fungal pathogens of trees, including *Eucalyptus* spp. Ironically, very little is known regarding the diversity or biology of *Ceratocystis* species on *Eucalyptus* spp. in Australia where most of these trees are native. The aim of this study was to survey for *Ceratocystis* spp., and their possible insect associates on eucalypts in Australia and thus, to establish a foundation of knowledge regarding these fungi on the continent. Collections were made in three states of Australia from wounds on trees, as well as from nitidulid beetles associated with these wounds. *Ceratocystis* spp. were identified based on morphology and multigene sequence comparisons. Of the 54 isolates obtained, two previously unknown species of *Ceratocystis* were found and these are described here as *C. corymbiicola* sp. nov. and *C. tyalla* sp. nov. Furthermore, the distribution of *C. pirilliformis* is expanded to include *Eucalyptus* spp. in Tasmania.

2.1. INTRODUCTION

The genus *Ceratocystis* includes important insect-associated pathogens of agricultural and forestry crops, worldwide (Kile 1993, Roux & Wingfield 2009). Disease symptoms associated with infection by these fungi include stem cankers, root and fruit rot, wood stain and vascular wilt. The type species of the genus, *C. fimbriata* Ellis & Halsted is best known as the causal agent of sweet potato black rot disease (Halsted 1890, Halsted & Fairchild 1891). However, in the past two decades *Ceratocystis* spp. have emerged as important threats to plantation forestry trees, causing diseases of *Eucalyptus* spp. and *Acacia mearnsii* de Wild trees in non-native plantation situations (Roux & Wingfield 2009). Important examples include *C. fimbriata s.l.* reported to cause wilt and death of *Eucalyptus* spp. in the Republic of Congo, Uganda, Uruguay and Brazil (Barnes *et al.* 2003a, Roux *et al.* 1999, 2000, 2001), and *C. albifundus* De Beer, Wingfield & Morris which causes a serious wilt disease of plantation-grown *Acacia mearnsii* De wild trees in South Africa (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux & Wingfield 2009).

The eucalypts include more than 700 tree species distributed in the genera, *Eucalyptus* L'Her., *Corymbia* K.D. Hill & L.A.S. Johnson and *Angophora* Cav. (Hill & Johnson 1995). In Australia, numerous indigenous fungi are found on these trees and none have been reported to impart major disease in native forest situations (Park *et al.* 2000, Keane *et al.* 2000). In contrast, where eucalypts are planted as non-natives in plantations, there have been numerous reports of diseases of these trees (Keane *et al.* 2000, Wingfield *et al.* 2008, Wingfield 2003). These include disease caused by or associated with *Ceratocystis* spp. (Roux & Wingfield 2009).

A number of *Ceratocystis* species have been reported from Australia infecting wounds on *Eucalyptus* trees. These include *C. eucalypti* Z.Q. Yuan & Kile that causes vascular stain on *E. regnans* F. Muell., *E. sieberi* L.A.S. Johnson and *E. globoidea* Blakely (Kile *et al.* 1996), *C. pirilliformis* causing sap-stain on *Eucalyptus* spp. (Barnes *et al*. 2003b, Kamgan Nkuekam *et al.* 2009), *C. moniliformopsis* Yuan & Mohammed infecting *E. obliqua* L'Her (Yuan & Mohammed 2002), *C. moniliformis* (Hedgcock) Moreau from *E. grandis* (Hill) Maiden (Fouche *et al.* 2007) and *C. atrox* M. Van Wyk & M.J. Wingfield found in the galleries of *Phoracantha acanthocera* Macleay (Coleoptera: Cerambycidae) infesting *E. grandis* (Van Wyk *et al.* 2007). These reports have all been from very limited studies. Based also on the recent descriptions of numerous previously undescribed species of *Ceratocystis* from *Eucalyptus* elsewhere in the world, it seems that numerous species await discovery on eucalypts in Australia. A recent study on *C. pirilliformis*, using polymorphic simple sequence repeat (SSR) markers, for example, suggests that this fungus

was introduced to South Africa (Kamgan Nkuekam *et al.* 2009) and it might be native to Australia (Barnes *et al.* 2003b, Kamgan Nkuekam *et al.* 2009) where it was first discovered.

Most *Ceratocystis* spp. are vectored by sap-feeding nitidulid (Coleoptera: Nitidulidae) and Dipteran flies forming an association that is considered a non-specific (Cease & Juzwik 2001, Moller & Devay 1968). However, some *Ceratocystis* spp., such as the conifer pathogens, *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter and *C. rufipenni* Wingfield, Harrington & Solheim are consistently vectored by specific bark beetles (Harrington & Wingfield 1998, Wingfield *et al*. 1997) indicating specific vector-fungus association. There have been no detailed studies regarding insect associations with *Ceratocystis* spp. in Australia although it is likely that most of these fungi are vectored casually by nitidulids and flies.

This study represents a collaborative effort between researchers from Australia and South Africa focused on increasing knowledge pertaining to *Ceratocystis* spp. on native eucalypts in Australia. The intention was thus to provide an improved baseline for quarantine procedures and to predict threats of *Ceratocystis* disease outbreaks, globally. Wounds on eucalypt trees in the eastern part of Australia were thus investigated for the presence of these fungi and their nitidulid insect associates. All isolates were identified using a combination of morphological characteristics and DNA sequence data. Their potential pathogenicity on *Eucalyptus* was investigated in greenhouse inoculation studies.

2.2. MATERIALS AND METHODS

2.2.1. Source of samples and isolations

Collections of *Ceratocystis* spp. from *Eucalyptus* spp. and *Corymbia* spp. in Australia were made over a six-week period between March and April 2008. Because *Ceratocystis* spp. require wounds for infection, and previous studies have shown stem wounds are commonly infected with these fungi (Kile *et al.* 1996, Barnes *et al.* 2003b, Roux *et al.* 2004, Kamgan Nkuekam *et al.* 2009), surveys were focused on wounds resulting from harvesting as well as stem-boring insects. Plantations and forests in Tasmania, New South Wales and the southern part of Queensland were visited. In Tasmania, numerous localities near Burnie, Tarraleah and Geevestown were surveyed. In New South Wales (NSW), commercial plantations and native eucalypt forests between Sydney in the south and up to the border with Queensland were included. Some key areas sampled in NSW included Pine creek State forest, Wattagan State forest, Wedding Bells State forest, Crab-tree

plantation, Ingalba State forest and other localities around these areas. In the state of Queensland, samples were collected from *Eucalyptus* trees growing around Brisbane.

Samples were collected from stumps of felled trees in all areas surveyed in Tasmania. In NSW, wounds included stumps of felled trees, galleries of cossid moths (Lepidoptera: Cossidae) and cerambycid beetles, as well as excavations made on the trees by parrots scavenging for larvae of insects infesting the trees. In Queensland, samples were collected from artificially induced wounds and from wounds made by birds feeding on wood-boring larvae. Pieces of bark or wood were collected from wounds and stored in separate brown paper bags for each tree, after they had been examined with a 20X magnification lens for the presence of *Ceratocystis* fruiting structures. All the samples were placed in separate brown paper bags for each tree or stump and transported to the laboratory in plastic bags that also served as moist chambers to induce sporulation. Samples from individual trees were maintained separately in brown bags and samples from specific localities were grouped together into a single plastic bag.

To obtain information on possible nitidulids vectoring of *Ceratocystis* spp. in Australia, Nitidulidae were collected from wounds on eucalypt trees with fungal mats. Insects were collected using an aspirator (Fergusson 1982). Insects were sucked into the aspirator as they emerged from the area between the bark flaps and the wood of wounded trees. Living insects were transferred to cylindrical and labeled glass containers containing a piece of tissue paper that served to reduce contact and conflict among insects. In the laboratory, insects collected were inactivated by cooling the glass containers in a box containing ice. The insects were grouped according to morphological characteristics using an Axiocam stereo microscope (Carl Zeiss Ltd., Germany). Representatives of each insect group were preserved in 70% ethanol prior to identification, by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food & Agriculture, United States of America.

Isolation and purification of fungi from wood samples followed methodology described in Kamgan Nkuekam *et al.* (2008a). Nitidulid beetles not retained for species identification were used to determine the presence of *Ceratocystis* spp. on their bodies. This was done by squashing individual insects between two slices of carrot (Moller & Devay 1968) and incubating them for five days at 25ºC. Fungi were isolated by transferring fruiting bodies (spore masses, mycelium, ascomata) growing on the carrots to 2% malt extract agar (MEA: 20 gl^{-1} malt extract and 15 gl^{-1} agar, Biolab, Midrand, South Africa and 1000 ml sterile deionised water) containing 0.05 gl^{-1} of streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany).

Isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative cultures have also been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

2.2.2. Morphological characterization

Ceratocystis isolates were incubated at 25ºC until sporulation and then grouped into morphotypes based on colour (Rayner 1970) and macro-morphology. Morphological structures including ascomata and ascospores, phialides, conidia from selected isolates representing each morphotype were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope. Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and ten measurements were made for additional isolates. Measurements were noted as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum).

In addition scanning electron microscopy (SEM) was used to observe conidia and conidiophores of some isolates. For this purpose, specimens were prepared following protocols described by Grobbelaar *et al.* (2009). Finally the specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater and examined using a JEOL JSM-840 scanning electron microscope.

2.2.3. Growth in culture

One isolate of each of the purported new species found in this study was used for growth studies in culture. Disks of agar (9 mm diam.) bearing mycelium of the test isolates were transferred from the actively growing margins of seven-day-old cultures and placed upside down at the centres of 90 mm Petri dishes containing 2% MEA. The plates were incubated in the dark for 10 days at temperatures ranging from 5ºC to 35ºC at 5 degree intervals. Five replicate plates were used for each isolate at each temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed (Kamgan Nkuekam *et al.* 2008a, b).

2.2.4. DNA sequence comparisons

All isolates representing each morpho-group of the *Ceratocystis* spp. collected in this study were used in DNA sequence comparisons. Spore drops from single apices of ascomata or conidiophores in pure cultures was collected and grown on 2% MEA for 7-10 days. Mycelium was scraped from the surface of the actively growing cultures and then transferred to 1.5 ml Eppendorf tubes using a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified on an Eppendorf Mastercycler (Merck, Germany) using primers ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5') (White *et al*., 1990). Part of the β-tubulin gene (BT1) and the transcription elongation factor-1α gene (TEF) were also amplified using the primers βt1a (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and βt1b (5'-GACGAGATCGTTCATGTTGAACTC-3') (Glass & Donaldson 1995), EF1F (5'-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs *et al*. 2004), respectively.

The PCR reaction mixtures as well as the thermal cycling used in this study were the same as described previously (Kamgan Nkuekam *et al*. 2008a). Aliquots of 5 µl of the PCR products were stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA), separated on a 1% agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich, Steinheim, Germany), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). 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, California, USA). Sequencing PCRs were prepared as described by Kamgan Nkuekam *et al*. (2008a) and both DNA strands were sequenced.

A preliminary identity for the *Ceratocystis* isolates was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov). Sequences of both strands for each isolate were examined visually and combined using the programme Sequence Navigator. Sequences were then aligned automatically using Mafft ver. 5.851 (Katoh *et al*. 2002) and analyzed using PAUP 4.0b10 (Swofford 1998). Additional sequences of related *Ceratocystis* species were obtained from the GenBank database. PAUP 4.0b10 was used to

construct phylogenetic trees from the data matrices using heuristic search option of the maximum parsimony method (Swofford 1998). Confidence levels for the phylogenies were estimated with the bootstrap method (Felsenstein 1985).

Bayesian analyses were performed with MrBayes V3.1 (Ronquist & Heuelsenbeck 2003), based on Markov Chain Monte Carlo (MCMC) methods. The best-fit model of evolution was determined using MrModeltest V2.2 (Nylander 2004) and included for each gene partition in MrBayes. Four simultaneous MCMCs were run for 1 000 000 generations and trees were sampled every 100th generation. The burn-in procedure in MrBayes V3.1 was used to discard trees that formed before the point of convergence, and the posterior probability in the majority rule concensus trees were calculated by MCMC sampling in MrBayes V3.1, using the best-fit model of evolution mentioned above.

The level of polymorphism in sequence variation between closest related species was analyzed with the software program MEGA V4 (Molecular Evolutionary Genetics Analysis) (Tamura *et al.* 2007). Sequences for each gene region considered were examined to determine the number of fixed base pair differences that separate closest related taxa. Allele networks were constructed with the software TCS (Clement *et al.* 2000) to illustrate the relationship between isolates of closely related species.

2.2.5. Pathogenicity tests

Pathogenicity tests were conducted in a quarantine greenhouse using *Eucalyptus grandis* clone TAG5. Five strains of each of two *Ceratocystis* species obtained in this study were used in the inoculation experiment. Ten trees, approximately two-years-old (~1 cm diameter), were inoculated with each test strain and five trees of the same age were inoculated with a sterile agar disc to serve as controls. Test strains included isolates (CMW28917, CMW28920, CMW28925, CMW28928, CMW28932) belonging to the *C. moniliformis s.l.* species complex, and isolates (CMW29120, CMW29275, CMW29349, CMW29354, CMW29546) belonging to the *C. fimbriata s.l.* species complex (Table 1). Greenhouse conditions included an average temperature of 25ºC and natural day/night conditions of about 13 hours daylight and 11 hours darkness. Inoculations were done using the same technique as described before by Kamgan Nkuekam *et al.* (2008a). Six weeks (42 days) after inoculation, the lengths of lesions, including the original wound on the bark surface as well as in the cambium of each tree were measured. Results were processed using the statistical software package SAS® V8 running under VM/CMS on the main frame computer at the University

of Pretoria, where lesion lengths were analyzed using the GLM procedure. Re-isolations were made from the lesions to confirm that the lesions had resulted from the effects of the test fungi.

2.3. RESULTS

2.3.1. Source of samples and isolations

A wide variety of *Ceratocystis* isolates arose from collections in New South Wales, Queensland and Tasmania. These fungi were isolated from harvesting and stem boring insect wounds on two eucalypt genera (*Eucalyptus* and *Corymbia*) spanning nine different species. These trees species included *E. nitens* Deane & Maiden, *E. globulus* Labill, *E. pilularis* Sm., *E. saligna* Sm., *E. dunnii* Maiden, *E. grandis* Hill ex Maiden, *E. grandis x camaldulensis*, *E. tereticornis* and *C. variegata* (F.Muell.) K.D.Hill & L.A.S.Johnson (Table 1). A total of 54 isolates were obtained from wounds on 200 trees sampled across the three states of Australia. Apart from discoloration of the wood around the wounds, no diseases symptoms were observed on the trees.

Nitidulidae were found only at Ingalba State forest in New South Wales. A limited number (30) of insects were collected from *E. pilularis* trees. These insects represented two groups based on morphology and included *Brachypeplus binotatus* Murray (13 insects) and *Brachypeplus planus* Erichson (10 insects). The remaining specimens were characteristic of *Carpophilus* spp. (7 insects) but could not be identified to species level. *Ceratocystis* spp. were isolated from six nitidulid beetles using the carrot baiting technique. These represented four *Ceratocystis* isolates based on cultural characteristics on MEA.

2.3.2. Morphological identification

Ceratocystis spp. collected in this study could broadly be assigned to three morphological groups based on colony morphology and the type of fruiting bodies produced on MEA (Table 1). Morphogroup A included species resembling those in the *C. moniliformis s.l.* species complex. This group, consisting of 18 cultures, was characterized by fast growing isolates with a strong fruity (banana) odor, ascomata exuding sticky spore drops containing hat-shaped ascospores typical of *Ceratocystis* spp., echinulate ascomatal bases and ascomatal necks with disciform bases. These isolates covered the entire surface of the 60mm Petri dishes within three days at 25ºC.

Morpho-group B isolates (Table 1) included species resembling those in the *C. fimbriata s.l.* species complex. There were of 33 isolates in this group and they were slow-growing and had granular edges. Limited numbers of ascomata were produced in culture. These isolates had no spines on the

ascomatal bases, they lacked disc-like bases on the ascomatal necks and produced hat-shaped ascospores. Isolates in this morpho-group could be further placed in two sub-groups based on morphological characteristics. One of these sub-groups from NSW, where it was collected from both insects and trees, was lighter-coloured and ascospore drops were produced abundantly. This was in comparison to isolates that originated from *Eucaltyptus* trees in Tasmania, and which were recognized as representing *C. pirilliformis* based on the morphological characteristics described by Barnes *et al.* (2003b).

2.3.3. DNA sequence comparisons

All isolates of *Ceratocystis* in morpho-group A (*C. moniliformis s.l.*) were sequenced and selected isolates (CMW21598, CMW28917, CMW28920, CMW28925, CMW28928, CMW28932) (Table 2) including representatives from each host that were used in phylogenetic analyses (Figure 1). All isolates generated amplicons of about 600, 550 and 850 bps for the ITS, BT1 and TEF gene regions, respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.283, suggesting that the data from the three gene regions could be combined. Comparison of these isolates with those of previously published species in GenBank by analyses of the combined data sets in PAUP resulted in a total of 1130 characters including gaps, with 960 constant characters, five variable characters (parsimony-uninformative) and 165 parsimony informative characters.

Phylogenetic analysis using parsimony and the heuristic search option resulted in 419 best trees with a consistency index (CI) and retention index (RI) value of 0.670 and 0.892, respectively. Isolates from Australia formed a well-resolved clade (Figure 1), supported by a bootstrap value of 98%, separate from any of the described species in the *C. moniliformis s.l.* species complex, suggesting that they represent an undescribed species. The closest phylogenetic neighbor of these isolates was *C. moniliformis*.

In separate analyses using both parsimony and Bayesian computations across the ITS, BT1 and TEF gene regions, respectively, only representatives of Morpho-group A and their most closely related phylogenetic neighbors were considered to confirm their species delimitation. In addition, the multilocus nucleotide polymorphisms showing differences among these taxa, as well as the number of fixed base pair differences across the three gene regions, were computed. In these analyses, representatives of Morpho-group A formed a well resolved clade clearly separated from its sister clades (Table 3) (Figures 3A, 3B, 3C). This separation was supported by a number of polymorphic nucleotide sites found across the three gene regions (Tables 4.1, 4.2). A number of

fixed base pair differences were also found (Tables 5, 6, 7) across the three gene regions considered and these were consistent with results based on concatenated analyses and morphology.

Isolates (CMW29120, CMW29275, CMW29354, CMW29349, CMW29546, CMW29549) in Morpho-group B1, and Morpho-group B2 (CMW29111, CMW29112, CMW29119, CMW29355) generated amplicons of about 600, 550 and 850 bps for parts of the ITS, BT1 and TEF gene regions, respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.273, suggesting that the data from the three gene regions could be combined. Comparison of these isolates with those from GenBank and automatic alignment using Mafft, followed by analyses in PAUP, resulted in a total of 1289 characters including gaps, with 630 constant characters, 166 variable characters (parsimony-uninformative) and 493 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option resulted in 734 best trees with a consistency index (CI) and retention index (RI) value of 0.692 and 0.889, respectively. Isolates resolved into two different clades within the larger *C. fimbriata s.l* group. Isolates representing Morpho-group B1 formed a well-resolved clade (Figure 2), supported by a bootstrap value of 85%, separate from any of the described species in the *C. fimbriata s.l.* species complex. The closest phylogenetic neighbor of these isolates was *C. atrox*. Isolates from Morpho-group B2 clustered with strains of *C. pirilliformis*, consistent with results based on morphological identification.

Bayesian analyses for representatives of Morpho-group B1 and Morpho-group B2 and their most closely related phylogenetic neighbors showed that representatives of Morpho-group B1 formed a well resolved clade, clearly separated from its sister clades (Table 3), *C. atrox* and *C. polychroma*, based on the ITS and BT1 gene regions (Figures 4A, 4B, 4C). This separation was supported by a number of fixed base pair differences found (Tables 9, 10, 11) and these were consistent with results based on concatenated analyses. Analyses of the TEF sequence data did not provide separation between these species.

Representatives of Morpho-group B2 and their most closely related *Ceratocystis* neighbors were intermingled in unrooted phylogenetic trees for all three gene regions considered (Figures 4A, 4B, 4C). Within the ITS gene region, there was only one informative site distinguishing between *C. zombamontana* and *C. pirilliformis*. *C. polyconidia* and *C. obpyriformis* in contrast fall into a well resolved clade, clearly separated from other taxa in phylogenetic analyses based on the ITS gene region (Figure 4A). In the BT sequence data set, *C. pirilliformis* and *C. zombamontana* had identical sequences (Figure 4B) while in the TEF, *C. pirilliformis*, *C. zombamontana* and *C.*

polyconidia all shared 100% homology (Figure 4C). These results were consistent with data from concatenated analyses and suggest that *C. pirilliformis* and *C. zombamontana* represent the same species based on DNA phylogenies, differing only in their ITS sequence data with three to seven random (non-fixed) base pair differences (Tables 12, 13).

Seven haplotypes were identified within the *C. pirilliformis s.l.* clade in multilocus analysis using TCS (Table 14, Figure 5). *C. polyconidia* (CMW23807, CMW23808) formed a single haplotype distinct from other species in the *C. pirilliformis s.l.* clade (Figure 5). Species including *C. pirilliformis* (CMW6569, CMW6579), *C. zombamontana* (CMW15235, CMW15236), *C. polyconidia* (CMW23809, CMW23818) and isolates collected from Australia (CMW29111, CMW29112, CMW29119, CMW29355) formed a single allelic network in which isolates were intermingled (Figure 5).

2.3.4. Taxonomy

Based on morphological studies and multigene sequence phylogenies, two *Ceratocystis* spp. emerged as distinct taxa, clearly separated from other related *Ceratocystis* reference strains. These included a *Ceratocystis* sp. residing in the *C. moniliformis s.l.* group and one in the *C. fimbriata s.l.* species complex. Furthermore, sequence data showed that *C. zombamontana* and *C. pirilliformis* represent the same species and should be reduced to synonymy. The following descriptions are provided for them.

Ceratocystis tyalla Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 6), **MB519980**.

***Etymology*:** The name is derived to the aboriginal name for eucalypts in Australia.

Coloniae mellicolores in MEA in 3 diebus in 30°C ad 36.17 mm crescentes. Bases ascomatum brunneae vel nigrae, globosae vel obpyriformes, spinis indumentoque hypharum fuscis. Colla ascomatum atrobrunnea hyphis ostiolaribus divergentibus. Bases collorum basin versus ornamentis disciformibus. Asci evanescentes. Ascosporae pileiformes hyalinae non septatae, vaginis vestitae. Anamorpha *Thielaviopsis* conidiophoris phialidicis hyalinis tubulosis, colliculis visibilibus (1.5-) 2.5-6 (-8.5) µm. Conidia hyalina non septata biformia; oblonga extremis obtusis (5.0-) 6.5-8.5 (-9.5) x (2.0-) 2.0-2.5 (-3.0) µm, bacilliformiaque basibus rotundatis (8.5-) 9.5-11.0 (-12.0) x (1.5-) 1.5-2.5 (-3.5) µm.

Colonies honey (19''b) coloured on MEA, reverse honey (19''b) coloured, almost brown. Colony diameters reaching 36.17 mm in 3 days on MEA at 30ºC. Optimal growth at 30ºC, growth

at 35ºC with colony diameters reaching 33.8mm in 3 days. No growth at 5ºC. Mycelium forming thick mats on agar with some white aerial mycelia. Hyphae septate, not constricted at septa. *Ascomata* scattered over the surface of the colonies or embedded in mycelium. *Ascomatal* bases brown to black, globose to obpyriform (124.5-) 143.0-176.0 (-195.5) µm long and (117.0-) 136.0-167.0 (-177.5) µm wide, with dark conical spines, (5.5-) 7.5-13.0 (-18.0) µm long and hyphal hair. *Ascomatal* necks dark brown (428.6-) 466.5-607.6 (-772.5) µm long, middle of necks (18.0-) 19.0-22.5 (-24.0) µm wide, tips of necks (9.0-) 11.0-13.5 (-15.0) µm wide, producing sticky and hyaline spore drops at the tips of divergent *ostiolar hyphae*, (15-) 18-25 (-29) µm long. Neck bases ornamented with disc shape, (37.0-) 43.0-52.5 (-59.5) µm wide at bases. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (3.5-) 4.0-4.5 (-5.0) x (2.0-) 2.0-2.5 (-3.0) µm, accumulating in round, straw yellow (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (15.0-) 18.0-27.0 (-35.5) x (2.0-) 2.5-3.0 (-4.0) µm; colarettes visible (1.5-) 2.5-6.0 (-8.5) µm. *Conidia* hyaline, aseptate, two types, oblong with obtuse ends (5.0-) 6.5-8.5 (-9.5) x (2.0-) 2.0-2.5 (-3.0) µm and bacilliform with rounded bases (8.5-) 9.5-11.0 (-12.0) x (1.5-) 1.5-2.5 (-3.5) µm. Chlamydospores (aleurioconidium) not observed.

Specimens examined: Australia, New South Wales, Marsden State Forest, isolated from cut stump of *Eucalyptus dunnii*, 19/03/2008, G. Kamgan Nkuekam & A. Carnegie, holotype PREM60434, living culture CMW28932, CBS128703

Additional specimens: Australia, New South Wales, Cairncross State Forest, isolated from *E. pilularis* stumps, 18/03/2008, G. Kamgan Nkuekam & A. Carnegie, paratype, living culture CMW28925/PREM60436/CBS127211, Wattagans State Forest, isolated from *E. saligna* stumps, 17/03/2008, G. Kamgan Nkuekam & A. Carnegie, CMW28928/PREM.60435/CBS128342, Pine Creek State Forest, isolated from *E. grandis* stumps, 18/03/2008, G. Kamgan Nkuekam & A. Carnegie, CMW28917, CMW28920.

Ceratocystis corymbiicola Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 7) **MB519979**.

***Etymology*:** The name reflects the host Corymbia.

Coloniae olivaceo-bubalinae in MEA in 10 diebus in 30°C ad 20 mm crescentes. Bases ascomatum nigrae, globosae sine spinis nec ornamentis. Colla ascomatum nigra hyphis ostiolaribus divergentibus. Asci evanescentes. Ascosporae pileiformes, vaginis vestitae, non septatae. Anamorpha *Thielaviopsis* conidiophoris phialidicis tubulosis hyalinis, sine colliculis. Conidia biformia; bacilliformia basibus rotundatis (11.0-) 15.0-21.5 (-27.5) x (3.0-) 3.5-4.5 (-5.5) µm,

bacilliformiaque extremis obtusis (7.5-) 8.5-12.0 (-14.5) x (3.5-) 4.0-5.5 (-6.5) µm. Chlamydosporae (aleurioconidium) ovoideae laeves singulae terminales, iuventute hyalinae, maturitate nigrescentes (8.5-) 11.0-14.0 (-16.5) x (6.5-) 8.0-11.0 (-16.5) µm.

Colonies olivaceous Buff (21'''d) on MEA, reverse smoke grey (21''''d). Colony diameters reaching 20 mm in 10 days on MEA at 30ºC. Optimal growth at 30ºC, no growth at 10ºC. Colony surfaces scattered with black ascomata. Mycelium immersed and superficial, with white-grey aerial mycelia. Hyphae septate, not constricted at septa. *Ascomatal* bases black, globose (159-) 189-241 (-290) µm long and (160.5-) 185.5-237.5 (-272.5) µm wide. Spines or ornamentations absent. *Ascomatal* necks black (603-) 755-1009 (-1098) µm long, bottom of necks smooth (43-) 55-71 (-77) µm wide, middle of necks (20.0-) 27.5-34.5 (-38.5) µm wide, tips of necks (13.0-) 15.5-19.5 (-22.0) µm wide. *Ostiolar hyphae* present, divergent (22.5-) 42.0-58.5 (-67.5) µm long. *Asci* evanescent. *Ascospores* hat-shaped, invested in sheaths, aseptate (4.5-) 5.0-5.5 (-6.0) µm long and (2.5-) 3.0-3.5 (-4.0) µm wide. Ascospores accumulating in round or disk-shaped, Buff (19''d) colored spore drops.

Anamorph state: *Thielaviopsis*. *Conidiophores* occurring singly, phialidic (17.0-) 38.5-83.0 (-109.0) x (2-) 3-5 (-6) µm, tubular with thin bases making them almost constricted at septa, hyaline, colarettes absent. Two types of *Conidia* produced; bacilliform-shaped conidia with round bases (11.0-) 15.0-21.5 (-27.5) x (3.0-) 3.5-4.5 (-5.5) µm, and bacilliform-shaped conidia with obtuse ends (7.5-) 8.5-12.0 (-14.5) x (3.5-) 4.0-5.5 (-6.5) µm, often produced in chains. *Chlamydospores* (aleurioconidium) ovoid, smooth, formed singly, terminal, hyaline when young, becoming black when mature (8.5-) 11.0-14.0 (-16.5) x (6.5-) 8.0-11.0 (-16.5) µm.

Specimens examined: Australia, New South Wales, Wedding Bells State Forest, isolated from cut stumps of *Corymbia variegata*, 19/03/2008, G. Kamgan Nkuekam & A. Carnegie, holotype PREM60431, living culture CMW29120/CBS127215.

Additional specimens: Australia, New South Wales, Ingalba State Forest, isolated from *Brachypeplus planus* infesting *Eucalyptus pilularis* stumps, 18/03/2008, G. Kamgan Nkuekam & A. Carnegie, paratype, living culture CMW29354/PREM 60432/CBS127217. Isolated from *E. pilularis* stumps CMW29349/PREM60433/CBS127216. Wattagans State Forest, New South Wales. Isolated from *E. saligna* stumps, 17/03/2008, CMW29275/PREM60442, Ingalba State Forest, isolated from *Carpophylus* sp. infesting *Eucalyptus pilularis* tree, 18/03/2008, CMW29546, Dyraba State Forest, isolated from *E. grandis x camaldulensis* stumps, 20/03/2008, CMW29549.

2.3.5. Pathogenicity tests

Six weeks after inoculation, *E. grandis* trees were assessed for disease development based on the length of lesions seen on the bark or at the cambial surfaces. *Ceratocystis tyalla* and *C. corymbiicola* produced very small lesions on both the bark (Figure 8) and the cambial surfaces (Figure 9). Trees showed no signs of disease, and re-isolation did not yield cultures of either *C. tyalla* or *C. corymbiicola*. Significant differences ($P<0.0001$) in lesion lengths were, however, found between *C. tyalla* and *C. corymbiicola* when compared to the control inoculations (Figures 8, 9).

2.4. DISCUSSION

This study reports on the most extensive survey of *Ceratocystis* spp. on eucalypts in their country of origin. Three species of *Ceratocystis* were collected in Australia from either wounds on eucalypt trees or from nitidulid beetles collected from wounds on trees. Two of these are previously undescribed fungal species for which the names *C. tyalla* and *C. corymbiicola* have been provided. Furthermore, the host and geographic ranges of *C. pirilliformis* have been expanded to include *Eucalyptus* trees in Tasmania.

Three *Ceratocystis* spp., two in the *C. fimbriata s.l.* species complex and one in the *C. moniliformis s.l.* species complex emerged from this study. These comprise two species described for the first time in this study and *C. pirilliformis*. *Ceratocystis tyalla* sp. nov. grouped in a unique sub-clade, most closely related to *C. moniliformis* in phylogenetic analyses. Similar to *C. moniliformis* and other fungi in the *C. moniliformis s.l.* species complex, it grows rapidly in culture, produces hat-shaped ascospores and has short conical spines on the ascomatal bases. *Ceratocystis tyalla* could, however, be distinguished from *C. moniliformis*, and other related fungi, by the fact that it produces a disc shape like structure at the base of the ascomatal necks that is less well defined than in other species of the group, giving an appearance of smooth neck bases. Phylogenetic inference based on three gene regions also clearly distinguished this taxon from its relatives.

The second previously undescribed *Ceratocystis* sp. collected from eucalypts in Australia, *C. corymbiicola* sp. nov. grouped in the *C. fimbriata s.l.* species complex. It was most closely related to *C. atrox* and *C. polychroma* in both the combined ITS, BT and TEF data sets as well as when these gene regions were analysed separately. Bootstrap support for the unique clade containing *C. corymbiicola* was, however, low in the concatenated tree. Morphological differences could,

however, be detected to support the description of *C. corymbiicola* as a unique species, distinct from *C. atrox*, a species described recently from Australia (Van Wyk *et al.* 2007). *C. corymbiicola* produces chlamydospores, which have not been found in *C. atrox*. In addition, *C. corymbiicola* produces two types of bacilliform conidia and only one type of phialide, while *C. atrox* produces cylindrical and barrel-shaped conidia, and two types of phialides (Van Wyk *et al.* 2007). The conidia of *C. corymbiicola* are unique in that many of them have swellings, even when young.

Inoculation studies with *C. tyalla* and *C. corymbiicola* under greenhouse conditions showed that these fungi are not serious pathogens of *Eucalyptus* trees. Neither of them gave rise to significant lesions or any disease symptoms of young *E. grandis* trees. This is not surprising for *C. tyalla* since species in the *C. moniliformis s.l.* group are not known as pathogens of trees (Davidson 1935). Although *C. corymbiicola* resides in a group of *Ceratocystis* spp. known to include important plant pathogens, it also did not produce any signs of disease in the inoculation trial.

Both previously undescribed *Ceratocystis* spp. collected in this study could have much wider distributions and host ranges than found in this study. The discovery of these fungi infecting different eucalypt genera spanning several different species suggests that eucalypts are probably their primary hosts. The fact that neither of them were associated with disease, or caused significant lesions in green house inoculation trials suggests that they are native to Australia. Furthermore, isolating *C. corymbiicola* from nitidulid beetles suggests that these insects could be potential vectors of the fungus in Australia, similar to what is known for these fungi from other continents.

It was not surprising to find *C. pirilliformis* infecting wounds of *Eucalyptus* trees in Australia. The fungus was collected from cut stumps of *E. nitens* and *E. globulus* in Tasmania and a single isolate came from a wound made by the giant wood moth infesting an *E. grandis* tree in NSW. This fungus was first described from wounds on *E. nitens* in Australia (Barnes *et al.* 2003b) and has subsequently also been found on *Eucalyptus* spp. in South Africa (Roux *et al.* 2004, Kamgan Nkuekam *et al.* 2009). Results of this study expand the host and geographic range of this fungus. It is suspected that *C. pirilliformis* is native to Australia, based on its wide distribution (Tasmania to Queensland) in the country and the fact that it appears not to cause disease on eucalypts in Australia (Barnes *et al.* 2003b, Kamgan Nkuekam *et al.* 2009). A recent population diversity study of *C. pirilliformis* isolates collected in South Africa showed a low level of diversity for isolates in South Africa and suggested high population diversity for the few isolates available from Australia (Kamgan Nkuekam *et al.* 2009). However, larger populations of *C. pirilliformis* from Australia are required to fully elucidate the origin of this fungus.

C. zombamontana, recently described from *Eucalyptus* trees in Malawi (Heath *et al.* 2009) was found to be phylogenetically similar to *C. pirilliformis* based on DNA sequence data of five gene regions. Strains of the two species have identical BT and TEF sequences. Strains of *C. zombamontana*, including the ex-type strain, have identical ITS1, ITS2 and 5.8S sequences as many strains of *C. pirilliformis* collected in the current study. A number of morphological differences were reported between the two species and these mostly included chlamydospores produced by *C. pirilliformis* and absent in *C. zombamontana*, and flask-shaped primary phialides produced by *C. zombamontana* compared to the cylindrical to lageniform phialides of *C. pirilliformis* (Heath *et al.* 2009). The presence or absence of chlamydospores should probably not be considered an important taxonomic characteristic as they are survival structures to enable these fungi to survive adverse conditions such as those found in the soil or in dead plant tissue (Accordi 1989). The haplotype network drawn from combined ITS, BT and TEF sequence data did not show separation between *C. pirilliformis* and *C. zombamontana*. The two species resided within the same allele tree where isolates of both the two species were intermingled with each other supporting the view that they probably represent a single species. However, prior to reducing them to synonymy, detailed comparisons particularly including their morphology and including a greater number of isolates should be conducted before they are reduced to synonymy.

Brachypeplus binotatus and *B. planus* the two nitidulid species identified in this study that show indication of unspecific association with *Ceratocystis* spp., have been reported from Australia previously. Numerous other species of Nitidulidae including *Carpophilus* spp. are also known to occur in Australia in various localities and habitats (Masters 2009). However, nitidulid beetles have not previously been found in association with *Ceratocystis* spp. in Australia. The initial results reported here may suggest that these insects are probably involved in overland spread of these fungi and further study of this association is likely to yield interesting insights into the biology of *Ceratocystis* spp. and their vectors in that country.

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Table 1. List of *Ceratocystis* isolates and their morphogroups collected during surveys in Eastern and Southern Australia.

Morphogroups	States	Area	Hosts	Isolate Number	Fungal species	Coordinates	Collectors
A	NSW	Marsden State Forest	*E. dunnii* stumps	28930, 28931, 28932, 28933	*C. tyalla*	S30º 14, 503' E152º 38, 745'	GNK. Kamgan & AJ. Carnegie
	"	Wattagans State Forest	*E. saligna* stumps	28927, 28928,28929	"	S33º 02, 738' E151º 19, 402'	"
	"	Cairncross State Forest	*E. pilularis* trees	28923, 28924, 28925, 28926	"	S31º 21, 275' E152º 45, 671'	"
	"	Pine Creek State Forest	*E. grandis* stumps	28920, 28921, 28922	"	S31º 21, 361' E152º 45, 009'	"
	"	Pine Creek State Forest	*E. grandis* stumps	28917, 28918, 28919	"	S30º 23, 545' E152º 56, 975'	"
	QLD	Brisbane	*E. tereticornis*	21598	"	NA	J. Roux & GS. Pegg
B1	QLD	Brisbane	*E. tereticornis*	21599, 21600, 21601, 21602	*C. corymbiicola*	NA	"
	NSW	Wedding bells State Forest	*C. variegata*	29120, 29121, 29122, 29123, 29124, 29347, 29348, 29428	"	S30º 03, 498' E153º 10, 266'	GNK. Kamgan & AJ. Carnegie
	"	Wattagans State Forest	*E. saligna*	29275, 29276, 29434	"	S33º 02, 738' E151º 19, 402'	"
	"	Dyraba State Forest	*E. grandis x camaldulensis* trees	29549	"	S28º 48, 522' E152º 50, 370'	"
	"	Nabiac	*E. grandis* trees	29344, 29345, 29346	"	S32º 05, 411' E152º 21, 838'	"
	"	KEW	*E. grandis x camaldulensis* trees	29547	"	S31º 36, 954' E152º 44, 096'	"
	"	Ingalba State Forest	*E. pilularis* stumps	29349	"	S30º 47, 970' E152º 51, 706'	"
	"	Ingalba State Forest	*Carpophilus* sp. infesting E. pilularis tree	29353, 29546	"	S30º 47, 970' E152º 51, 706'	"
	"	Ingalba state forest	*Brachypeplus planus* infesting *E. pilularis* tree	29354	"	S30º 47, 970' E152º 51, 706'	"
B2	NSW	Crab tree plantation	*E. grandis* trees, infested by giant wood moths and damaged by	29355	*C. pirilliformis*	S30º 08, 345' E153º 06, 123'	"

		cocketou bird				
Tasmania	Burnie	*E. nitens* stumps	29111, 29112, 29113, 29114	“	S41º 10, 185’ E145º 45, 441’	GNK. Kamgan & Caroline Mohammed
“	Burnie	*E. globulus* stumps	29119	“	S41º 08, 783’ E145º 48, 435’	“
“	Tarraleah	*E. nitens* stumps	29092, 29093, 29094	“	S11º 15, 846’ E024º 19, 115’	“

Table 2. List of *Ceratocystis* isolates and their GenBank accession numbers sequenced in this study (*) or used for DNA sequence comparisons.

Isolates designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
C. acacivora	CMW22562	EU588655	ITS	NA	*Acacia mangium*	M. Tarigan	Indonesia
		EU588635	BT	"	"	"	"
		EU588645	EF	"	"	"	"
	CMW22563	EU588656	ITS	NA	"	"	"
		EU588636	BT	"	"	"	"
		EU588646	EF	"	"	"	"
C. albifundus	CMW4068	DQ520638	ITS	NA	*Acacia mearnsii*	J. Roux	South Africa
		EF070429	BT	"	"	"	"
		EF070400	EF	"	"	"	"
	CMW5329	AF388947	ITS	NA	*A. mearnsii*	J. Roux	Uganda
		DQ371649	BT	"	"	"	"
		EF070401	EF	"	"	"	"
C. atrox	CMW19383	EF070414	ITS	CBS120517	*E. grandis*	MJ. Wingfield	Australia
		EF070430	BT	"	"	"	"
		EF070402	EF	"	"	"	"
	CMW19385	EF070415	ITS	CBS120518	*E. grandis*	MJ. Wingfield	Australia
		EF070431	BT	"	"	"	"
		EF070403	EF	"	"	"	"
C. bhutanensis	CMW8399	AY528959	ITS	CBS115772, BH 8/8	*Picea spinulosa*	T. Kirisits & DB. Chhetri	Bhutan
		AY528964	BT	"	"	"	"
		AY528954	EF	"	"	"	"
	CMW8215	AY528958	ITS	CBS114290, PREM57805	*P. spinulosa*	T. Kirisits & DB. Chhetri	Bhutan
		AY528963	BT	"	"	"	"
		AY528953	EF	"	"	"	"
C. caryae	CMW14793	EF070424	ITS	CBS114716	*Carya cordiformis*	J. Johnson	USA
		EF070439	BT	"	"	"	"
		EF070412	EF	"	"	"	"
	CMW14808	EF070423	ITS	CBS115168	*C. ovata*	"	USA
		EF070440	BT	"	"	"	"
		EF070411	EF	"	"	"	"
C. colombiana	CMW5751	AY177233	ITS	CBS121792	*Coffea arabica*	M. Marin	Colombia
		AY177225	BT	"	"	"	"
		EU241493	EF	"	"	"	"
	CMW5761	AY177234	ITS	CBS121791	"	B. Castro	"
		AY177224	BT	"	"	"	"
		EU241492	EF	"	"	"	"

C. corymbiicola	*CMW29120	HM071902	ITS	CBS127215	*C. variegata*	GNK. Kamgan	"
		HM071914	BT	"	"	"	"
		HQ236453	EF	"	"	"	"
	*CMW29354	HM071907	ITS	CBS127217	*B. planus*	"	"
		HM071919	BT	"	"	"	"
		HQ236455	EF	"	"	"	"
	*CMW29349	HM071905	ITS	CBS127216	*E. pilularis*	"	"
		HM071917	BT	"	"	"	"
		HQ236456	EF	"	"	"	"
	*CMW29275	HM071903	ITS	NA	*E. saligna*	"	"
		HM071915	BT	NA	"	"	"
		HQ236454	EF	NA	"	"	"
	*CMW29546	HM071904	ITS	NA	*Carpophilus* sp.	"	"
		HM071916	BT	NA	"	"	"
		HQ236457	EF	NA	"	"	"
	*CMW29549	HM071906	ITS	NA	*E. grandis x camaldulensis*	"	"
		HM071918	BT	NA	"	"	"
		HQ236458	EF	NA	"	"	"
C. eucalypticola	CMW11536	FJ236723	ITS	CBS124016	*Eucalyptus* sp.	M. van Wyk	South Africa
		FJ236783	BT	"	"	"	"
		FJ236753	EF	"	"	"	"
	CMW10000	FJ236722	ITS	CBS124019	*Eucalyptus* sp.	M. van Wyk	"
		FJ236782	BT	"	"	"	"
		FJ236752	EF	"	"	"	"
C. fimbriata	CMW1547	AF264904	ITS	NA	*Ipomoea batatas*	ECH. McKenzie	Papua N. Guinea
		EF070443	BT	"	"	"	"
		EF070395	EF	"	"	"	"
	CMW15049	DQ520629	ITS	CBS141.37	*I. batatas*	CF. Andrus	USA
		EF070442	BT	"	"	"	"
		EF070394	EF	"	"	"	"
C. fimbriatomima	CMW24174	EF190963	ITS	CBS121786	*Eucalyptus* sp.	MJ. Wingfield	Venezuela
		EF190951	BT	"	"	"	"
		EF190957	EF	"	"	"	"
	CMW24176	EF190964	ITS	CBS121787	*Eucalyptus* sp.	"	"
		EF190952	BT	"	"	"	"
		EF190958	EF	"	"	"	"
C. inquinans	CMW21106	EU588587	ITS	NA	*A.mangium*	M. Tarigan	Indonesia
		EU588666	BT	"	"	"	"
		EU588674	EF	"	"	"	"
	CMW21107	EU588588	ITS	"	"	"	"
		EU588667	BT	"	"	"	"

		EU588675	EF	"	"	"	"
C. larium	CMW25436	EU881908	ITS	CBS122607	*Styrax benzoin*	MJ. Wingfield	Indonesia
		EU881896	BT		"	"	"
		EU881902	EF		"	"	"
	CMW25437	EU881909	ITS	NA	"	"	"
		EU881897	BT		"	"	"
		EU881903	EF		"	"	"
C. manginecans	CMW13851	AY953383	ITS	"	*Mangifera indica*	M. Deadman	Oman
		EF433308	BT	"		"	"
		EF433317	EF	"		"	"
	CMW13852	AY953384	ITS	"	*Hypocryphalus mangifera*	"	"
		EF433309	BT	"		"	"
		EF433318	EF	"		"	"
C. microbasis	CMW21115	EU588592	ITS	"	*A.mangium*	M. Tarigan	Indonesia
		EU588671	BT	"	"	"	"
		EU588679	EF	"	"	"	"
	CMW21117	EU588593	ITS	"	"	"	"
		EU588672	BT	"	"	"	"
		EU588680	EF	"	"	"	"
C. moniliformis	CMW9590	AY431101	ITS	CBS116452	*Eucalyptus grandis*	J. Roux	South Africa
		AY528985	BT	"	"	"	"
		AY529006	EF	"	"	"	"
	CMW8379	AY529005	ITS	NA	*Cassia fistula*	MJ. Wingfield	Bhutan
		AY528995	BT	"	"	"	"
		AY529016	EF	"	"	"	"
C. moniliformopsis	CMW10214	AY528999	ITS	CBS115792, ORB 33	*E. sieberi*	MJ. Dudzinski	Australia
		AY528988	BT	"	"	"	"
		AY529009	EF	"	"	"	"
	CMW9986	AY528998	ITS	CBS109441	*E. obliqua*	ZQ. Yuan	Australia
		AY528987	BT	"	"	"	"
		AY529008	EF	"	"	"	"
C. neglecta	CMW17808	EF127990	ITS	CBS121789	*Eucalyptus* sp.	MJ. Wingfield	Colombia
		EU881898	BT			"	"
		EU881904	EF			"	"
	CMW18194	EF127991	ITS	CBS121017	*Eucalyptus* sp.	"	"
		EU881899	BT			"	"
		EU881905	EF			"	"
C. oblonga	CMW23802	EU245020	ITS	CBS122820	*A. mearnsii*	RN. Heath	South Africa
		EU244992	BT	"	"	"	"
		EU244952	EF	"	"	"	"
	CMW23803	EU245019	ITS	CBS122291	*A. mearnsii*	RN. Heath	South Africa

		EU244991	BT	"	"	"	"
		EU244951	EF	"	"	"	"
C. obpyriformis	CMW23807	EU245004	ITS	CBS122608	*A. mearnsii*	RN. Heath	South Africa
		EU244976	BT	"	"	"	"
		EU244936	EF	"	"	"	"
	CMW23808	EU245003	ITS	CBS122511	"	"	"
		EU244975	BT	"	"	"	"
		EU244935	EF	"	"	"	"
C. omanensis	CMW11048	DQ074742	ITS	CBS115780, PREM57815	*Mangifera indica*	AO. Al-Adawi	Oman
		DQ074732	BT	"	"	"	"
		DQ074737	EF	"	"	"	"
	CMW11046	DQ074739	ITS	CBS118112, PREM57814	*M. indica*	AO. Al-Adawi	Oman
		DQ074729	BT	"	"	"	"
		DQ074734	EF	"	"	"	"
C. papillata	CMW8850	AY233866	ITS	CBS121794	*Citrus x tangelo*	MJ. Wingfield	Colombia
		AY233875	BT			"	"
		EU241485	EF			"	"
	CMW8856	AY233867	ITS	CBS121793	*Citrus lemon*	"	"
		AY233874	BT			"	"
		EU241484	EF			"	"
C. pirilliformis	CMW6569	AF427104	ITS	PREM57322, DAR75993	*E. nitens*	MJ. Wingfield	Australia
		DQ371652	BT	"	"	"	"
		AY528982	EF	"	"	"	"
	CMW6579	AF427105	ITS	PREM57323, DAR75996	*E. nitens*	MJ. Wingfield	Australia
		DQ371653	BT	"	"	"	"
		AY528983	EF	"	"	"	"
	*CMW29111	NA	ITS	NA	*E. nitens*	GNK. Kamgan	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29112	NA	ITS	NA	*E. nitens*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29119	NA	ITS	NA	*E. globulus*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29355	NA	ITS	NA	*E. grandis*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"

C. platani	CMW14802	DQ520630	ITS	CBS115162	Platanus occidentalis	TC. Harrington	USA
		EF070425	BT	"	"	"	"
		EF070396	EF	"	"	"	"
	CMW23918	EU426554	ITS	NA	"	MJ. Wingfield	Greece
		EU426555	BT	"	"	"	"
		EU426556	EF	"	"	"	"
C. polychroma	CMW11424	AY528970	ITS	CBS115778, PREM57818	*Syzygium aromaticum*	ECY. Liew & MJ. Wingfield	Indonesia
		AY528966	BT	"	"	"	"
		AY528978	EF	"	"	"	"
	CMW11436	AY528971	ITS	CBS115777, PREM57819	*S. aromaticum*	ECY. Liew & MJ. Wingfield	Indonesia
		AY528967	BT	"	"	"	"
		AY528979	EF	"	"	"	"
C. polyconidia	CMW23809	EU245006	ITS	CBS122289	*A. mearnsii*	RN. Heath	South Africa
		EU244978	BT	"	"	"	"
		EU244938	EF	"	"	"	"
	CMW23818	EU245007	ITS	CBS122290	"	"	"
		EU244979	BT	"	"	"	"
		EU244939	EF	"	"	"	"
C. populicola	CMW14789	EF070418	ITS	CBS119.78	*Populus* sp	J. Gremmen	Poland
		EF070434	BT	"	"	"	"
		EF070406	EF	"	"	"	"
	CMW14819	EF070419	ITS	CBS114725	*Populus* sp.	T. Hinds	USA
		EF070435	BT	"	"	"	"
		EF070407	EF	"	"	"	"
C. savannae	CMW17300	EF408551	ITS	CBS121151	*Acacia nigrescens*	GNK. Kamgan & J. Roux	South Africa
		EF408565	BT	"	"	"	"
		EF408572	EF	"	"	"	"
	CMW17297	EF408552	ITS	CBS121021	*Combretum zeyheri*	GNK. Kamgan & J. Roux	South Africa
		EF408566	BT	"	"	"	"
		EF408573	EF	"	"	"	"
C. smalleyi	CMW14800	EF070420	ITS	CBS114724	*Carya cordiformis*	G. Smalley	USA
		EF070436	BT	"	"	"	"
		EF070408	EF	"	"	"	"
C. tanganyicensis	CMW15992	EU244999	ITS	CBS122293	*A. mearnsii*	RN. Heath & J. Roux	Tanzania
		EU244971	BT	"	"	"	"
		EU244931	EF	"	"	"	"
	CMW15999	EU244998	ITS	CBS122294	"	"	"

		EU244970	BT	“	“	“	“
		EU244939	EF	“	“	“	“
C. tribiliformis	CMW13015	AY529004	ITS	CBS115949	*Quercus* sp.	MJ. Wingfield	Indonesia
		AY528994	BT	“	“	“	“
		AY529015	EF	“	“	“	“
	CMW13013	AY529003	ITS	CBS115866	*Quercus* sp.	MJ. Wingfield	Indonesia
		AY528993	BT	“	“	“	“
		AY529014	EF	“	“	“	“
C. tsitsikammensis	CMW14276	EF408555	ITS	CBS121018	*Rapanea melanophloeos*	GNK. Kamgan & J. Roux	South Africa
		EF408569	BT	“	“	“	“
		EF408576	EF	“	“	“	“
	CMW14278	EF408556	ITS	CBS121019	*R. melanophloeos*	GNK. Kamgan & J. Roux	South Africa
		EF408570	BT	“	“	“	“
		EF408577	EF	“	“	“	“
C. tyalla	*CMW28932	HM071900	ITS	CBS128703	*E. dunnii*	GNK. Kamgan	Australia
		HM071913	BT	“	“	“	“
		HQ236452	EF	“	“	“	“
	*CMW28928	HM071898	ITS	CBS128342	*E. saligna*	“	“
		HM071912	BT	“	“	“	“
		HQ236451	EF	“	“	“	“
	*CMW28925	HM071897	ITS	CBS127211	*E. pilularis*	“	“
		HM071911	BT	“	“	“	“
		HQ236450	EF	“	“	“	“
	*CMW21598	HM071901	ITS	NA	*E. tereticornis*	“	“
		HM071908	BT	NA	“	“	“
		HQ236447	EF	NA	“	“	“
	*CMW28917	HM071899	ITS	NA	*E. grandis*	“	“
		HM071909	BT	NA	“	“	“
		HQ236448	EF	NA	“	“	“
	*CMW28920	HM071896	ITS	NA	*E. grandis*	“	“
		HM071910	BT	NA	“	“	“
		HQ236449	EF	NA	“	“	“
C. variospora	CMW20935	EF070421	ITS	CBS114715	*Quercus alba*	J. Johnson	USA
		EF070437	BT	“	“	“	“
		EF070409	EF	“	“	“	“
	CMW20936	EF070422	ITS	CBS114714	*Q. robur*	D. Houston	USA
		EF070438	BT	“	“	“	“
		EF070410	EF	“	“	“	“
C. virescens	CMW3276	DQ061281	ITS	NA	*Quercus* sp.	T. Hinds	USA
		AY528990	BT	“	“	“	“
		AY529011	EF	“	“	“	“

C. zombamontana	CMW15235	EU245002	ITS	CBS122297	*Eucalyptus* spp.	RN. Heath & J. Roux	Malawi
		EU244974	BT	"	"	"	"
		EU244934	EF	"	"	"	"
	CMW15236	EU245000	ITS	CBS122296	"	"	"
		EU244972	BT	"	"	"	"
		EU244932	EF	"	"	"	"

Table 3. Information on maximum parsimony trees for each gene region analyzed for *C. tyalla* and *C. corymbiicola*.

Tree Statistics	*C. tyalla*			*C. corymbiicola*				
	ITS	**BT1**	**TEF**	**ITS**	**BT1**	**TEF**	**RPB2**	**BT2**
Total number of characters	448	440	311	535	496	188	313	463
No. of variable characters	1	8	1	5	4	3	53	43
No. of informative characters	1	8	34	91	33	13	4	1
No. of most parsimonious trees	1	1	1	62	2	4	1	1
Tree length	1	8	35	114	40	17	57	45
Consistency index (CI)	1	1	1	0.921	0.950	0.941	1	1
Retention index (RI)	1	1	1	0.984	0.986	0.986	1	1

Table 4.1. Summary of polymorphic nucleotides found within the ITS and BT1 gene regions generated for phylogenetic analyses and showing differences between *C. tyalla* strains and closest related taxa.

Isolates designation	ITS			BT1							
	263	437	158	163	166	191	290	341	386	395	470
CMW9590-*C. moniliformis*	C	C	C	T	T	A	T	G	C	T	G
CMW8379-*C. moniliformis*	.	.	.	.	.	.	.	.	.	.	.
CMW21598-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW28917-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW28920-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW28925-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW28928-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW28932-*C. tyalla*	T	.	.	A	.	.	.	.	.	.	.
CMW13015-*C. tribiliformis*	.	T	G	.	G	C	C	A	T	C	A
CMW13013-*C. tribiliformis*	.	T	G	.	G	C	C	A	T	C	A

Table 4.2. Summary of polymorphic nucleotides found within the TEF gene region generated for phylogenetic analyses and showing differences between *C. tyalla* strains and closest related taxa.

Isolates designation	TEF																																
	6	7	25	29	31	35	37	42	48	71	103	120	126	133	165	168	191	197	209	216	228	229	238	241	245	248	257	266	277	278	289	291	295
CMW9590-*C. moniliformis*	T	G	A	G	T	A	C	T	-	G	A	-	T	A	T	T	A	C	A	T	G	A	T	G	C	T	C	A	G	G	G	A	G
CMW8379-*C. moniliformis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW21598-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	G	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW28917-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW28920-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW28925-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW28928-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW28932-*C. tyalla*	C	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.
CMW13015-*C. tribiliformis*	C	A	T	A	G	G	T	C	T	A	G	.	C	G	G	C	G	T	G	C	A	C	C	A	A	C	T	T	T	A	T	G	T
CMW13013-*C. tribiliformis*	C	A	T	A	G	G	T	C	T	A	G	.	C	G	G	C	G	T	G	C	A	C	C	A	A	C	T	T	T	A	T	G	T

Table 5. Number of fixed base pair across the ITS gene region showing differences between *C. tyalla* and closest related taxa.

Isolates designation	CMW9590	CMW8379	CMW21598	CMW28917	CMW28920	CMW28925	CMW28928	CMW28932	CMW13015	CMW13013
CMW9590-*C. moniliformis*	0									
CMW8379-*C. moniliformis*	0	0								
CMW21598-*C. tyalla*	1	1	0							
CMW28917-*C. tyalla*	1	1	0	0						
CMW28920-*C. tyalla*	1	1	0	0	0					
CMW28925-*C. tyalla*	1	1	0	0	0	0				
CMW28928-*C. tyalla*	1	1	0	0	0	0	0			
CMW28932-*C. tyalla*	1	1	0	0	0	0	0	0		
CMW13015-*C. tribiliformis*	1	1	1	1	1	1	1	1	0	
CMW13013-*C. tribiliformis*	1	1	1	1	1	1	1	1	0	0

Table 6. Number of fixed base pair across the BT1gene region showing differences between *C. tyalla* and closest related taxa.

Isolates designation	CMW9590	CMW8379	CMW21598	CMW28917	CMW28920	CMW28925	CMW28928	CMW28932	CMW13015	CMW13013
CMW9590-*C. moniliformis*	0									
CMW8379-*C. moniliformis*	0	0								
CMW21598-*C. tyalla*	1	1	0							
CMW28917-*C. tyalla*	1	1	0	0						
CMW28920-*C. tyalla*	1	1	0	0	0					
CMW28925-*C. tyalla*	1	1	0	0	0	0				
CMW28928-*C. tyalla*	1	1	0	0	0	0	0			
CMW28932-*C. tyalla*	1	1	0	0	0	0	0	0		
CMW13015-*C. tribiliformis*	8	8	9	9	9	9	9	9	0	
CMW13013-*C. tribiliformis*	8	8	9	9	9	9	9	9	0	0

Table 7. Number of fixed base pair across the TEF gene region showing differences between *C. tyalla* and closest related taxa.

Isolates designation	CMW9590	CMW8379	CMW21598	CMW28917	CMW28920	CMW28925	CMW28928	CMW28932	CMW13015	CMW13013
CMW9590-*C. moniliformis*	0									
CMW8379-*C. moniliformis*	0	0								
CMW21598-*C. tyalla*	7	7	0							
CMW28917-*C. tyalla*	6	6	1	0						
CMW28920-*C. tyalla*	6	6	1	0	0					
CMW28925-*C. tyalla*	6	6	1	0	0	0				
CMW28928-*C. tyalla*	6	6	1	0	0	0	0			
CMW28932-*C. tyalla*	6	6	1	0	0	0	0	0		
CMW13015-*C. tribiliformis*	32	32	27	26	26	26	26	26	0	
CMW13013-*C. tribiliformis*	32	32	27	26	26	26	26	26	0	0

Table 8.1. Summary of polymorphic nucleotides found within the ITS gene regions generated for phylogenetic analyses and showing differences between *C. corymbiicola* strains and closest related taxa.

Isolates designation	ITS																												
	13	17	19	31	61	62	69	88	97	113	120	182	185	186	202	203	261	388	389	390	391	392	393	394	395	418	419	443	463
CMW11424-*C. polychroma*	G	-	T	G	G	G	-	-	C	-	-	T	-	-	A	-	A	C	-	-	-	-	-	-	-	-	-	T	-
CMW11436-*C. polychroma*	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW19383-*C. atrox*	.	.	.	.	T	T	.	.	.	A	.	.	G	G	G	.	.	T	T	T	T	T	T	T	C	.	.	.	.
CMW19385-*C. atrox*	.	.	.	C	T	T	.	.	.	A	.	.	G	G	G	.	.	T	T	T	T	T	T	T	C	.	.	C	.
CMW29120-*C. corymbiicola*	A	.	C	.	.	.	.	G	T	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	C
CMW29275-*C. corymbiicola*	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	C
CMW29354-*C. corymbiicola*	A	A	C	.	.	.	.	.	T	.	C	.	.	.	.	A	.	T	T	.	.	.	.	.	C	C	T		C
CMW29349-*C. corymbiicola*	A	.	C	.	.	.	.	.	T	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	C
CMW29546-*C. corymbiicola*	A	A	C	.	.	.	.	.	T	.	C	.	.	.	.	A	.	T	T	.	.	.	.	.	C	C	T	.	C
CMW29549-*C. corymbiicola*	A	.	C	.	.	.	G	G	T	.	C	A	.	.	.	.	C	T	.	.	.	.	.	.	C	.	.	.	C

Table 8.2. Summary of polymorphic nucleotides found within the BT1 and TEF gene regions generated for phylogenetic analyses and showing differences between *C. corymbiicola* strains and closest related taxa.

Isolates designation	BT1								TEF				
	22	146	174	206	229	234	244	468	42	80	141	142	143
CMW11424-*C. polychroma*	T	A	C	G	T	T	C	C	A	C	T	T	T
CMW11436-*C. polychroma*	.	.	.	.	.	.	.	.	.	.	T	T	.
CMW19383-*C. atrox*	C	G	T	.	C	.	T	T	C	.	T	T	.
CMW19385-*C. atrox*	C	G	T	.	C	.	T	T	.	.	T	T	.
CMW29120-*C. corymbiicola*	.	.	.	.	C	G	T	.	.	T	T	.	.
CMW29275-*C. corymbiicola*	.	.	.	.	C	G	T	.	.	.	.	.	.
CMW29354-*C. corymbiicola*	.	.	.	.	C	G	T	.	.	.	.	.	.
CMW29349-*C. corymbiicola*	.	.	.	.	C	G	T	.	.	.	.	.	.
CMW29546-*C. corymbiicola*	.	.	.	.	C	G	T	.	.	.	.	.	.
CMW29549-*C. corymbiicola*	.	.	.	A	C	G	T	.	.	.	.	.	.

Table 9. Number of fixed base pair across the ITS gene region showing differences between *C. corymbiicola* and closest related taxa.

Isolates designation	CMW11424	CMW11436	CMW19383	CMW19385	CMW29120	CMW29275	CMW29354	CMW29349	CMW29546	CMW29549
CMW11424-*C. polychroma*	0									
CMW11436-*C. polychroma*	0	0								
CMW19383-*C. atrox*	4	4	0							
CMW19385-*C. atrox*	4	4	2	0						
CMW29120-*C. corymbiicola*	8	8	18	20	0					
CMW29275-*C. corymbiicola*	5	5	15	17	3	0				
CMW29354-*C. corymbiicola*	12	12	20	21	6	7	0			
CMW29349-*C. corymbiicola*	7	7	17	19	1	2	5	0		
CMW29546-*C. corymbiicola*	12	12	20	22	6	7	0	5	0	
CMW29549-*C. corymbiicola*	11	11	21	23	3	6	9	4	9	0

Table 10. Number of fixed bases pairs across the BT1gene region showing differences between *C. corymbiicola* and closest related taxa.

Isolates designation	CMW11424	CMW11436	CMW19383	CMW19385	CMW29120	CMW29275	CMW29354	CMW29349	CMW29546	CMW29549
CMW11424-*C. polychroma*	0									
CMW11436-*C. polychroma*	0	0								
CMW19383-*C. atrox*	6	6	0							
CMW19385-*C. atrox*	6	6	0	0						
CMW29120-*C. corymbiicola*	3	3	5	5	0					
CMW29275-*C. corymbiicola*	3	3	3	5	0	0				
CMW29354-*C. corymbiicola*	3	3	5	5	0	0	0			
CMW29349-*C. corymbiicola*	3	3	5	5	0	0	0	0		
CMW29546-*C. corymbiicola*	3	3	5	5	0	0	0	0	0	
CMW29549-*C. corymbiicola*	4	4	5	6	1	1	1	1	1	0

Table 11. Number of fixed base pair across the TEF gene region showing differences between *C. corymbiicola* and closest related taxa.

Isolates designation	CMW11424	CMW11436	CMW19383	CMW19385	CMW29120	CMW29275	CMW29354	CMW29349	CMW29546	CMW29549
CMW11424-*C. polychroma*	0									
CMW11436-*C. polychroma*	0	0								
CMW19383-*C. atrox*	3	3	0							
CMW19385-*C. atrox*	3	3	0	0						
CMW29120-*C. corymbiicola*	1	1	2	1	0					
CMW29275-*C. corymbiicola*	0	0	1	0	1	0				
CMW29354-*C. corymbiicola*	0	0	1	0	1	0	0			
CMW29349-*C. corymbiicola*	0	0	1	0	1	0	0	0		
CMW29546-*C. corymbiicola*	0	0	1	0	1	0	0	0	0	
CMW29549-*C. corymbiicola*	0	0	1	0	1	0	0	0	0	0

Table 12. Number of fixed base pair across the ITS gene region showing differences between species in *C. pirilliformis s.l.* clade of *Ceratocystis*.

Isolates designation	ITS														
	19	23	94	95	110	121	122	157	176	192	193	283	350	352	421
CMW15236-*C. zombamontana*	T	-	G	-	T	-	-	T	-	T	T	G	A	A	-
CMW15235-*C. zombamontana*	.	.	.	-	.	-	-	.	.	.	.	.	.	.	-
CMW6569-*C. pirilliformis*	C	.	.	G	.	T	T	-	.	-	-	.	.	.	T
CMW6579-*C. pirilliformis*	C	.	.	-	.	T	T	-	A	-	-	.	.	.	-
CMW29112	C	.	.	G	.	T	T	-	.	-	-	.	.	.	T
CMW29111	C	.	.	G	.	T	T	-	.	-	-	.	.	.	T
CMW29119	C	.	-	-	.	-	-	-	.	-	-	.	.	.	T
CMW29355	C	.	.	-	.	-	-	.	.	.	.	.	.	.	-
CMW23808-*C. obpyriformis*	C	A	.	G	G	T	T	-	.	-	-	A	.	.	-
CMW23807-*C. obpyriformis*	C	A	.	G	G	T	T	-	.	-	-	A	.	.	-
CMW23809-*C. polyconidia*	C	.	.	-	.	T	T	-	.	-	-	.	C	C	T
CMW23818-*C.polyconidia*	C	.	.	-	.	T	T	-	.	-	-	.	C	C	T

Table 13. Number of fixed base pair across the ITS gene region showing differences between species in *C. pirilliformis s.l.* clade of *Ceratocystis*.

Isolates designation	CMW15236	CMW15235	CMW6569	CMW6579	CMW29112	CMW29111	CMW29119	CMW29355	CMW23808	CMW23807	CMW23809	CMW23818
CMW15236-*C. zombamontana*	0											
CMW15235-*C. zombamontana*	0	0										
CMW6569-*C. pirilliformis*	8	8	0									
CMW6579-*C. pirilliformis*	7	7	3	0								
CMW29112	8	8	0	3	0							
CMW29111	8	8	0	3	0	0						
CMW29119	6	6	4	5	4	4	0					
CMW29355	1	1	6	5	6	6	4	0				
CMW23808-*C. obpyriformis*	10	10	4	5	4	4	8	8	0			
CMW23807-*C. obpyriformis*	10	10	4	5	4	4	8	8	0	0		
CMW23809-*C. polyconidia*	9	9	3	4	3	3	5	7	7	7	0	
CMW23818-*C.polyconidia*	9	9	3	4	3	3	5	7	7	7	0	0

Table 14. Number of haplotypes and their frequencies amongst species in *C. pirilliformis s.l.* clade of *Ceratocystis*.

Haplotype numbers	Frequencies	Isolate numbers	Haplotype designation
1	2	CMW6569	*C. pirilliformis*
1	2	CMW29111	Australia
2	1	CMW6579	*C. pirilliformis*
3	1	CMW29112	Australia
4	1	CMW29119	″
5	3	CMW29355	″
5	3	CMW15235	*C. zombamontana*
5	3	CMW15236	″
6	2	CMW23807	*C. obpyriformis*
6	2	CMW23808	″
7	2	CMW23809	*C. polyconidia*
7	2	CMW23818	″

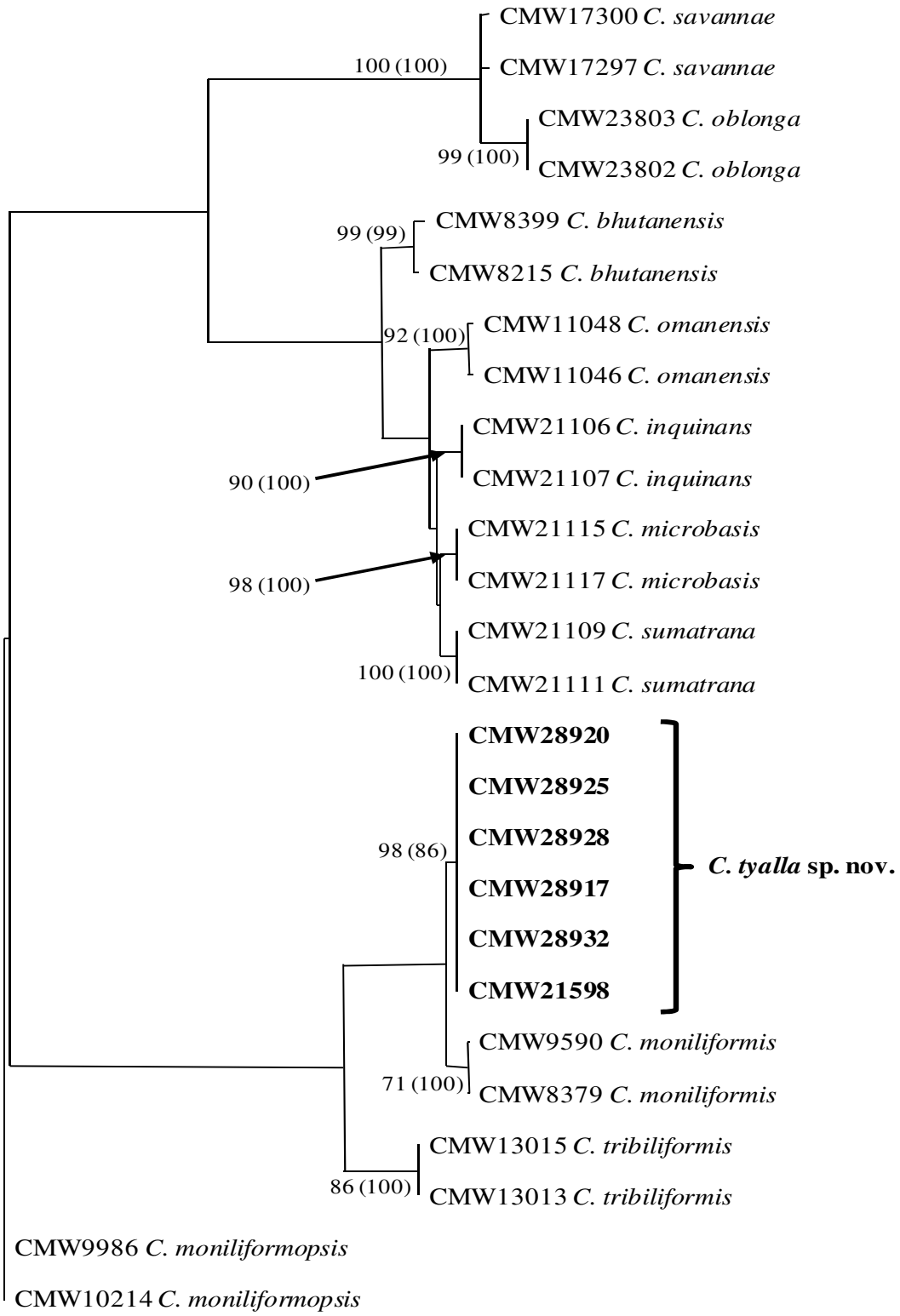

Figure 1: Phylogenetic tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between *C. tyalla* sp. nov. from *Eucalyptus* trees in Australia and other *Ceratocystis* spp. resembling *C. moniliformis*. *C. virescens* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with posterior probability values in brackets.

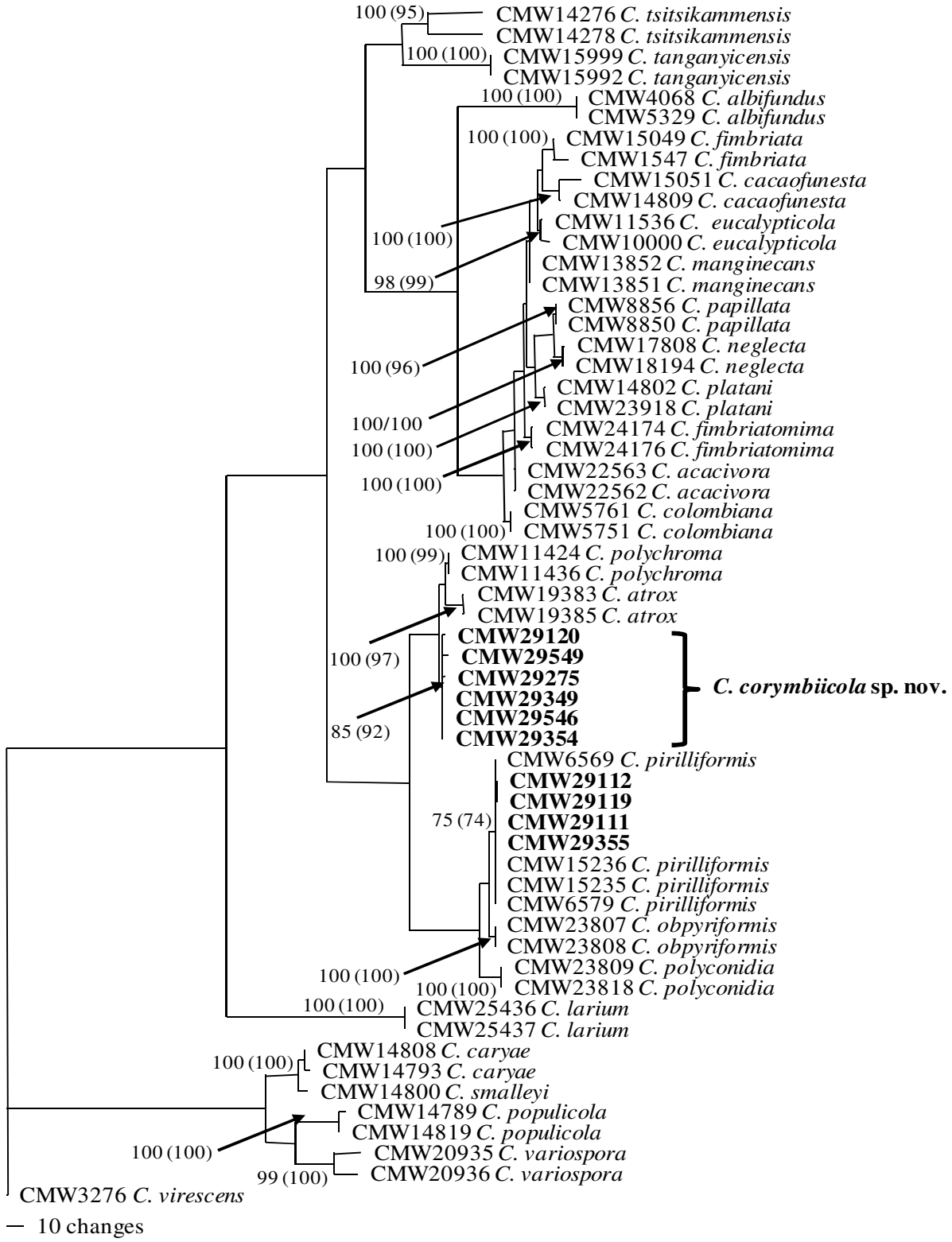

Figure 2: Phylogenetic tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between *C. corymbiicola* sp. nov. from eucalypt trees in Australia and other *Ceratocystis* spp. resembling *C. fimbriata*. *C. virescens* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with the posterior probability values in brackets.

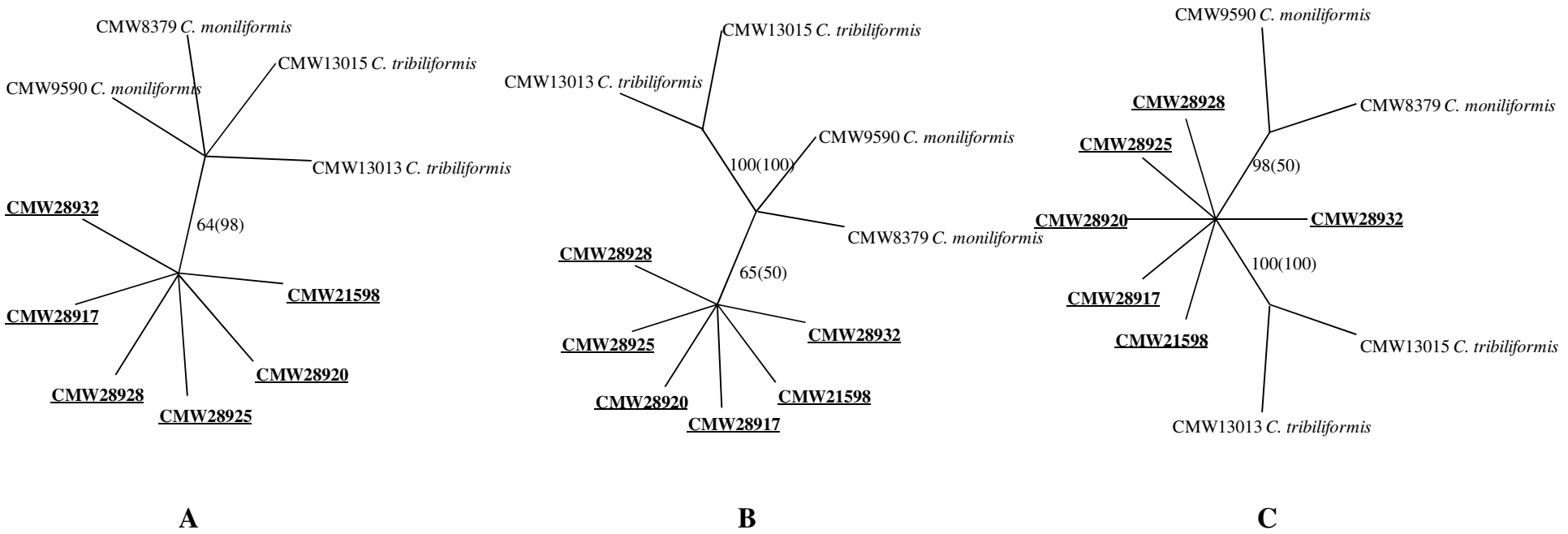

Figure 3: Unrooted maximum parsimony tree produced from a heuristic search of the ITS (A) BT (B) and TEF (C) sequence data respectively, showing the relationship between *C. tyalla* sp. nov. (isolates underlined) from *Eucalyptus* trees in Australia and its most closely related neighbor in the *C. moniliformis s.l.* species complex. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with Bayesian posterior probability values in brackets.

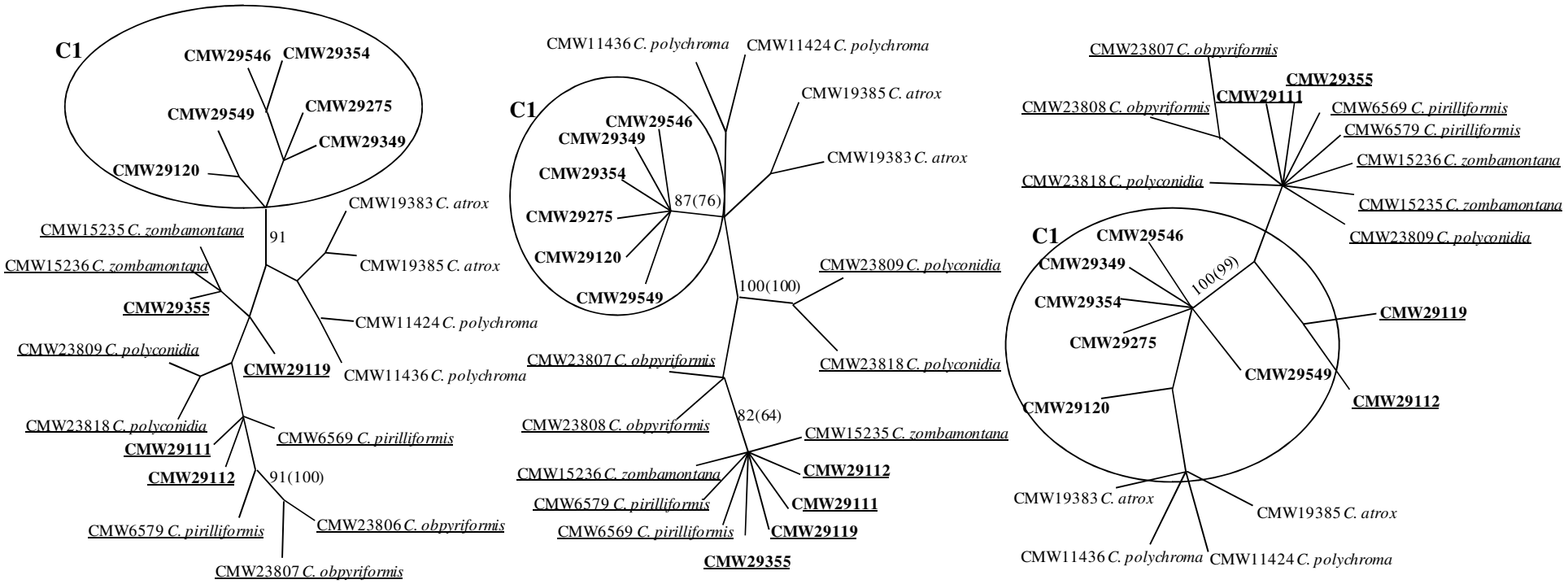

Figure 4: Unrooted maximum parsimony tree produced from a heuristic search of the ITS (Fig. A) BT (Fig. B) and TEF (Fig. C) sequence data respectively, showing the relationship between *C. corymbiicola* sp. nov. (C1) from *Eucalyptus* trees in Australia and its most closely related neighbor in the *C. fimbriata s.l.* species complex and the diversity within the *C. pirilliformis s.l.* species complex (isolates underlined). All isolates sequenced are in bold type. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with Bayesian posterior probability values in brackets.

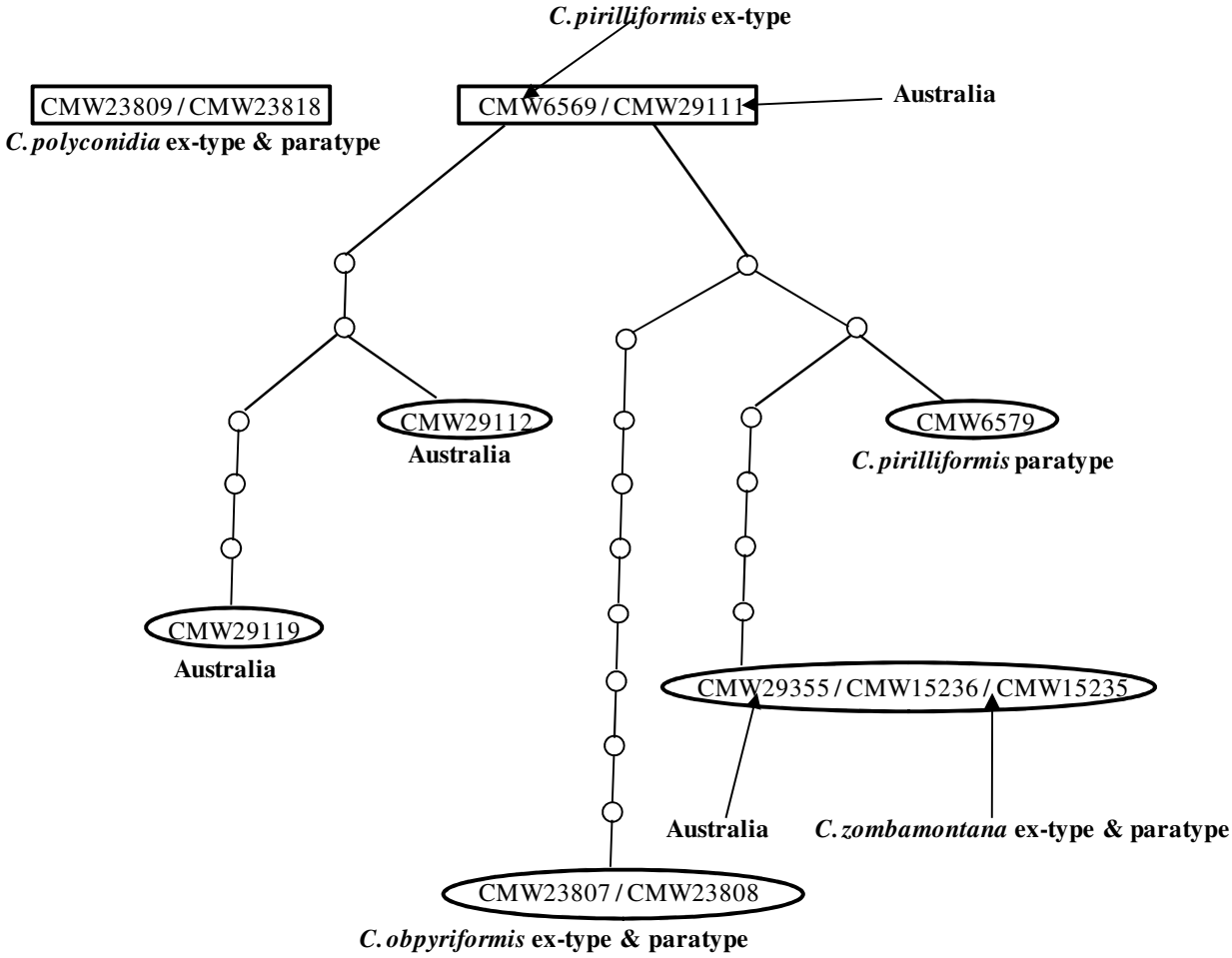

Figure 5: Allele networks produced from combined ITS, BT and TEF sequence data, showing the relationship between species in the *C. pirilliformis s.l.* clade comprising the type strains of *C. pirilliformis* (CMW6569, CMW6579), *C. zombamontana* (CMW15235, CMW15236), *C. polyconidia* (CMW23809, CMW23818), *C. obpyriformis* (CMW23807, CMW23808) and other isolates collected from Australia (CMW29111, CMW29112, CMW29119, CMW29355) and identified as *C. pirilliformis* in this study. *C. polyconidia* is represented as very different and separated from other species while *C. pirilliformis* is very variable and intermingled with *C. zombamontana*. *C. obpyriformis* grouped within the allele of *C. pirilliformis*, but formed a single haplotype different from this species.

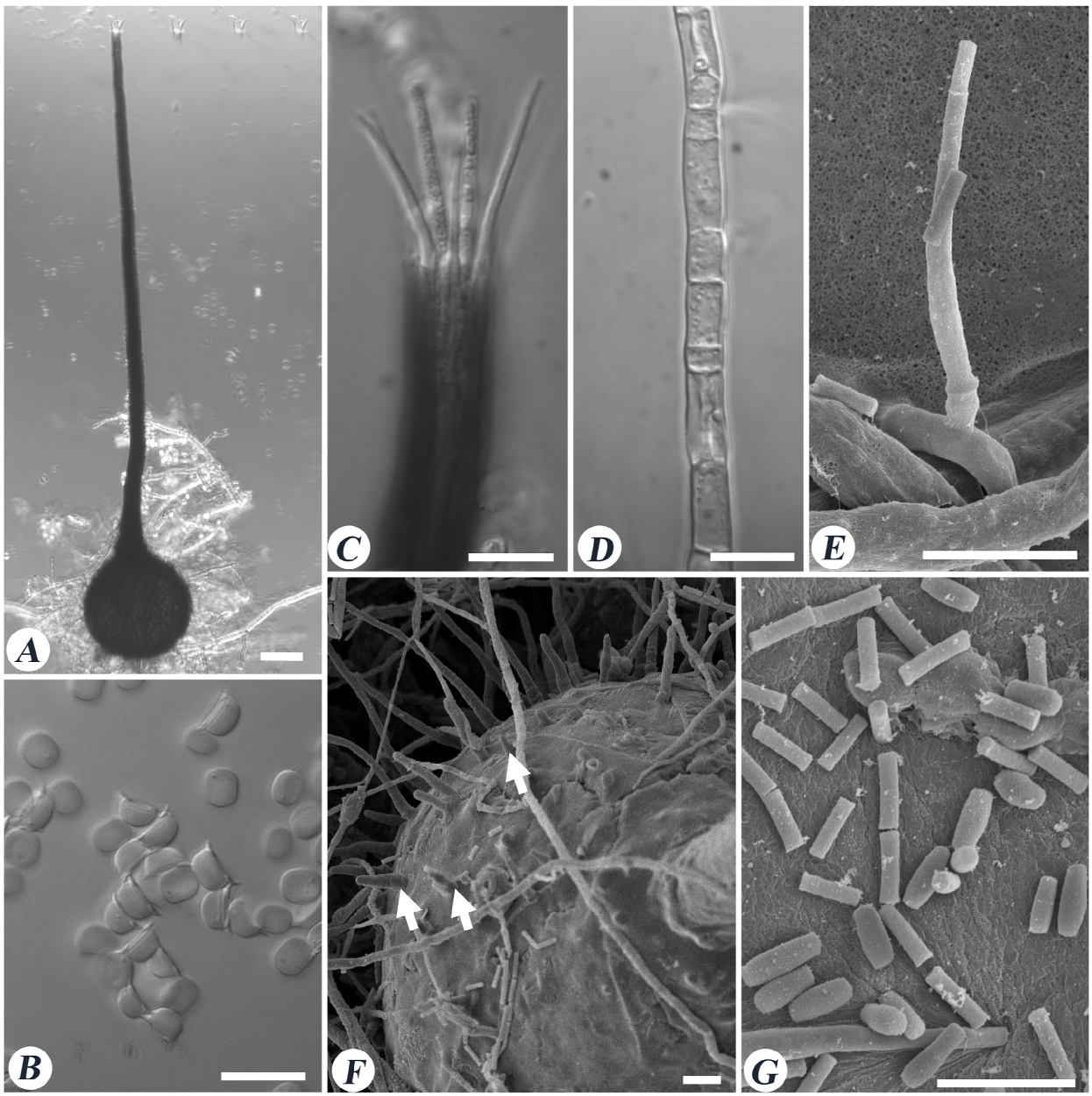

Figure 6: Morphological characteristics of *Ceratocystis tyalla* sp. nov. A) Globose ascomatal base (scale bar = 50 µm), B) Hat-shaped ascospores (scale bar = 10 µm) C) Divergent ostiolar hyphae (scale bar = 10 µm), D) Septate hyphae (scale bar = 10 µm) E) Phialidic conidiogenous cell with emerging conidia (scale bar = 10 µm), F) Ascomatal base with conical spines (scale bar = 10 µm), G) Oblong conidia with obtuse end and bacilliform shaped conidia (scale bar = 10 µm).

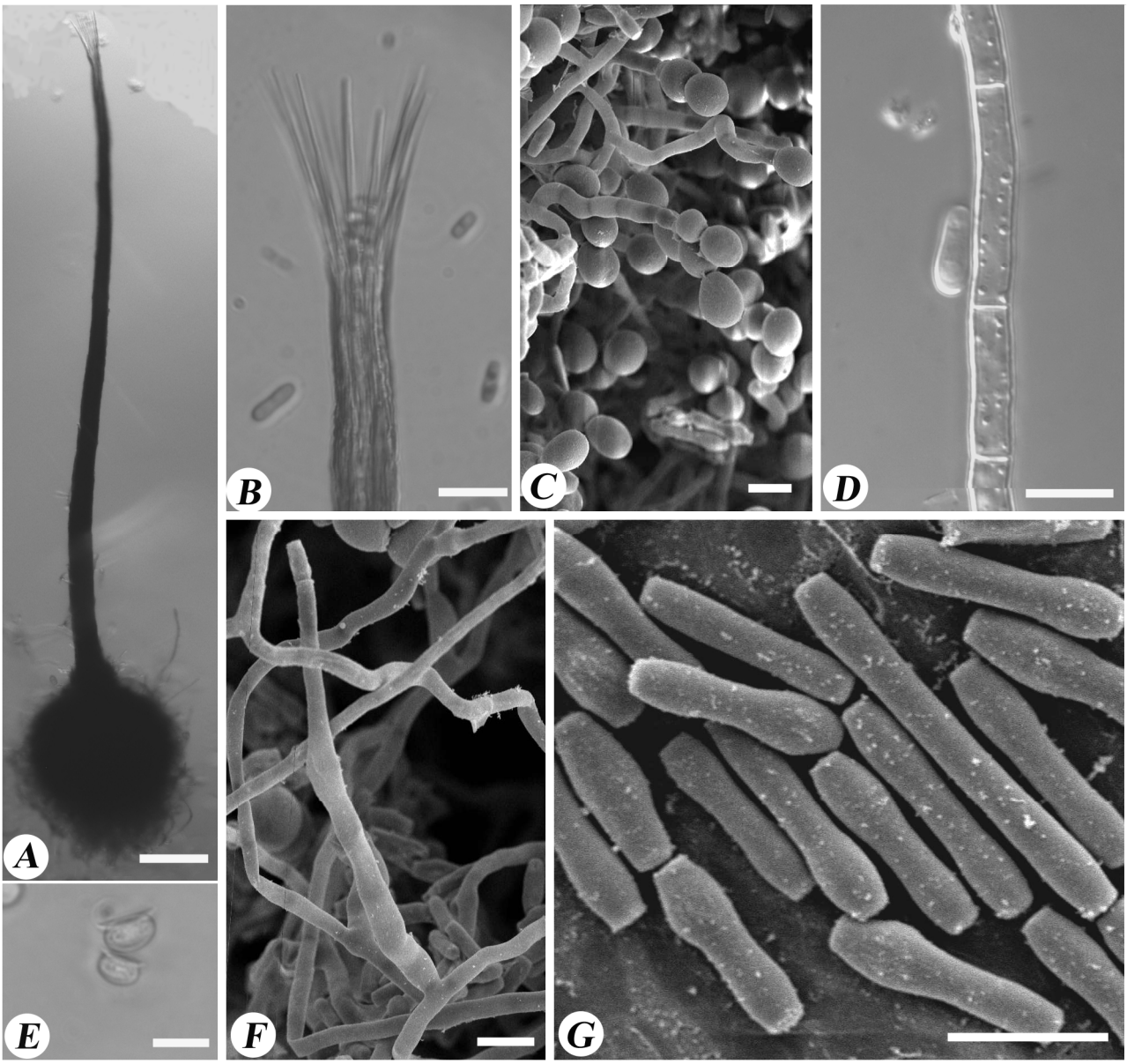

Figure 7: Morphological characteristics of *Ceratocystis corymbiicola* sp. nov. A) Globose ascomatal base (scale bar = 100 µm), B) Divergent ostiolar hyphae (scale bar = 20 µm), C) Ovoid chlamydospores (scale bar = 10 µm), D) Septate hyphae (scale bar = 10 µm), E), Hat-shaped ascospores in side view (scale bar = 10 µm), F) Phialidic conidiogenous cell with emerging bacilliform conidia (scale bar = 10 µm), G) Bacilliform shaped conidia with obtuse end and bacilliform shaped conidia with round bases (scale bar = 10 µm).

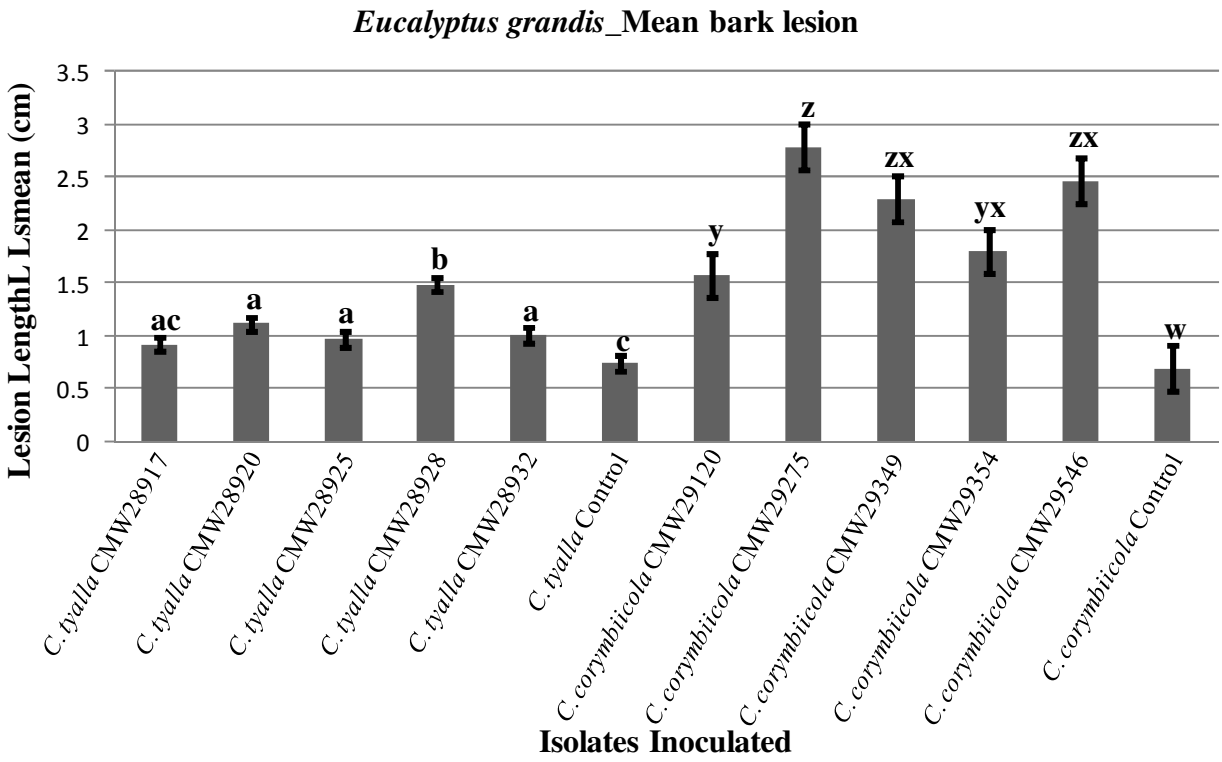

Figure 8: Vertical bar chart showing results of inoculation trial (bark lesion) with *C. tyalla* isolates (CMW28917, 28920, 28925, 28928, 28932) on *E. grandis* trees. Lsmean = 1.05, R = 0.54, CV = 21.2, P<0.0001, Significance level = 0.05. Average lesion lengths (0.93-1.49) cm; and with *C. corymbiicola* isolates (CMW29120, 29275, 29349, 29354, 29546) on *E. grandis* trees. Lsmean = 1.94, R = 0.54, CV = 34.45, P<0.0001, Significance level = 0.05. Average lesion lengths (1.58-2.79) cm. Lsmeans with similar letters are not statistically significant while those with different letters are statistically significant.

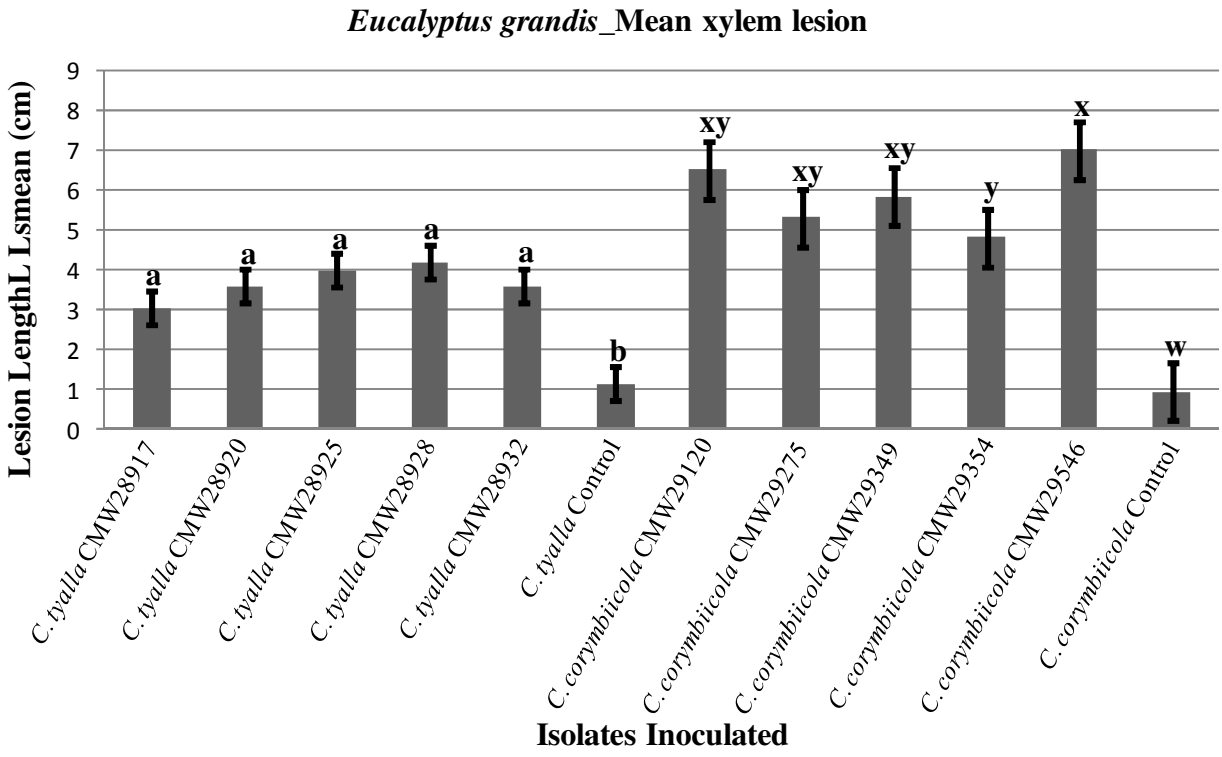

Figure 9: Vertical bar chart showing results of inoculation trial (xylem lesion) with *C. tyalla* (CMW28917, 28920, 28925, 28928, 28932) on *E. grandis* trees. Lsmean = 3.27, R = 0.39, CV = 40.97, P<0.0001, Significance level = 0.05. Average lesion lengths (3.05-4.2) cm; and with *C. corymbiicola* (CMW29120, 29275, 29349, 29354, 29546) on *E. grandis* trees. Lsmean = 5.06, R = 0.46, CV = 44.76, P<0.0001, Significance level = 0.05. Average lesion lengths (4.8-7) cm. Lsmeans with similar letters are not statistically significant while those with different letters are statistically significant.

CHAPTER III

Ophiostoma species (Ophiostomatales, Ascomycota), including two new taxa on eucalypts in Australia.

ABSTRACT

The genus *Ophiostoma* accommodates ascomycetes in the order Ophiostomatales, some of which are important pathogens of trees. Although these fungi are well-known in the northern Hemisphere, very little is known regarding their occurrence or importance in Australia. The aim of this study was to collect *Ophiostoma* spp. infecting wounds on *Eucalyptus* spp. in Australia, where most of these trees are native. Collections were made in three states of Australia and the isolates were identified using morphological and multigene sequence comparisons. Of the 76 isolates collected, two previously unknown species of *Ophiostoma* were found and these are described here as *O. tasmaniense* sp. nov. and *O. undulatum* sp. nov. In addition, *O. quercus* and *O. tsotsi* are reported for the first time from eucalypts in Australia and the distribution of *Pesotum australiae* is expanded to include eucalypts in Tasmania. In pathogenicity tests, very small lesions were observed in both the bark and xylem of *E. grandis* trees, suggesting that none of the collected species are pathogens of *Eucalyptus* spp.

3.1. INTRODUCTION

The Ophiostomatales are ascomycetes that include *Ophiostoma* with *Pesotum* J.L. Crane & Schokn. and *Sporothrix* Hektoen & C.F. Perkins anamorphs, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. with *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs and *Grosmannia* Goid. with *Leptographium* Lagerb. & Melin anamorphs (Upadhyay 1981, Zipfel *et al*. 2006). Together with *Ceratocystis* (Microascales) these genera are commonly referred to as Ophiostomatoid fungi and they produce fruiting structures with similar morphologies adapted to insect dispersal (Wingfield *et al*. 1993). Sexual fruiting structures of these fungi are mostly ascomata with long necks, exuding sticky spores at their apices that attach easily to the bodies of passing insects, which transport them to new substrates (Upadhyay 1981, Jacobs & Wingfield 2001, Wingfield *et al.* 1993). Asexual forms are erect conidiophores with sticky spores at their apices (*Hyalorhinocladiella*, *Pesotum* and *Leptographium*) or dry spores (*Sporothrix*) that can be wind dispersed (Crane & Schoknecht 1973, Malloch & Blackwell 1993, Jacobs & Wingfield 2001).

Ophiostoma H. & P. Sydow species with their *Pesotum* and *Sporothrix* anamorphs include important pathogens of trees such as *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, responsible for two Dutch elm disease pandemics that have devastated elm trees in Europe and the United States of America (Sinclair & Lyon 2005). Numerous other *Ophiostoma* spp. are agents of sapstain, especially in conifer logs and lumber causing discoloration and reducing its commercial value (Seifert 1993). A well known example in South Africa on hardwoods include *O. quercus* (Georgev.) Nannf. causing sapstain on *E. grandis* (Hill) Maiden trees in South Africa (De Beer *et al.* 2003).

Ophiostomatoid fungi require wounds to infect trees and they are commonly vectored by arthropods. Most *Ophiostoma* spp. depend on bark beetles or weevils for their dispersal and typically have a mutualistic relationship with these insects (Mathiesen-Käärik 1953, Whitney 1982, Paine *et al.* 1997, Kirisits 2004). In this regard, they also have a close association with phoretic mites associated with these insects (Bridges & Moser 1986, Levieux *et al*. 1989, Moser 1997, Malloch & Blackwell 1993). This association with timber-infesting insects makes the Ophiostomatoid fungi especially well adapted for accidental introduction into new environments.

The eucalypts include more than 700 tree species distributed in three genera, *Eucalyptus* L'Her., *Corymbia* K.D. Hill & L.A.S. Johnson and *Angophora* Cav. (Hill & Johnson 1995). Almost all eucalypts are endemic to Australia with only a few species that are restricted to some islands in

southern Asia (Potts & Pederick 2000). In their native range, *Eucalyptus* species constitute approximately 78% of forests while more than 85% of hardwood plantations are comprised of *Eucalyptus* spp. (Anonymous 2009). In the rest of the world, eucalypts have been planted for more than 200 years and presently numerous species are grown in plantations covering more than 20 million hectares and in more than 60 countries (Iglesias-Trabado & Wilstermann 2008). In this regard they represent a significant element of the economies of countries where they provide fuel, paper pulp and construction timber.

At least ten fungal species residing in the Ophiostomatales are known from trees in Australia, including the human pathogen *Sporothrix schenkii* Hektoen & C.F. Perkins (Conias & Wilson 1998), and two species isolated from moldy hay, *Ophiostoma stenoceras* (Robak) Nannf. and *O. nigrocarpum* (R.W.Davidson) de Hoog (O'Reilly & Altman 2006). Almost all the other species have been isolated from stained and/or bark beetle infested non-native *Pinus* spp. These included *Ophiostoma ips* (Rumb.) Nannf. (Stone & Simpson 1987, 1989, 1991), *Ceratocystiopsis minuta* (Siem.) Upadh. & Kendrick (Stone & Simpson 1989), *Grosmannia huntii* (R.C. Rob.-Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. (Jacobs *et al.* 1998), *O. quercus*, *O. floccosum* Mathiesen, *Pesotum fragrans* (Mathiesen) Okada & Seifert (Harrington *et al.* 2001), an unidentified species of *Leptographium* (Vaartaja 1967) and *Pesotum* (reported as *Graphilbum*) (Stone & Simpson 1991). To the best of our knowledge, the only member of the Ophiostomatales reported from a host tree native to Australia is *Pesotum australiae* Kamgan Nkuekam, Jacobs & Wingfield that occurs on wounds on native *Acacia mearnsii* de Wild (as *'P. australi'* Kamgan Nkuekam *et al.* 2008a). Based on the recent descriptions of numerous previously undescribed species of *Ophiostoma* from hardwoods elsewhere in the world, it is highly likely that numerous species await discovery in Australia and especially on eucalypts that dominate the natural landscape. Knowledge of these species on eucalypts in their native range would be valuable in the development of quarantine strategies intended to limit the movement of wood-inhabiting pathogens.

This investigation represents a collaborative effort between researchers from Universities and Forestry Departments in Australia and South Africa. The aim was to increase available knowledge pertaining to the Ophiostomatales occurring in Australia. Wounds on eucalypt trees in the eastern part of Australia were thus inspected for the presence of these fungi. All isolates were identified using a combination of morphological and DNA sequence data. Their potential pathogenicity to *E. grandis* was tested in greenhouse inoculation studies.

3.2. MATERIALS AND METHODS

3.2.1. Collection of samples

Surveys for Ophiostomatales infecting *Eucalyptus* and *Corymbia* spp. in Australia were conducted between March and April in 2008. Samples were collected from stumps as well as from wounds made by stem boring insects (e.g. *Phoracantha* spp.). Sampling was undertaken in the states of Tasmania, New South Wales and the southern part of Queensland. In Tasmania, areas sampled included numerous localities near Burnie, Tarraleah and Geevestown. In New South Wales (NSW), commercial plantations and native eucalypt forests between Sydney and up to the border with Queensland were visited. Some key areas sampled in NSW included Pine Creek State forest, Wattagan State forest, Wedding Bells State forest, Crab-Tree plantation, Ingalba State forest and other localities around these main areas. In the state of Queensland, samples were collected from *Eucalyptus* trees grown close to South East Queensland.

After determining the presence of *Ophiostoma* fruiting structures (mycelia, ascomata, synnemata) with a 20X magnification lens, pieces of wood were cut from trees and placed in separate brown paper bags for each tree sampled. All the samples in bags were placed in larger plastic bags to prevent desiccation and to promote sporulation and transported to the laboratory.

Isolations and purification of fungi from wood samples were done as described by Kamgan Nkuekam *et al.* (2008a, b). Ten additional isolates collected in Australia during previous surveys were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI, www.fabinet.up.ac.za) at the University of Pretoria. These isolates originated from felled *Eucalyptus grandis* logs infested by cerambycid beetles near Brisbane. All isolates used in this study were deposited in the culture collection (CMW) of the Tree Pathology Co-operative Programme (TPCP), South Africa, and representative cultures have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa. Representative isolates of species closely related to the Australian species isolated in the present study, and for which insufficient DNA sequence data were available from Genbank, were obtained for reference purposes from CMW (Table 1). These included those for *O. borealis* G. Kamgan Nkuekam, H. Solheim & Z.W. de Beer**,** *O. denticiliatum* Linnakoski, Z.W de Beer & M.J. Wingf., *O. karelicum* Linnakoski, Z.W. de Beer & M.J. Wingf. and *P. australiae*.

3.2.2. Culture characteristics and morphology

Isolates resembling fungi in the Ophiostomatales were grouped into morphotypes based on features of their cultures on Oatmeal Agar Media (OMA: 30 g Oats, 20 g Biolab agar and 1000 ml deionised water). Spore drops of conidia or ascospores from single apices of conidiophores or ascomata in pure cultures were transferred to OMA to promote sporulation for morphological comparisons. Cultures were incubated at 25ºC until sporulation and then grouped into morphotypes based on colour (Rayner 1970) and macro-morphology. Fruiting structures (ascomata and ascospores; synnemata and conidia) of selected isolates representing each of the morphotypes were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and ten measurements were made for additional isolates. The means were then calculated for relevant morphological structures. Measurements were noted as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum).

Scanning electron microscopy (SEM) was used to observe fruiting bodies (conidia, conidiophores) of the asexual states of the fungi. For this purpose, specimens were prepared as described by Grobbelaar *et al.* (2010). The specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater (Emitech K550X, Ashford, England) and examined using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

3.2.3. Growth in culture

Three isolates of each undescribed species (Table 1) identified in this study were used for growth studies in culture. This was done on MEA at seven different temperatures between 5-35ºC following the technique described by Kamgan Nkuekam *et al.* (2008a).

3.2.4. DNA sequence comparisons

Representative isolates of each morphological group of fungi were selected for DNA sequence comparisons. Spore drops from single apices of ascomata or conidiophores in pure cultures was collected and grown on 2% Malt Extract Agar (MEA) for 7-10 days. Mycelium was scraped from the surfaces of the actively growing cultures and then transferred to 1.5 ml Eppendorf tubes using a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified on an Eppendorf Mastercycler (Merck, Germany) using primers ITS1 and ITS4 (White *et al*. 1990). Part of the β-tubulin gene (BT) and the transcription elongation factor-1α gene (TEF) were also amplified using the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995), and EF1F and EF2R (Jacobs *et al*. 2004) respectively.

The PCR reaction mixtures and thermal cycling conditions were the same as those described previously (Kamgan Nkuekam *et al*. 2008a, b). A 5 µl aliquot of the PCR products were stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA), separated on a 1% agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). Sequencing reactions were performed using the Big Dye cycle sequencing kit with Amplitaq DNA polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturer's protocols on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing PCR's were prepared as described by Kamgan Nkuekam *et al*. (2008b) and both DNA strands were sequenced for each gene region. Sequences for both strands of each isolate were examined visually and consensus sequences assembled using Sequence Navigator (Applied Biosystems Division, Perkin Elmer, Foster City, CA, USA.).

A preliminary identity for the isolates was obtained by performing a standard nucleotide BLAST against the GenBank database (http://www.ncbi.nlm.nih.gov). Data sets comprising sequences obtained in the present study, as well as reference sequences of related species obtained from GenBank, were compiled in MEGA 4.0.2 (Tamura *et al.* 2007). Sequences were then aligned automatically using the online version of MAFFT 6 (Katoh *et al.* 2002). Data for the three gene regions sequenced were analyzed separately from each other, and three types of analyses were done for each data set. Firstly PAUP 4.0b10 (Sinauer Associates, Sunderland, Massachusetts) was used to construct phylogenetic trees from the data matrices using heuristic search option of the maximum parsimony (MP) method. Confidence levels of the phylogenies were estimated with 1000 bootstrap replicates. Additionally, Bayesian analyses were performed with MrBayes 3.1 (Ronquist & Heuelsenbeck 2003), based on Markov Chain Monte Carlo (MCMC) methods. Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (http://www.abc.se/~nylander/). The models applied to the ITS, BT and TEF data sets were (GTR+I+G), (SYM+G) and (GTR+G) respectively. Four simultaneous MCMCs were run for 1 000 000 generations and trees were sampled every 100th generations. The burn-in procedure in MrBayes

3.1 was used to discard the first trees that formed before the point of convergence, and 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. Lastly, maximum likelihood (ML) analyses were conducted online using PhyML 3.0 (Guindon & Gascuel 2003). The AIC was used in Modeltest 3.7 (Posada & Crandall 1998) to select appropriate substitution models for the three data sets. For ITS, the selected model was GTR+I+G (gamma Shape parameter=0.6097; Pinvar=0.6101), for the BT it was TVM+G (gamma Shape parameter=0.1904, Pinvar=0) and GTR+G (gamma Shape parameter=1.0065, Pinvar=0) for the TEF data set.

3.2.5. Pathogenicity tests

Pathogenicity tests were conducted in a quarantine greenhouse using *Eucalyptus grandis* clone TAG5. This clone was selected as it has been shown to be susceptible to a range of fungal pathogens in previous studies. Two strains each (Table 1), for four of the fungal species identified in this study were used in the inoculation experiment. Ten trees, approximately two-years-old (~1 cm diameter), grown in plastic bags, were inoculated with each test strain and two other trees of the same age were inoculated with a sterile agar disc to serve as controls. Inoculations were done at an average temperature of 25ºC and natural day/night lighting conditions of about 13 hours daylight and 11 hours darkness, using the same technique as described before by Kamgan Nkuekam *et al.* (2008b). Six weeks (42 days) after inoculation, the lengths of lesions on the bark surface and in the xylem of each tree were measured. Re-isolations were made from the lesions to meet the requirements of Koch's postulates. All data were processed using the statistical software package SAS® V8 running under VM/CMS on the main frame computer at the University of Pretoria, where lesion lengths were analyzed using GENMOD with a POISSON distribution and a link function of log.

3.2.6. Mating compatibility

To obtain sexual states for isolates collected in this study that did not produce teleomorphs on wood or in culture (Table 1), isolates were paired in all possible combinations on water agar (20 gl^{-1} agar, Biolab, Midrand, South Africa and 1000 ml sterile deionised water) supplemented with sterile *Eucalyptus* chips (Kamgan Nkuekam *et al.* 2008a, Grobbelaar *et al.* 2010a). Petri dishes were incubated at 25ºC for one month and monitored weekly for the presence of ascomata producing ascospores.

In a separate experiment, strains of *Pesotum australiae* (CMW6606, CMW6589, CMW6588, CMW6590) collected from wounds of *A. mearnsii* trees, and identified in a previous study

(Kamgan Nkuekam *et al.* 2008a), were crossed in the same manner with a strain (CMW29101) collected from a wound on a *Eucalyptus* tree in this study. These latter pairings were done in an attempt to stimulate production of ascomata for *P. australiae*, which have not previously been seen for this species.

3.3. RESULTS

3.3.1. Collection of samples

A total of 66 isolates resembling species in the Ophiostomatales were collected from 200 eucalypt trees sampled during the surveys in New South Wales, Queensland and Tasmania. Samples from which fungi were isolated included wounds on two eucalypt genera, *Eucalyptus* and *Corymbia* and including eight different species. These tree species included *E. nitens* Deane & Maiden, *E. globulus* Labill, *E. pilularis* Sm., *E. saligna* Sm., *E. agglomerate* Maiden, *E. dunnii* Maiden, *E. grandis* and *C. variegata* (F.Muell.) K.D.Hill & L.A.S. Johnson. Apart from some discoloration of the wood around the wounds, there were no other symptoms suggestive of a disease problem.

3.3.2. Culture characteristics and morphology

Isolates could be assigned to two morphological groups based on colony morphology and the type of fruiting bodies produced on either MEA or OMA. Isolates collected in 2008 all shared similar culture morphology (Table 1, Group A), with no sexual states observed and resembling species of *Pesotum*. Colonies of these isolates sporulated best on OMA. They produced only synnemata, with creamy to white spore drops. No ascomata were produced in culture. Isolates collected in Queensland, near Brisbane could be grouped in a second morphological group (Table 1, Group B), which was characterized by slower growth than those in group A. Ascomata with long necks, exuding creamy ascospore drops, were often produced in culture. These isolates produced white mycelial mats with undulating colony surfaces and *Sporothrix* anamorphs in culture.

3.3.3. DNA sequence comparisons

Representative isolates from different hosts and geographic locations were selected for DNA sequencing (Table 1). All of these isolates generated fragments of approximately 600 bps for the ITS, 400 bps for the BT and 800 bps for the TEF gene regions. Blast searches in the GenBank data base showed that all isolates belonged in the genus *Ophiostoma* and its related anamorph genus, *Pesotum*. Alignments of ITS sequence data for isolates from Australia with those of related *Ophiostoma* spp. from GenBank, resulted in a total of 589 characters including gaps, with 489 constant characters, 20 parsimony uninformative characters and 80 parsimony informative

characters. The aligned BT data set consisted of a total of 241 characters including gaps, with 146 constant characters, nine parsimony uninformative characters and 86 parsimony informative characters. The TEF data set consisted of a total of 552 characters including gaps, with 92 constant characters, 21 parsimony uninformative characters and 439 parsimony informative characters. For reference species, TEF sequences were not available in GenBank and they were consequently generated in this study (Table 1).

Phylogenetic analyses using maximum parsimony (MP) generated 144 trees for the ITS, 273 trees for the BT, and 1117 trees for the TEF data sets respectively. The Consistency Index (CI) values for the three data sets were 0.778; 0.634 and 0.679 respectively, while the Retention Index (RI) values were 0.918; 0.914 and 0.925 respectively. For each data set, a 50% majority rule tree obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as from maximum likelihood analyses (ML). For each data set, a ML tree is presented (Figures 1, 2, 3) indicating at the relevant nodes, MP bootstrap values, Bayesian posterior probabilities, and ML bootstrap values respectively.

Isolates representing both morphological groups could not be resolved fully in the phylogenetic analyses using the ITS gene region (Figure 1) and were scattered within the tree and intermingled with species such as *O. quercus*, *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf., *O. denticiliatum* and *P. australiae*. However, within this complex, isolates representing Group B formed a distinct clade, which was most closely related to *O. quercus*, with 100% support in all three analyses.

In the BT tree (Figure 2), Group A isolates separated into four lineages. The first set of isolates grouped with strains of *O. quercus* (A1). The second set of isolates formed a well supported clade (A2), distinct from, but most closely related to *O. quercus*. A single isolate grouped with the two *Pesotum australiae* isolates (A3), while the fourth set of isolates grouped in a well-supported lineage with strains of *O. tsotsi* (A4). As was the case for the ITS region, isolates in group B formed a well supported, distinct clade, but this time most closely related to *P. australiae*.

Analyses of the TEF data set gave results similar to those obtained with the BT data set (Figure 3). These data confirmed with good support the distinction of especially clades A2 and B2 from respectively *O. tsotsi* and *P. australiae*. Furthermore, TEF data of *O. denticiliatum*, *P. australiae*, *O. karelicum* and *O. borealis* isolates separate all four these species into lineages which are distinct from other species in the complex.

3.3.4. Mating compatibility

The *Pesotum* isolates (Table 1, Group A2) that did not produce ascomata in culture and that were paired in all possible combinations, produced sexual fruiting bodies typical of the genus *Ophiostoma* on *Eucalyptus* chips. The crosses between the reference *P. australiae* isolates (Table 1) from a previous study, and the single isolate (CMW29101) identified as *P. australiae* based on DNA sequences from the present study, did not produce ascomata.

3.3.5. Taxonomy

Based on morphological examinations and multigene sequence phylogenies, two *Ophiostoma* spp. from Australia emerged as distinct and previously undescribed species. The following descriptions are provided for them.

Ophiostoma undulatum Kamgan-Nkuek., M.J. Wingf. and Jol. Roux sp. nov. (Fig. 4). **MB518956**

***Etymology*:** The name refers to the undulating mycelium of colonies on growth media.

Coloniae albae vel bubalinae in MEA in 10 diebus in 25°C ad 16 mm crescentes. Mycelium tegetes crassas undulantes circulares concentricas in superficie agaris formans. Bases ascomatum nigrae, globosae sine decoribus. Colla ascomatum nigra sine hyphis ostiolaribus. Ascosporae reniformes non septatae, hyalinae (5-) 5.5-6.0 (-6.5) x (1.5-) 1.5-2.0 (-2) µm. Anamorpha *Sporothrix* conidiophoris hyalinis apicem versus ramosis. Conidia non septata, hyalina oblonga vel cylindrica (5-) 5.5-7.0 (-8) x (2-) 2.5-3.0 (-3.5) µm.

Colonies white to Buff (19''f) on MEA. Reverse Hazel (17'''b). Colony diameters reaching 16 mm in 10 days on MEA at 25°C. Optimal growth at 25°C. No growth at 5°C and at 35°C. Mycelia forming thick, undulating, circular and concentric mats on agar surface. *Ascomata* produced by only a few strains, scattered over the colony surface and embedded within mycelium, producing white, creamy spore drops at the neck apices. *Ascomatal* necks black (68.5-) 181.5-348.0 (-378.0) µm long. Ascomatal bases black, globose (34.5-) 66.5-135.0 (-193.5) µm long and (35.0-) 69.0-140.5 (-206.0) µm wide, without ornamentations. Neck bases smooth. *Ostiolar hyphae* absent. *Asci* rarely seen, evanescent, deliquescing early in the development. *Ascospores* reniform, aseptate, hyaline (5.0-) 5.5-6.0 (-6.5) x (1.5-) 1.5-2.0 (-2.0) µm.

Anamorph: *Sporothrix,* conidiophores, hyaline, branched towards the apex, (9.5-) 16.0-27.0 (-34.0) x (1.5-) 2.0-2.5 (-3.0) µm. Conidia, aseptate, hyaline, oblong to cylindrical (5.0-) 5.5-7.0 (-8) x (2.0-) 2.5-3.0 (-3.5) µm.

Specimens examined: Australia, state of Queensland, isolated from wet *E. grandis* stems infested by *Phoracantha* beetles, August 2005, Michael J. Wingfield, holotype PREM60443, living culture CMW19396 = CBS127183.

Additional specimens: Australia, state of Queensland, isolated from *E. grandis* stems infested by *Phoracantha* beetles, August 2005, Michael J. Wingfield, paratype, living culture CMW19394 = PREM60438 = CBS127182, CMW19397 = PREM60437 = CBS127184.

Ophiostoma tasmaniense Kamgan-Nkuek., Jol. Roux and Z. W. de Beer sp. nov. (Fig. 5). **MB518955**

***Etymology*:** The name refers to the state of Tasmania, Australia, where the fungus was first collected.

Coloniae in OMA fumeae, in MEA bubalinae in 10 diebus in 25°C ad 24 mm crescentes. Bases ascomatum nigrae, globosae cum pilis ostiolaribus. Colla ascomatum nigra cum hyphis ostiolaribus divergentibus. Ascosporae allantoideae non septatae (4.5-) 5.0-5.5 (-6.0) µm longae (1.0-) 1.0-1.5 (-2.0) µm latae. Anamorpha biformus. Gradus *Pesotum* conidiophoris erectis atrobrunneis apicem versus pallescentibus. Cellulae conidiogenae hyalinae acerosae. Conidia non septata, hyalina (3.5-) 4.0-4.5 (-5.0) µm x (1.0-) 1.0-1.5 (-2.0) µm. Gradi *Sporothricis* conidiophorae hyalinae cylindricae cum denticulis prominentibus. Conidia saepe septata, hyalina oblonga vel cylindrica interdum distincte pediformia (8.5-) 11.0-19.5 (-30.5) x (2.0-) 2.5-4.0 (-5.0) µm.

Colonies Smoke grey (21’’’’d) on OMA with conidiophores forming light-colored slimy heads, arranged in mostly circular rings or scattered over the colonies. On MEA *colonies* Buff (19’’d) with conidiophores forming cream-colored slimy heads scattered over the colonies, mostly surrounded by a mat of mycelia, reverse *colonies* Buff (19’’d) becoming Honey (19’’b) toward the middle of the plates. Colony diameters reaching 24 mm in 10 days on MEA at 25ºC. Optimal growth temperature 25ºC, no growth at 5ºC or at 35ºC.

Teleomorph state produced on *Eucalyptus* chips after random crossing between strains after about one month. *Ascomatal* bases black, globose (66.5-) 95.5-144.0 (-171.5) µm long and (71.0-) 98.5-159.0 (-197.5) µm wide, with hyphal hairs. *Ascomatal* necks black (101.0-) 499.5-1105.5 (-1274.0) µm long, neck bases smooth (29.0-) 34.5-46.0 (-53.0) µm wide, middle of necks (17.5-) 19.5-25.5 (-29.5) µm wide, tips of necks (10.5-) 11.5-17.5 (-22.0) µm wide. *Ostiolar hyphae* present, divergent (11.5-) 15.5-20.5 (-21.5) µm long. *Asci* evanescent. *Ascospores* alantoid in side

view, aseptate (4.5-) 5.0-5.5 (-6.0) µm long and (1.0-) 1.0-1.5 (-2.0) µm wide. Ascospores accumulating in round, creamy spore drops.

Anamorph: *Pesotum*, *conidiophores* synnematal, erect, dark brown at the bases, becoming lighter towards the apex, (472.0-) 553.5-708.5 (-774.5) µm long, (46.5-) 67.5-110.5 (-170.0) µm wide in the middle, (46.0-) 54.0-90.0 (-129.5) µm wide at the base. *Conidiophore heads* (160.0-) 198.0-328.0 (-452.0) µm across the widest part, light brown becoming hyaline towards the apex. *Conidiogenous cells*, hyaline, acerose (11.5-) 14.5-28.5 (-41.5) µm long, (1.0-) 1.0-1.5 (-2.0) µm wide, tapering towards the apex. *Conidia* produced through holoblastic, annellidic development. Conidia aseptate, hyaline, oblong to obovoid, accumulating in slimy heads on the apices of the synnemata, (3.5-) 4.0-4.5 (-5.0) µm x (1.0-) 1.0-1.5 (-2.0) µm.

Anamorph: *Sporothrix,* conidiophores, hyaline, cylindrical and branched, tapering towards the apex, (10.5-) 27.0-61.5 (-108.5) x (1.5-) 2.0-2.5 (-3.0) µm, prominent denticles present. *Conidia*, often septate, hyaline, oblong to cylindrical (8.5-) 11.0-19.5 (-30.5) x (2.0-) 2.5-4.0 (-5.0) µm in size.

Specimens examined: Australia, state of Tasmania, Tarraleah State forest, isolated from cut stumps of *Eucalyptus nitens*, 7 March 2008, G. Kamgan Nkuekam, holotype PREM60439, living culture CMW29088 = CBS127212.

Additional specimens: Australia, state of Tasmania, Burnie, isolated from *E. globulus* stumps. 4 March 2008, G. Kamgan Nkuekam, paratype, living cultures CMW29115 = PREM60440 = CBS127213, CMW29116 = PREM60444 = CBS127214.

3.3.6. Distribution and host range of *Ophiostoma* spp.

Five *Ophiostoma* species were identified from areas sampled in three different states of Australia. In NSW, two *Ophiostoma* spp. were identified. These comprised *O. quercus*, collected from four eucalypt tree species (*E. grandis*, *E. dunnii*, *E. agglomerate* and *C. variegata*) and *O. tsotsi* that was found on two *Eucalyptus* spp. (*E. pilularis* and *E. grandis*). In Tasmania, three *Ophiostoma* spp. were collected, including *O. quercus* from two *Eucalyptus* spp. (*E. nitens* and *E. globulus*), *O. tasmaniense* collected from three *Eucalyptus* spp. (*E. globulus*, *E. nitens* and *E. saligna*), and *P. australiae* collected from *E. nitens*. In Queensland, only *O. undulatum* was found on *E. grandis* trees.

3.3.7. Pathogenicity tests

Six weeks after inoculation, *Eucalyptus* trees were assessed for lesion development seen on the bark or at the cambial surface. All four fungal species used for inoculation, *O. quercus*, *O. tsotsi*, *O. tasmaniense* sp. nov. and *O. undulatum sp. nov*., produced very small lesions on both the bark and the xylem of inoculated *E. grandis* trees. Because no lesions could be measured for many inoculations, lesion lengths were analyzed using GENMOD with a POISSON distribution and a link function of log. Significant differences ($P<0.0001$) in lesion lengths on either the bark or the xylem were, however, not found between the four fungal species and their replicates when compared with each other (Figures 6, 7). At the time when lesion lengths were recorded, trees were healthy with no signs of disease. All species other than *O. undulatum* were successfully isolated from the lesions. Control inoculations were covered with callus when the experiment was terminated and none of the test fungi were isolated from them.

3.4. DISCUSSION

In this study, five *Ophiostoma* spp. were identified from a survey of wounds on eucalypt trees in Australia. Two of these fungi were previously undescribed species for which the names *O. undulatum* and *O. tasmaniense* have been provided. *Ophiostoma tsotsi* and *O. quercus* are reported for the first time from *Eucalyptus* trees in Australia, and the host and geographic ranges of *P. australiae* are expanded to include *Eucalyptus nitens* in Tasmania. The four species (*O. quercus*, *O. tsotsi*, *O. tasmaniense* sp. nov. and *O. undulatum sp. nov*.) inoculated onto *E. grandis* trees caused very small lesions, suggesting that they are not pathogens.

All five species identified in this study fall within a group of fungi commonly known as the *O. piceae* complex. This complex was first recognized to include nine morphologically similar species (Harrington *et al*. 2001), and has been the subject of considerable taxonomic confusion (Przybyl & de Hoog 1989, Harrington *et al*. 2001). *Ophiostoma tasmaniense*, one of the new taxa described in this study, along with *O. tsotsi*, *O. quercus* and *P. australiae*, could not be differentiated in phylogenetic analyses using only the ITS gene regions. This is not surprising as ITS gene regions have previously been shown to be insufficient to differentiate closely related members of the *O. piceae* complex (Chung *et al.* 2006, Kamgan Nkuekam *et al.* 2008a, 2010) thus sequences for the BT gene region (Chung *et al.* 2006, Kamgan Nkuekam *et al.* 2008a, 2010) and more recently the TEF gene region (Grobbelaar *et al.* 2009) have been useful in distinguishing these species. Based on BT and TEF sequence data, *O. tasmaniense* described in this study is most closely related to *O.*

quercus. This expands the host and geographic ranges of members of the *O. piceae* complex in the Southern Hemisphere.

It was not surprising to find *P. australiae* infecting wounds of *Eucalyptus* trees in Australia. This fungus was recently described from wounds on native *Acacia mearnsii* trees near Cann River (Victoria), Australia (Kamgan Nkuekam *et al.* 2008a). Its teleomorph has not been seen and it could not be induced in this study by crossing it with different strains isolated from *A. mearnsii* and *Eucalyptus* trees. Isolation of this fungus from Tasmania, and from *Eucalyptus* spp., expands its host and geographic range. It provides further evidence that it is native to the continent, as surveys for Ophiostomatoid fungi on both *A. mearnsii* and *Eucalyptus* spp., in for example Africa, have not yielded this fungus.

Ophiostoma tsotsi, another species collected in this study, was for many years confused with *O. quercus*. Recent studies have, however, considered cryptic species in the *O. quercus* complex, using polymorphic sequence repeats (Grobbelaar *et al.* 2008) and multigene DNA sequence phylogenies (Grobbelaar *et al.* 2009) of isolates morphologically resembling *O. quercus*, originating from various hardwood trees in Africa. These data revealed a unique clade amongst these isolates, resulting in the description of *O. tsotsi* (Grobbelaar *et al.* 2010a). *Ophiostoma tsotsi* is known only from hardwood tree species, including *Eucalyptus* and *Acacia mearnsii* in southern Africa and China (Grobbelaar *et al.* 2010a, b). Its discovery on *Eucalyptus* trees in Australia expands its geographic range. The discovery also supports the view (Grobbelaar *et al.* 2010a, b) that its distribution is probably more extensive than is currently known. The origin of the species remains unknown and more extensive sampling and population genetic tools will be necessary to determine this.

Ophiostoma quercus has previously been reported from *P. radiata* in Australia (Harrington *et al.* 2001) and is known to occur on *Eucalyptus* spp. in Africa (De Beer *et al.* 2003). However, this study is the first to report the fungus on eucalypt species in Australia. *Ophiostoma quercus* has a cosmopolitan distribution and a wide host range including hardwood and softwood trees and has been reported from numerous countries, worldwide. The taxonomy of this fungus is, however, not fully resolved. It is suspected that there are cryptic species within what is now referred to as the *O. quercus* complex, based on the wide host and geographic ranges of isolates in this group. This is supported by the occurrence of sub-clades within the larger *O. quercus* clade in phylogenetic trees using part of the ITS, BT (Kamgan Nkuekam *et al.* 2008a, Grobbelaar *et al.* 2009, Kamgan Nkuekam *et al.* 2010, Linnakoski *et al.* 2009) and histone gene regions (Grobbelaar *et al.* 2009).

Isolates from Australia grouped in a number of different sub-clades of what is currently treated as the *O. quercus* complex (Kamgan Nkuekam *et al.* 2008a, 2010, Grobbelaar *et al.* 2009, Linnakoski *et al.* 2009) that clearly deserves further taxonomic study.

Ophiostoma tasmaniense is morphologically most similar to *O. quercus*. The two fungi produce only a *Pesotum* anamorph and a mycelial synanamorph (*Sporothrix*) in culture. The teleomorph state of *O. tasmaniense* was generated in this study by crossing different strains of the fungus in different combinations on agar plates supplemented with *Eucalyptus* chips, as was the case for *O. quercus* (Morelet 1992, De Beer *et al.* 2003). It is clearly heterothallic and mating tester strains for it were produced in this study. Based on morphology, *O. tasmaniense* differs from *O. quercus* in having substantially shorter ascomatal necks, and the synnematal conidia of *O. tasmaniense* emerged from conidiogenous cells that are much more clearly annellidic than those of *O. quercus*, which have a deceptive appearance of developing sympodially (Wingfield *et al.* 1991).

The newly described *O. undulatum* is phylogenetically most closely related to *P. australiae*. Unlike other members of the *O. piceae* complex, *O. undulatum* does not have a *Pesotum* anamorph, which is one of the morphological characters that was used to define the *O. piceae* complex (Harrington *et al.* 2001). *Ophiostoma undulatum*, although phylogenetically most closely related to *P. australiae*, does not share any morphological similarities with it. The new species produces a thick and undulating mycelial mats with ascomata embedded below the mycelium on both MEA and OMA. Furthermore, the anamorph state is a *Sporothrix*, in contrast to *P. australiae*, which has only a *Pesotum* anamorph (Kamgan Nkuekam *et al.* 2008a). Small differences were found in the growth rates of *O. undulatum* and *P. australiae* at optimum temperatures. Colonies of *O. undulatum* reached 16 mm in ten days at 25ºC, while in previous studies those of *P. australiae* reached 14 mm at the same temperature (Kamgan Nkuekam *et al.* 2008a). However, *O. undulatum* could grow at 30ºC with colonies reaching 11 mm in ten days, while *P. australiae* does not grow at 30ºC and higher (Kamgan Nkuekam *et al.* 2008a).

This study represents the most comprehensive consideration of fungi in the Ophiostomatales occurring on *Eucalyptus* and *Corymbia* trees in Australia. The results show that *Ophiostoma* spp. are more diverse on wounds on *Eucalyptus* trees in Australia than previously understood. Of the fungi collected in this study, *O. quercus* and *O. tsotsi* have been reported from trees in countries other than Australia. This suggests intercontinental movement of these fungi, most likely through infected wood. This study should provide a foundation for future studies considering trends in biodiversity and pathogenicity of *Ophiostoma* in Australia.

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Table 1. List of *Ophiostoma* isolates and their morphogroups collected during the surveys in Australia and sequenced in this study. References species for which TEF was sequenced for comparative studies are listed below the morphogroups.

Morpho group	ID	Hosts	CMW	Accession numbers			Collectors	Countries
				ITS	BT	TEF		
A1	*O. quercus*	*Corymbia variegata*	29552	NA	NA	NA	GNK. Kamgan	Australia
		Eucalyptus dunnii	[b.]29279	NA	NA	NA	"	"
		E. agglomerata	29277	"	"	"	"	"
		E. grandis	29299	GU797206	GU797194	GU797224	"	"
		E. nitens	29091	GU797207	GU797192	NA	"	"
			[b.]29096	NA	NA	NA	"	"
			29103	GU797203	GU797193	GU797221	"	"
		E. globulus	29117	NA	NA	NA	"	"
			29118	GU797204	GU797198	GU797222	"	"
			29356	NA	NA	NA	"	"
			24755	NA	NA	NA	MJ. Wingfield	"
			24750	GU797205	GU797195	"	"	"
			24743	NA	NA	NA	"	"
			24745	GU797208	GU797197	"	"	"
			24758	GU797202	GU797196	"	"	"
A2	*O. tasmaniense*	*E. globulus*	[a.b.c.]29115	GU797209	GU797190	GU797225	GNK. Kamgan	"
			[a.c.]29116	GU797212	GU797191	GU797226	"	"
		E. nitens	[c.]29086	NA	NA	NA	"	"
			[T.a.b.c.]29088	GU797211	GU797188	GU797223	"	"
			[c.]29099	GU797210	GU797189	GU797220	"	"
		E. saligna	29272	NA	NA	NA	"	"
A3	*P. australiae*	*E. nitens*	[c.]29101	GU797213	GU797184	GU797232	"	"
A4	*O. tsotsi*	*E. grandis*	29293	GU797216	GU797199	GU797229	"	"

		E. pilularis	29294	GU797214	GU797200	GU797228	"	"
			29432	NA	NA	NA	"	"
			29433	"	"	"	"	"
			[b]29539	"	"	"	"	"
			29540	GU797215	GU797201	GU797227	"	"
			[b]29541	NA	NA	NA	"	"
			29543	"	"	"	"	"
			29548	"	"	"	"	"
B	*O. undulatum*	*E. grandis*	[c]19392	NA	NA	NA	MJ. Wingfield	"
			[a.c]19394	GU797219	GU797185	GU797235	"	"
			[T.a.b.c]19396	GU797218	GU797186	GU797233	"	"
			[a.b.c]19397	GU797217	GU797187	GU797234	"	"
			19402	NA	NA	NA	"	"
Reference strains	*O. borealis*	*Tilia cordata*	17860	EF408594	GQ249311	GU930823	T. Kirisits	Austria
		Betula pubescens	[T]18966	EF408593	GQ249317	GU930822	GNK. Kamgan	Norway
	O. denticiliatum	*Scolytus ratzeburgi* from *Betula* sp.	[T]29493	FJ804490	FJ804502	GU930818	R. Linnakoski	"
		"	[T]29494	FJ804491	FJ804503	GU930819	"	"
	O. karelicum	"	[T]23099	EU443762	EU443773	GU930821	"	Russia
		"	23101	EU443763	EU443774	GU930820	"	Finland
	P. australiae	*Acacia mearnsii*	6589	EF408602	EF408605	GU797231	MJ. Wingfield	Australia
		"	[T]6606	EF408603	EF408606	GU797230	"	"

T = ex-type isolates

[a] Isolates used for growth studies

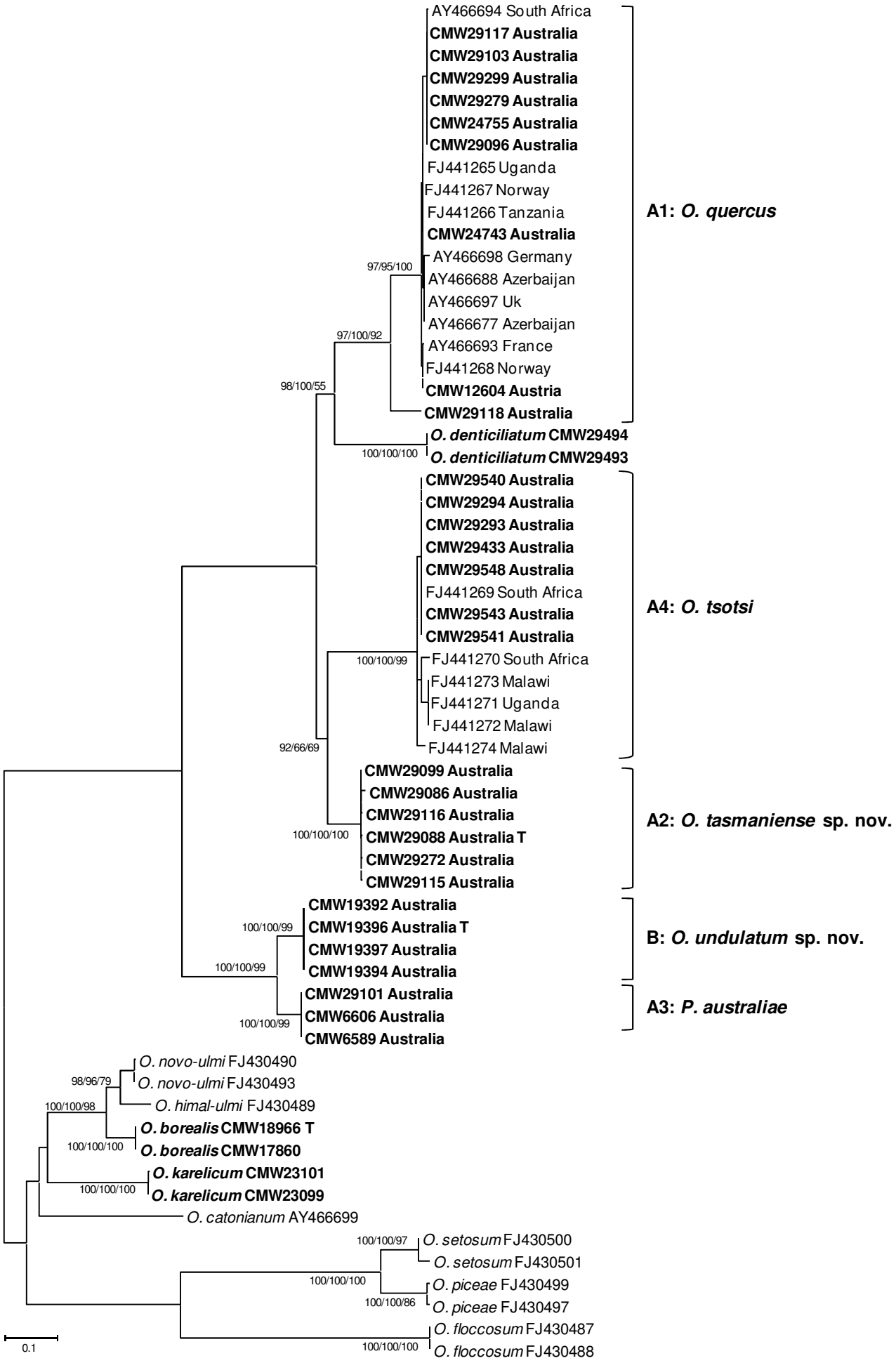
[b] Isolates used in pathogenicity trials

[c] Isolates used for mating compatibility trials

Figure 1: Phylogenetic tree produced from a maximum likelihood analysis of the ITS sequence data, showing the placement of *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia among known species of *Ophiostoma*. Isolates sequenced in this study are in bold type font. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node. T = ex-type isolates.

Figure 2: Phylogenetic tree produced from a maximum likelihood analysis of the BT sequence data, showing the relationship *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia with known species of *Ophiostoma*. Isolates sequenced in this study are in bold type font. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node. T = ex-type isolates.

Figure 3: Phylogenetic tree produced from a maximum likelihood analysis of the TEF sequence data, showing the relationship between *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia with known species of *Ophiostoma*. Isolates sequenced in this study are in bold type font. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node. T = ex-type isolates.



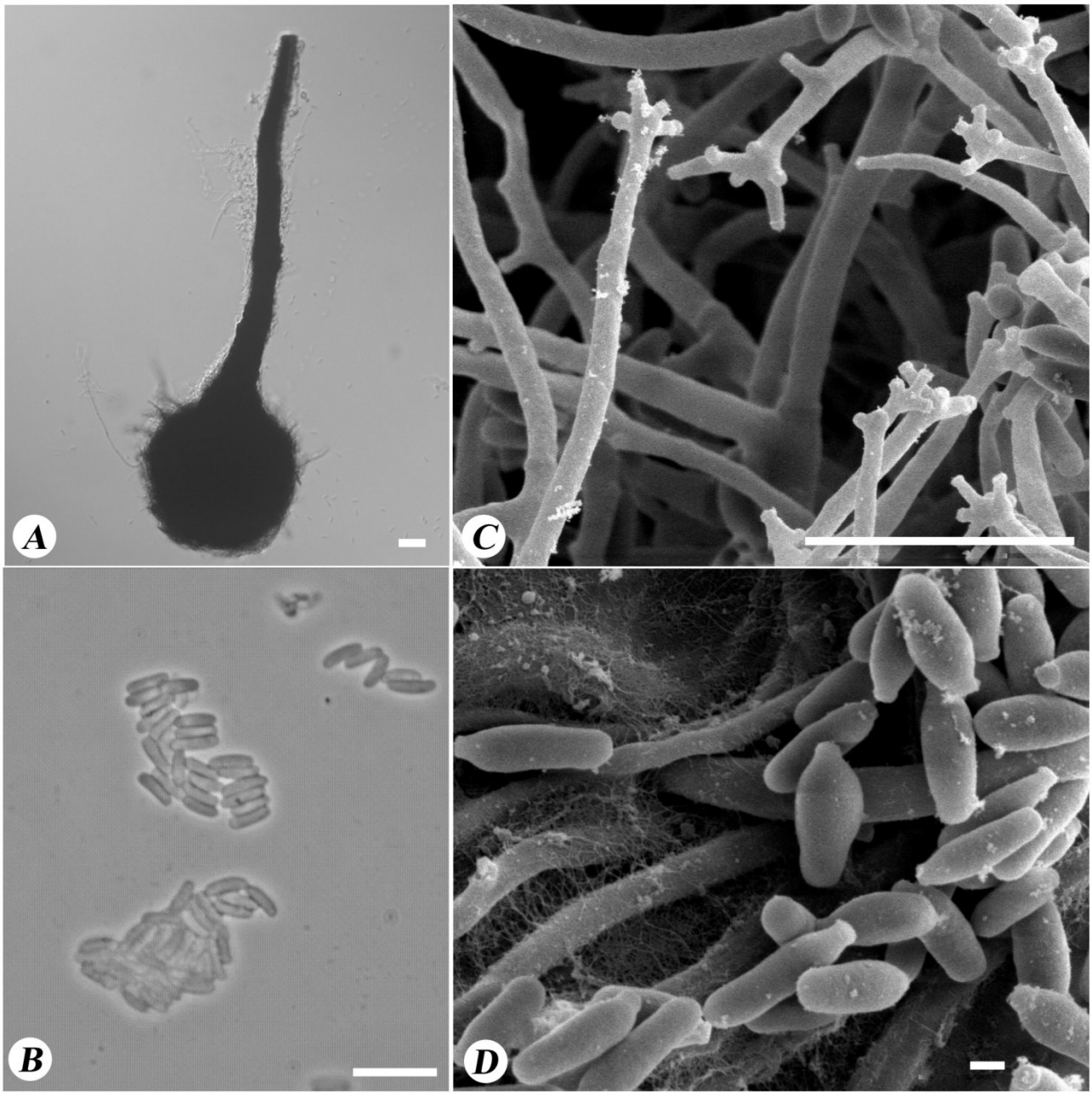


Figure 4: Morphological characteristics of *Ophiostoma undulatum* sp. nov. (A) globose ascomatal base and relatively short neck (scale bar = 20 µm), (B) reniform ascospores (scale bar = 10 µm), (C) conidiogenous cells with denticles (scale bar = 10 µm), (D) conidia, oblong to cylindrical, acerose (scale bar = 1 µm).

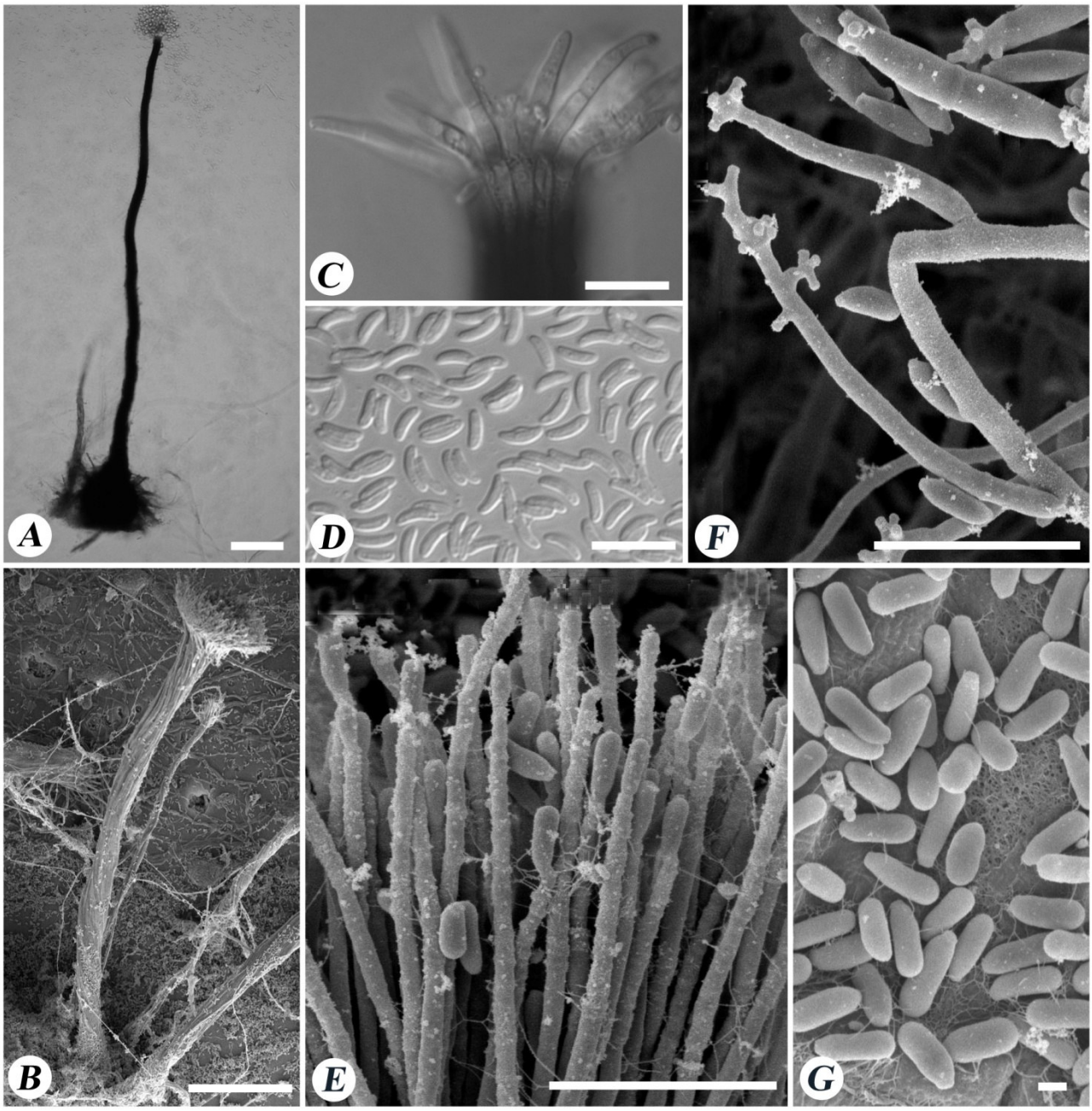


Figure 5: Morphological characteristics of *Ophiostoma tasmaniense* sp. nov. (A) globose ascomatal base (scale bar = 100 µm), (B) synnematal anamorph (scale bar = 100 µm), (C) divergent ostiolar hyphae (scale bar = 10 µm), (D) allantoid ascospores (scale bar = 10 µm), (E) conidiogenous cell with annelidic proliferation of conidia (scale bar = 10 µm), (F) *Sporothrix* conidiogenous cells with denticles and mycelial conidial (scale bar = 10 µm), (G) oblong to cylindrical conidia (scale bar = 1 µm).

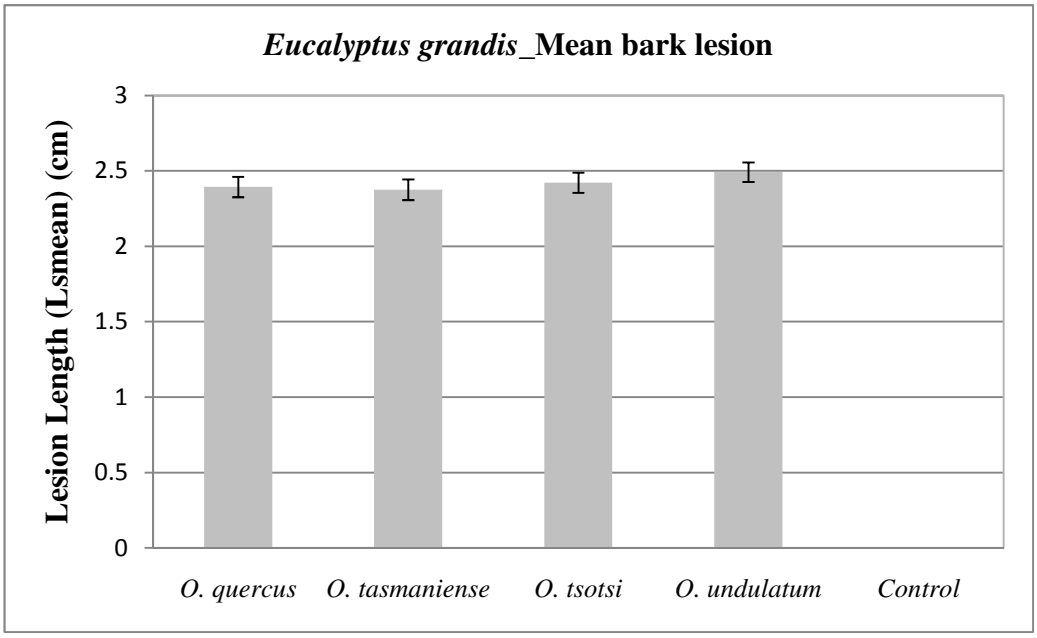


Figure 6: Vertical bar chart showing results of inoculation trial (bark lesion) with *O. quercus* (Lsmean = 2.3937), *O. tasmaniense* (Lsmean = 2.3749), *O. tsotsi* (Lsmean = 2.4216), *O. undulatum* (Lsmean = 2.4920) on *E. grandis* trees. Lsmean determined for two isolates used per species as indicated in Table 1, P<0.0001, Confidence limit = 95%.

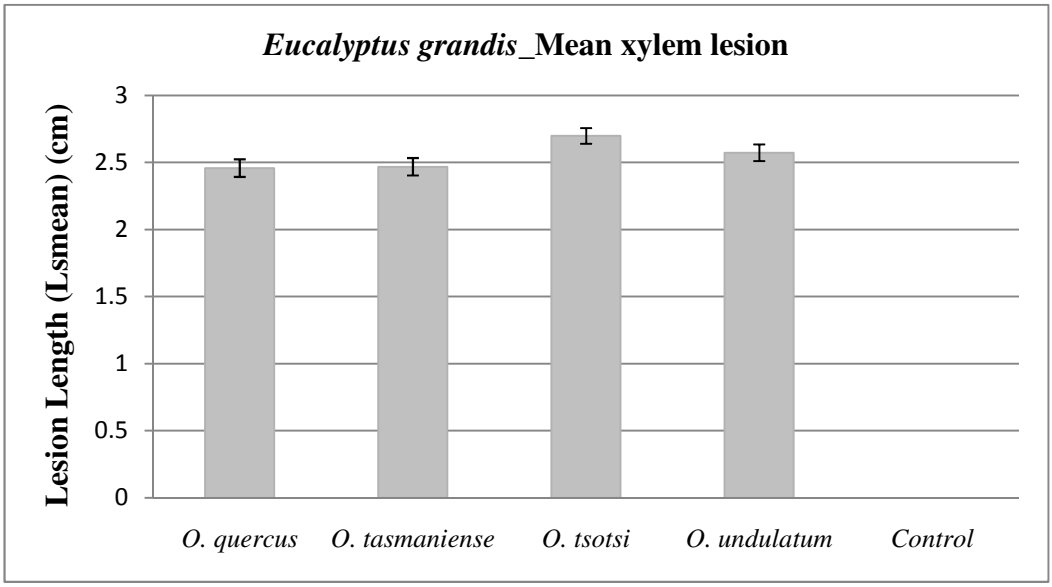


Figure 7: Vertical bar chart showing results of inoculation trial (xylem lesion) with *O. quercus* (Lsmean = 2.4582), *O. tasmaniense* (Lsmean = 2.4676), *O. tsotsi* (Lsmean = 2.6975) and *O. undulatum* (Lsmean = 2.5726) on *E. grandis* trees. Lsmean determined for two isolates used per species as indicated in Table 1, P<0.0001, Confidence limit = 95%.

CHAPTER IV

Ceratocystis species, including two new taxa, from *Eucalyptus* trees in South Africa.

ABSTRACT

Ceratocystis is an ascomycete fungal genus residing in the Microascales and includes important pathogens of trees including *Eucalyptus*. Species of *Ceratocystis* and their *Thielaviopsis* asexual states are typically associated with insects such as nitidulid beetles that spread them over long distances. *Eucalyptus* trees comprise a substantial component of the forestry industry in South Africa, however, limited information is available regarding *Ceratocystis* spp. that infect these trees. In this study, *Ceratocystis* spp. were collected from wounds on *Eucalyptus* spp. in all the major plantation regions of South Africa, as well as from insects associated with these wounds. Both morphology and multigene DNA sequence analyses, using three nuclear loci, were used to identify the *Ceratocystis* spp. collected. Of the 260 isolates collected, seven *Ceratocystis* spp. and two *Thielaviopsis* spp. were identified comprising *C. eucalypticola*, *C. pirilliformis*, *C. savannae*, *C. oblonga*, *C. moniliformis*, *T. basicola*, *T. thielavioides* and two new *Ceratocystis* spp. that are described here as *C. salinaria* sp. nov. and *C. decipiens* sp. nov. Insects associated with these *Ceratocystis* spp. were *Brachypeplus depressus* (Nitidulidae), *Carpophylus bisignatus*, *C. dimidiatus* (Nitidulidae), *Xyleborus affinis* (Scolytidae), *Litargus* sp. (Mycetophagidae) and a *Staphylinid* (Staphylinidae) species.

4.1. INTRODUCTION

Species of fungi in the genus *Ceratocystis* Ellis & Halsted (Ascomycetes: Microascales), and their *Thielaviopsis* Went anamorphs, includes important pathogens of agricultural and forestry crops (Kile 1993, Roux & Wingfield 2009). These fungi can cause diseases including stem cankers, root and fruit rot, as well as vascular wilts. The type species of *Ceratocystis*, *C. fimbriata* Ellis & Halsted, is the causal agent of sweet potato black rot disease (Halsted 1890, Halsted & Fairchild 1891). Other important diseases caused by *Ceratocystis* spp. include canker and death of plane trees caused by *C. platani* (Walter) Engelbrecht & Harrington (Walter *et al.* 1952, Panconesi 1999), oak wilt caused by *C. fagacearum* (Bretz) J. Hunt (Juzwik *et al.* 2008, Sinclair & Lyon, 2005) and wilt and death of *Acacia mearnsii* de Wild trees caused by *C. albifundus* De Beer, Wingfield & Morris (Morris *et al.* 1993, Wingfield *et al.* 1996).

Ceratocystis spp. require wounds to infect their hosts (Moller & Devay 1968, Walter *et al.* 1952, Kile 1993) and are associated with insects that act as their vectors. Most *Ceratocystis* spp. are vectored by sap-feeding nitidulids and flies in what is considered a non-specific association (Cease & Juzwik 2001, Moller & Devay 1968). Other *Ceratocystis* spp., such as *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter, *C. rufipenni* Wingfield, Harrington & Solheim and *C. fujiensis* M. J. Wingf., Yamaoka & Marin, occur on conifers and are vectored by bark beetles (Harrington & Wingfield 1998, Wingfield *et al*. 1997, Marin *et al.* 2005). There is, however, growing evidence that the association between some nitidulid beetles and *Ceratocystis* spp., such as *C. fagacearum* are not entirely casual as previously believed (Juzwik *et al*. 2004, Hayslett *et al.* 2007).

There have been increasing numbers of reports of *Ceratocystis* spp. infecting or causing diseases of *Eucalyptus* trees during the course of the last ten years (Roux & Wingfield 2009). Seven *Ceratocystis* spp. have been reported infecting wounds on non-native *Eucalyptus* trees in plantations worldwide. Four of these species occur in Africa. *C. atrox* M. Van Wyk & M.J. Wingfield (VanWyk *et al.* 2007a) and *C. eucalypti* Z.Q. Yuan & Kile are known only from Australia (Kile *et al.* 1996), *C. neglecta* M. van Wyk, Jol. Roux & C. Rodas from Colombia (Rodas *et al.* 2008), *C. fimbriatomima* M. van Wyk & M.J. Wingf. from Venezuela (Van Wyk *et al.* 2009), *C. zombamontana* R.N. Heath & Jol. Roux from Malawi (Heath *et al.* 2009a), *C. moniliformis* (Hedgcock) Moreau from South Africa and Tanzania (Heath *et al.* 2009a), *C. pirilliformis* I. Barnes & M.J. Wingf. from Australia and South Africa (Barnes *et al.* 2003a, Roux *et al.* 2004, Kamgan Nkuekam *et al.* 2009), and *C. eucalypticola* M. van Wyk & M.J. Wingf. (Van Wyk *et al.* 2011)

from Brazil, the Republic of Congo (Roux *et al.* 1999), Uganda (Roux *et al.* 2001), Uruguay (Barnes *et al.* 2003b) and South Africa (Roux *et al.* 2004). Of these, *C. eucalypticola* is the only species that has been shown to cause wilt and death of *Eucalyptus* trees in Brazil and the Republic of Congo (Roux *et al.* 1999), Uganda (Roux *et al.* 2001) and Uruguay (Barnes *et al.* 2003b).

Three *Ceratocystis* spp. have been reported from wounds on *Eucalyptus* trees in South Africa. These are *C. eucalypticola*, *C. moniliformis* and *C. pirilliformis* (Roux *et al.* 2004, Kamgan Nkuekam *et al.* 2009). *C. eucalypticola* and *C. pirilliformis* have not been associated with naturally dying *Eucalyptus* trees in South Africa, but artificial inoculation with these fungi in both the field and greenhouse resulted in distinct lesions, suggesting that they have the potential to kill *Eucalyptus* trees in South Africa (Roux *et al.* 2004). Other *Ceratocystis* spp. known from South Africa occur on non-native *A. mearnsii* (Morris *et al.* 1993, Wingfield *et al.* 1996) or indigenous trees (Roux *et al.* 2007, Kamgan Nkuekam *et al.* 2008). In this regard, there is growing concern that *Ceratocystis* spp. Can shift hosts such as is the case for *C. albifundus*, first isolated from native *Protea* spp. in South Africa (Gorter 1977) and later found causing disease on non-native *A. mearnsii* trees in plantations (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux & Wingfield 2009).

Very little is known regarding the insect associates of *Ceratocystis* spp. in South Africa. A recent study considering the epidemiology of the wattle wilt pathogen, *C. albifundus*, in South Africa, this fungus and *Ceratocystis oblonga* R. N. Heath & Jolanda Roux were isolated from three nitidulid beetle species, *Brachypeplus depressus* Erichson, *Carpophilus bisignatus* Boheman and *C. hemipterus* L. (Heath *et al.* 2009b). These insects were collected from both indigenous woodlands and from commercial plantations of non-native *A. mearnsii* trees, where they were either caught in insect traps or collected from beneath bark flaps on cut stumps (Heath *et al.* 2009b). The presence of these fungi on free-flying nitidulid beetles and on insects occurring on fungal mats growing under bark flaps suggested that nitidulid beetles are vectors of *C. albifundus* and *C. oblonga* on both native trees and *A. mearnsii* trees in its non-native range in South Africa (Heath *et al.* 2009b).

Previous studies of *Ceratocystis* spp. on *Eucalyptus* trees in South Africa have been limited to a small number of geographic and climatic areas and limited numbers of *Eucalyptus* spp. The recent discoveries of *C. neglegta*, *C. atrox* and *C. zombamontana* on *Eucalyptus* spp., together with numerous previously undescribed species from native trees in South Africa, suggested that additional species may occur on *Eucalyptus* trees in South Africa. This, together with the limited information regarding the biology and epidemiology of *Ceratocystis* spp. on *Eucalyptus* trees prompted this study aimed at expanding the base of knowledge of the diversity of *Ceratocystis* spp.

infecting *Eucalyptus* trees in the country. The nitidulid vectors of these fungi in commercial *Eucalyptus* plantations were also identified.

4.2. MATERIALS AND METHODS

4.2.1. Collection of Fungal isolates from trees

Ceratocystis spp. were collected from wounds on *Eucalyptus* trees in plantations in South Africa, over a two year period from February 2007 to December 2008. Collection sites covered the majority of the eucalypt growing areas of the country (Figure 1) and included localities near Louis Trichardt and Tzaneen (Limpopo Province), Lothair and Sabie (Mpumalanga Province), George, Cape Town and Stellenbosch (Western Cape Province), Kumbo and Lotobeni (Eastern Cape Province) and localities near KwaMbonambi and Pietermaritzburg (KwaZulu Natal Province). Samples were mainly collected from the stumps of freshly harvested *Eucalyptus* trees and from *Eucalyptus* logs either on the plantation floor or at the harbor.

Pieces of bark or wood were collected from cut stumps and transported to the laboratory as described by Kamgan Nkuekam *et al.* (2009). Isolation and purification of fungi from samples followed the protocol described by Kamgan Nkuekam *et al*. (2009). Isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative specimens have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

4.2.2. Collection of insect samples

Insects were collected from beneath bark flaps on cut stumps of *Eucalyptus* trees. This was done using an aspirator (Fergusson 1982). Insects were stored in separate Eppendorf tubes and transported to the laboratory following the method described by Kamgan Nkuekam *et al*. (2010). The insects were grouped based on morphological characteristics viewed using an Axiocam dissection microscope (Carl Zeiss Ltd., Germany). Representatives of each insect group were preserved in 70% ethanol prior to identification by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food and Agriculture, United State of America.

Isolation of fungi from insects was done using carrot baiting (Moller & Devay 1968, Heath *et al.* 2009b). Mycelium, ascomata or ascospores of putative *Ceratocystis* spp. were then transferred from the carrot surfaces to 2% malt extract agar (MEA: 20 gl^{-1} malt extract and 15 gl^{-1} agar, Biolab, Midrand, South Africa and 1000 ml sterile deionised water) containing 0.05 gl^{-1} of the antibiotic streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany).

4.2.3. Morphological characterization

Ceratocystis isolates were incubated at 25ºC until sporulation and then grouped based on colour (Rayner 1970) and macro-morphology on MEA. Morphological structures including ascomata and ascospores, phialides and conidia from isolates representing each morphotype were mounted in 80% lactic acid on glass microscope slides and examined using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen to represent the types of new species and ten measurements were made for additional isolates. Measurements were noted as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum).

Scanning Electron Microscopy (SEM) was used to examine spores and the asexual states of the *Ceratocystis* spp. Specimens were prepared for SEM as described by Grobbelaar *et al.* (2009). The specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater (Emitech K550X, Ashford, England) and examined using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

4.2.4. Growth in culture

Growth in culture was examined for two isolates of each new species identified in this study. A disk of agar (9 mm diam.) bearing mycelium of the test isolates was transferred from the actively growing margins of seven-day-old cultures and placed upside down at the centres of 90 mm Petri dishes containing 2% MEA. The plates were incubated in the dark for 10 days at temperatures ranging from 5ºC to 35ºC at 5 degree intervals. Five replicate plates were used for each isolate at each temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed.

4.2.5. DNA sequence comparisons

Single spore drops collected from the apices of ascomata in pure cultures were transferred to 2% MEA and allowed to grow for 7-10 days. Mycelium was scraped from the surfaces of the actively

growing cultures and transferred to 1.5 ml Eppendorf tubes using a sterile hypodermic needle. DNA was extracted from all isolates using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified on an Eppendorf Mastercycler (Merck, Germany) using primers ITS1 and ITS4 (White *et al*. 1990). Part of the β-tubulin gene (BT) and the transcription elongation factor-1α gene (TEF) were also amplified using the primers βt1a and βt1b (Glass & Donaldson 1995), EF1F and EF2R (Jacobs *et al*. 2004) respectively.

The PCR reaction mixtures as well as the thermal cycling were the same as those described previously (Kamgan Nkuekam *et al*. 2008). An 5 µl aliquot of the PCR products was pre-stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA) and separated on a 1% agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), following the manufacturer's instructions. The concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). 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, California, USA). Sequencing PCR was prepared as described by Kamgan Nkuekam *et al*. (2008) and both DNA strands were sequenced.

A preliminary identity for the isolates was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov). Sequences of both DNA strands for each isolate were examined visually and combined using the programme Sequence Navigator v. 1.01 (ABI PRISM, Perkin Elmer, Warrington, UK). Additional sequences of related *Ceratocystis* spp. and *Thielaviopsis* spp. were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) for comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Katoh & Toh 2008).

4.2.6. Phylogenetic analyses

Phylogenetic analyses of sequences for each group of isolates separated based on morphology were performed independently of each other. Phylogenetic analyses of data sets for each of the three nuclear loci (ITS, BT, TEF) were performed both separately and as combined data sets. For each

data set, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done.

MP analyses were performed in PAUP 4.0b10 (Swofford 1998), using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1000 replications).

Bayesian analyses based on Markov chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) as outlined previously (Kamgan Nkuekam *et al.* 2010). Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (http://www.abc.se/~nylander/). The best fit model of evolution applied to ITS, BT and TEF are summarized in Table 1. Burn-in values were determined using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) to discard trees that formed before the point of convergence, and the posterior probability in the majority rule consensus trees were calculated by MCMC sampling in MrBayes V3.1.2, using the best-fit model of evolution (Table 1).

Maximum likelihood (ML) analyses were conducted online using PhyML 3.0 (Guindon & Gascuel 2003). The AIC was used in Modeltest 3.7 (Posada & Crandall 1998) to select appropriate substitution models for the three data sets (Table 1).

4.2.7. Pathogenicity tests

The pathogenicity of two new *Ceratocystis* spp. identified in this study was tested in a quarantine greenhouse. Two strains of each species were used to inoculate ten approximately two-year-old (~1 cm diameter) *Eucalyptus grandis* clone TAG5 trees. Two additional trees of the same age were inoculated with a sterile agar disc to serve as controls. The experimental design and conditions for inoculation were the same as those described by Kamgan Nkuekam *et al.* (2008). Six weeks (42 days) after inoculation, the lengths of lesions on the bark surface and in the xylem of each tree were measured. Re-isolations were made from the lesions to meet the requirements of Koch's postulates. All data were processed using the statistical software package SAS® V8 running under VM/CMS on the main frame computer at the University of Pretoria, where lesion lengths were analyzed using the GLM procedure.

4.3. RESULTS

4.3.1. Collection of fungal isolates from trees

A total of one hundred *Ceratocystis* isolates were obtained from wounds of *Eucalyptus* trees sampled during the surveys. More than 300 trees were sampled in the process and isolates were obtained from all the areas sampled. This spanned six different Provinces and a wide variety of climatic conditions (Table 2). *Eucalyptus* spp. from which *Ceratocystis* isolates were obtained included six different species, namely *E. grandis* W. Hill ex Maiden, *E. nitens* H.Deane & Maiden, *E. saligna* Sm., *E. maculata* Hook., *E. cloeziana* F. Muell and *E. diversicolor* F. Muell (Table 2).

4.3.2. Fungal isolates from insects

A wide variety of insects were found in four of the six Provinces sampled during the surveys. More than 385 insects, spanning five genera in three different families were collected. Members of the Nitidulidae were the most common insects found with 255 specimens collected. These nitidulids were identified as *Brachypeplus depressus* Erichson (120 specimens), *Carpophilus bisignatus* Boheman (20 specimens) and *C. dimidiatus* Fabricius (25 specimens). Ninety other nitidulid beetles were of a *Carpophilus* sp. that could not be identified to species level. Other insects collected were residing in the Staphylinidae (100 specimens), *Lithargus* sp. (Coleoptera: Mycetophagidae) (10 specimens) and *Xyleborus affinis* (Coleoptera: Scolytidae) (20 specimens) (Table 3). A total of 160 isolates of *Ceratocystis* were obtained from the insects collected in this study (Table 3).

4.3.3. Morphological characterization

Based on colony colour and the morphology of the ascomata, ascospores, conidiogenous cells and conidia produced on MEA, three main morphological groups in *Ceratocystis s.l.* were identified. The first set of isolates produced colonies and structures typical of species in the *C. fimbriata sensu lato* (*s.l.*) species complex and these are referred to as the *C. fimbriata* group. These isolates could be further subdivided into two sub-groups. One of these sub-groups resembled those of *C. fimbriata s.s*, the other were typical of those of *C. pirilliformis* as described by Barnes *et al.* (2003a). The second set of isolates produced colonies with a morphology typical to those in the *C. moniliformis s.l.* complex and are treated as such. The third set of isolates produced colonies having only the asexual *Thielaviopsis* state of *Ceratocystis*.

4.3.4. DNA sequence comparisons and phylogenetic analyses

The ITS gene region for all isolates in the *C. fimbriata s.l.* group generated contigs of ~600 bp. For the BT and TEF gene regions, only a few representative isolates were selected for DNA sequencing.

Amplification of the DNA isolates generated contigs of ~550 and ~900 bps for the BT and TEF, respectively. A preliminary nucleotide Blast against the GenBank database using data sets from each of the three gene regions revealed that some of the isolates were similar to either *C. fimbriata s.s.*, *C. manginecans* or *C. eucalypticola* and the others were closely related to *C. pirilliformis*.

Comparison of the ITS, BT and TEF sequence data of selected isolates in the *C. fimbriata s.l.* group with those of related *Ceratocystis* spp. from GenBank (Table 4) showed that one group of isolates represented *C. eucalypticola* and the other *C. pirilliformis* (Figure 1). These results were confirmed using parsimony analysis, Bayesian analyses and maximum likelihood analyses (ML). Tree statistics for maximum parsimony analysis are summarized in Table 5. Comparison of sequence data for all three gene regions confirmed the identities of the isolates as either *C. pirilliformis* or *C. eucalypticola*. These isolates had identical BT sequence and the TEF sequences differed only in a small number of bases in multiple base repeat regions (data not shown). The results are similar to those in a study of Van Wyk *et al*. (2011) where, minor sequence variations was found in the ITS data of isolates identified as *C. eucalypticola* and the type of the fungus (Figures 1, 2).

Isolates resembling those in the *C. moniliformis* complex could be distinguished only based on sequence data for the BT (Figure 3) and TEF (Figure 4) gene regions, with no resolution obtained for the ITS gene region (data not shown). This is similar to what has previously been reported for the *C. moniliformis s.l.* complex (Van Wyk *et al.* 2006, Kamgan Nkuekam *et al.* 2008). Sequence data for the BT gene region grouped isolates into five clades (Figure 3, Table 4) that represented *C. savannae*, *C. moniliformis*, *C. oblonga* and two unidentified species and the identities were supported by Bayesian, MP and ML analyses. Isolates representing *C. moniliformis* were 100% identical to those of the type species. Minor differences of up to two base pairs were found between some isolates identified as *C. savannae* and the ex-type isolate of the fungus (Table 6). Some isolates in the larger *C. oblonga* clade differed from the ex-type isolate in up to three base pairs (Table 6). An unrooted tree showing relationships between the two unidentified species and their closest phylogenetic neighbors was also constructed (Figure 4).

Analyses of the TEF data set for the *C. moniliformis s.l.* isolates were not concordant with those for the BT data set, but also revealed five different clades (Figure 5, Table 4). These clades represented *C. savannae* and *C. oblonga*, which could not be differentiated from each other using TEF sequence data, *C. moniliformis* and three separate clades including isolates from eucalypts and insects. All clades were supported by Bayesian, MP and ML analyses (Figure 5, Table 4). There were three base pair differences between eucalypt isolates identified as *C. moniliformis* and the ex-type isolate

of the fungus. Minor variations were found among isolates identified as *C. savannae* and the ex-type species of the fungus. Isolates residing in Clade 1 of the *C. moniliformis s.l.* group, identified as representing an undescribed taxon based on the BT gene region split into two well-supported clades in the TEF analyses, different from other *Ceratocystis* reference strains (Figure 5). A total of 11 bp differences (10 indels and 1 fixed bp) separated the two clades (Table 7). Isolates residing in Clade 2 formed a single well resolved and highly supported clade in the TEF tree and thus similar to the results for the BT (Figure 3, 5). Analyses of a TEF data set including only *C. savannae*, *C. oblonga* and their closest relatives and visualization of results in an unrooted tree confirmed the unique nature of Clade 2 isolates. Isolates of *C. savannae* and *C. oblonga* could not be distinguished from each other, while a set of isolates from eucalypts resided in two different clades (Figure 6). For the TEF data set, all analyses, including Bayesian, MP as well as ML were concordant.

Parsimony analysis of the combined dataset for the ITS, BT and TEF gene regions for isolates in the *C. moniliformis s.l.* group and including related *Ceratocystis* spp. from GenBank (Table 4) resolved the isolates into five different clades (Figure 7, Table 4). Sequence discordance found in TEF data sets remained present except that the new species that split in two clades in the TEF tree, resided in a larger well-supported clade showing considerable sequence variation among isolates within the clade (Figure 7). These data were confirmed by the 50% majority rule tree obtained from Bayesian analyses, a bootstrap tree obtained from MP as well as from ML analyses.

Isolates residing in the group where only a *Thielaviopsis* state was present were considered, only based on sequence data for only the ITS gene region. This generated a contig of ~500 bp. A preliminary nucleotide Blast against the GenBank database using data sets from the ITS gene regions confirmed that isolates reside in *Thielaviopsis*. Comparison of the ITS data set for these isolates with sequences for other *Thielaviopsis* spp. in GenBank using parsimony analysis resulted in a phylogenetic tree where one set of the isolates grouped with *T. basicola* (Berk. Et Br.) Ferr (AF275490, AF275494), and the second set of isolates grouped with *T. thielavoides* (Peyr.) A.E. Paulin, T.C. Harr. & McNew (AF275487, AF275488) strains with 100% bootstrap support at nodes (Figure 8).

4.3.5. Taxonomy

Based on phylogenetic analyses of sequence data for a number of gene regions, two previously unknown *Ceratocystis* spp. are recognized on *Eucalyptus* spp. and insects associated with these trees in South Africa. These two fungi reside in the larger *C. moniliformis s.l.* species complex, and

were clearly separated based on sequence data for reference strains of other species in this group. The following descriptions are provided for them.

Ceratocystis salinaria Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 9) **MB519695**

Etymology*: *Salinaria "pertaining to salt-works" used by Vitruvius (1st Century AD) and reflects the fact that the fungus was found in the Soutpansberg area, famous for its salt pans in South Africa.

Coloniae supra bubalinae, infra mellinae, usque ad 45.5 mm diametro post 3 dies in MEA in 25°C. Bases *ascomatum* atrobrunneae, globosae vel obpyriformes, spinis fuscis conicis et pilis hypharum munitis. Colla *ascomatum* atrobrunnea, in apicibus *hypharum ostiolorum* divergentium guttas sporarum viscidas hyalinas facientes. Bases collorum basin versus discis munitae. *Asci* evanescentes. *Ascosporae* pileiformes hyalinae non septatae, vaginis munitae. Anamorpha *Thielaviopsis, conidiophoris* in mycelio singulis phialidicis hyalinis tubularibus, sine colliculis. *Conidia* hyalina non septata, biformia, aut oblonga (5.0-) 5.5-7.5 (-9.0) x (1.5-) 2.0-3.0 (-3.5) µm aut bacilliformia basibus rotundatis (6.5-) 7.5-9.5 (-10.5) x (1.0-) 1.5-2.5 (-3.0) µm.

Colonies colour Buff (19''d), ascomata produced rapidly and abundantly, scattered over the colonies and embedded within aerial mycelium, giving colonies a Honey (19''b) coloured appearance. Aerial mycelium distributed evenly across the colonies giving cultures a fluffy appearance. Reverse colonies Honey (19''b). Colony diameter reaching 45.5 mm in 3 days on MEA at 25ºC. Optimal growth at 25ºC, growth at 30ºC with colony diameter reaching 43 mm in 3 days. No growth at 5ºC or 37ºC. Mycelium forming thick mat on agar. Hyphae smooth, not constricted at septa. *Ascomata* scattered over the colonies. *Ascomatal* bases dark brown, globose to obpyriform (138.0-) 189.0-247.5 (-272.0) µm long and (124.0-) 155.5-204.5 (-232.5) µm wide, with dark conical spines (5.0-) 6.5-9.0 (-11.0) µm and hyphal hair. *Ascomatal* necks dark brown (297.5-) 379.5-499.5 (-592.0) µm long, middle of necks (19.0-) 23.5-28.5 (-31.5) µm wide, tips of necks (11.5-) 12.0-16.5 (-23.0) µm wide, producing sticky and hyaline spore drops at the tips of divergent *ostiolar hyphae* (19.5-) 24.5-100.5 (-123.5) µm long and with disc-like (disciform) bases, (43.0-) 55.0-73.5 (-88.0) µm wide at bases. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (4.5-) 5.0-5.5 (-6.0) x (2.5-) 3.0-3.5 (-4.0) µm, accumulating in round, *straw yellow* (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (18.5-) 20.5-28.5 (-39.5) x (2.0-) 2.5-3.0 (-3.5) µm, colarettes absent. *Conidia* hyaline, aseptate, two

types, oblong (5.0-) 5.5-7.5 (-9.0) x (1.5-) 2.0-3.0 (-3.5) µm and bacilliform-shaped with rounded bases (6.5-) 7.5-9.5 (-10.5) x (1.0-) 1.5-2.5 (-3.0) µm. Chlamydospores (aleuroconidia) not observed.

Specimen examined: South Africa, Limpopo Province, Soutpansberg area (S23º 02,350', E030 º 14,209'), isolated from stumps of *Eucalyptus maculata*, 18/06/2007, G. Kamgan Nkuekam & J. Roux, holotype PREM60557, living culture CMW25911 = CBS129733

Additional specimens: South Africa, Limpopo Province, Soutpansberg area, from stumps of *Eucalyptus saligna*, 17/12/2008, G. Kamgan Nkuekam & J. Roux, paratype, living culture CMW30702=PREM60558, from stumps of *Eucalyptus saligna*, 17/12/2008, G. Kamgan Nkuekam & J. Roux, paratype, living culture CMW30703=PREM60559 = CBS129734.

Ceratocystis decipiens Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 10) **MB519696**

Etymology*: *Decipiens, the latin word for "deceiving" and referring to the fact that the fungus would be seen as a single species based on BT or two species based on TEF sequence data.

Coloniae in MEA supra bubalinae, infra margine mellinae, medio versus subisabellinae, usque ad 39 mm diametro post 3 dies in 30°C. Bases *ascomatum* atrobrunneae, globosae vel obpyriformes, spinis fuscis conicis et pilis hypharum munitis. Colla *ascomatum* atrobrunnea, in apicibus *hypharum ostiolorum* divergentium guttas sporarum viscidas hyalinas facientes. Bases collorum basin versus discis munitae. *Asci* evanescentes. *Ascosporae* pileiformes hyalinae non septatae, vaginis munitae. Anamorpha *Thielaviopsis, conidiophoris* in mycelio singulis phialidicis hyalinis tubularibus, sine colliculis. *Conidia* hyalina non septata, biformia, aut oblonga (4.5-) 5.5-6.5 (-7.5) x (1.5-) 2.0-2.5 (-3.5) µm aut bacilliformia basibus rotundatis (5.0-) 5.5-7.5 (-10.5) x (1.0-) 1.5-2.0 (-2.5) µm.

Colonies Buff (19''d) coloured, ascomata often absent or produced late in small quantities, scattered over the colonies. Reverse colonies Honey (19''b) from the edge, turning nearly Isabelline (17''i) towards the center. Colony diameters reaching 39 mm in 3 days on MEA at 30ºC. Optimal growth at 30ºC, no growth at 35ºC or at 5ºC. Mycelium forming thick mat on agar, becoming fluffy towards the center. Hyphae septate, not constricted at septa. *Ascomata* scattered over the surface of the colonies or embedded in mycelium. *Ascomatal* bases dark brown, globose to obpyriform (132.5-) 167.5-216.5 (-258.5) µm long and (108.5-) 162.5-218.0 (-244.0) µm wide, with dark conical spines (5.0-) 5.5-11.5 (-16.5) µm and hyphal hair ornamentations. *Ascomatal* necks dark brown (355.0-) 401.0-500.5 (-596.5) µm long, middle of necks (17.5-) 21.0-25.5 (-27.5) µm wide, tips of necks (9.5-) 11.0-13.0 (-16.0) µm wide, producing sticky, hyaline spore drops at the tips of

divergent *ostiolar hyphae* (13.0-) 15.5-24.5 (-35.5) µm long and with disc-like (disciform) bases (47.0-) 58.5-86.5 (-102.5) µm wide at bases. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (4.0-) 4.5-5.0 (-5.5) x (2.0-) 2.5-3.0 (-3.5) µm, accumulating in round, straw yellow (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (15.5-) 21.5-30.5 (-35.0) x (2.0-) 2.5-3.5 (-4.0) µm, colarettes absent. *Conidia* hyaline, aseptate, two types, oblong (4.5-) 5.5-6.5 (-7.5) x (1.5-) 2.0-2.5 (-3.5) µm and bacilliform-shaped (5.0-) 5.5-7.5 (-10.5) x (1.0-) 1.5-2.0 (-2.5) µm. Chlamydospores (aleuroconidia) not observed.

Specimen examined: South Africa, Limpopo Province, Soutpansberg area (S23º 02,350', E030 º 14,209'), isolated from *Staphylinid* sp. obtained from stumps of a *Eucalyptus saligna* tree, 17/12/2008, G. Kamgan Nkuekam & J. Roux, holotype PREM60560, living culture CMW30855 = CBS129736.

Additional specimens: South Africa**,** Limpopo Province, Soutpansberg area, from wound on *Eucalyptus cloeziana*, 21/06/2007, G. Kamgan Nkuekam & J. Roux, paratype, living culture CMW25918=PREM60561 = CBS129735, from wound on *Eucalyptus maculata*, 21/06/2007, G. Kamgan Nkuekam & J. Roux, paratype, living culture CMW25914=PREM60562 = CBS129737.

In all, nine *Ceratocystis* spp. identified in this study were collected in six Provinces spanning various climatic conditions in South Africa. These include two *Thielaviopsis* spp. and seven *Ceratocystis* spp., two (*C. decipiens* sp. nov. and *C. salinaria* sp. nov.) of which are described in this study. *C. eucalypticola* was the most wide spread species, occurring in four of the sampled Provinces (Figure 13, Tables 2, 3). It was obtained from *E. grandis* trees and from a wide variety of insects (Table 2, 3). *C. pirilliformis* was collected in three Provinces on four *Eucalyptus* spp. (*E. cloeziana*, *E. diversicolor*, *E. grandis*, *E. saligna*) and from *B. depressus* (Table 2, 3). *C. oblonga* was found in two Provinces (Limpopo and Mpumalanga) on *E. grandis* trees. It was also obtained from *B. depressus*, *C. bisignatus* and *C. dimidiatus* (Table 3). *C. moniliformis* was also found in both the Limpopo and Mpumalanga Provinces on two *Eucalyptus* spp. (*E. grandis*, *E. maculata*) and from a staphylinid beetle (Table 2, 3). *C. salinaria* was found in two Provinces (Limpopo, Western Cape) on three *Eucalyptus* spp. (*E. cloeziana*, *E. maculata*, *E. saligna*), while *C. decipiens* was found exclusively in the Limpopo Province on two *Eucalyptus* spp. (*E. cloeziana*, *E. maculata*) and on a staphylinid beetle (Table 2, 3). *C. savannae* was also found in Limpopo Province, on two *Eucalyptus* spp. (*E. cloeziana*, *E. maculata*) and on *B. depressus*, *C. bisignatus*, *C. dimidiatus* and a staphylinid beetle (Table 2, 3). *T. basicola* and *T. thielavoides* were isolated from nitidulid beetles collected in the Eastern Cape and KwaZulu Natal Provinces, respectively.

4.3.6. Pathogenicity tests

Six weeks after inoculation, *Eucalyptus* trees were assessed for lesion development in the bark and cambium. *Ceratocystis salinaria* and *C. decipiens* produced very small lesions on both the bark (Figure 11) and in the cambium (Figure 12). At the time of assessment, trees showed no signs of disease and neither *C. salinaria* nor *C. decipiens* could be isolated from the small lesions associated with their inoculation.

4.4. DISCUSSION

Seven *Ceratocystis* spp. and two *Thielaviopsis* spp. were identified in this study from various insects and *Eucalyptus* spp. grown in six Provinces of South Africa. Two of the *Ceratocystis* spp., *C. eucalypticola* and *C. pirilliformis* are members of the *C. fimbriata s.l.* species complex. The five other *Ceratocystis* spp. reside in the *C. moniliformis s.l.* species complex. These include *C. savannae*, *C. oblonga*, *C. moniliformis* and two new taxa for which the names *C. salinaria* and *C. decipiens* have been provided. Other than these, *T. basicola* and *T. thielavioidea* were also obtained. In pathogenicity tests, *C. salinaria* and *C. decipiens* resulted in only small lesions on both the bark and the xylem of young *Eucalyptus* trees and they are, therefore, not considered pathogens of *Eucalyptus* trees in South Africa.

Separation of *Ceratocystis* sp. based on phylogenetic inference from DNA sequence data is becoming increasingly difficult as new species are described. During the course of the past ten years, sequence data for the ITS, TEF, BT gene regions have been used to distinguish species in the *C. fimbriata* and *C. moniliformis* complexes from each other(Van Wyk *et al.* 2006, 2007a, b, Kamgan Nkuekam *et al.* 2008). In this regard, the ITS gene region has provided the most information for species in the *C. fimbriata s.l* clade and the TEF and BT regions are most informative for species in the *C. moniliformis s.l.* complex (Van Wyk *et al.* 2006, Kamgan Nkuekam *et al.* 2008). In this study, we used both separate analyses for different gene regions as well as combined analyses to infer phylogeny of members of the *C. moniliformis s.l.* and *C. fimbriata s.l.* species complex. This made it possible to avoid errors in interpretation that could arise from using either separate analyses or combined analyses exclusively as suggested by Huelsenbeck *et al.* (1996)

Delimiting species within *C. moniliformis s.l.* is especially problematic. This is not only due to lack of concordance between gene regions (Van Wyk *et al.* 2006, Kamgan Nkuekam *et al.* 2008), but could also due to the presence of pseudogenes that are often present in more than one copy. In this

study, isolates of *C. decipiens*, formed a single clade based on BT sequences, but two distinct clades based on TEF sequences and they were identical based on ITS sequences. Because of these inconsistencies we have opted for a conservative approach where these isolates have been described as representing a single species. Similarly, isolates identified as representing *C. oblonga* were identical to the ex-type and other isolates of *C. savannae* based on TEF and ITS sequences, but differed from this species based on BT sequences. Differences were also found amongst *C. oblonga* isolates collected in the current study and those representing the ex-type isolate of the species both in the TEF and BT gene regions, suggesting that they represent closely related but distinct taxa. We have, however, refrained from describing them as distinct as more robust markers should be used to support this decision.

The two new *Ceratocystis* spp. described in this study, *C. salinaria* and *C. decipiens*, are members of the *C. moniliformis s.l.* species complex. Except for differences in growth rates and minor differences in the measurements of the ascomatal bases, ascomatal necks, ostiolar hyphae, conidiophores, conidia, ascospores, these fungi are virtually indistinguishable from each other based on morphology. Similar to other members of the *C. moniliformis s.l.* species complex, *C. salinaria* and *C. decipiens* are characterized by fast growing cultures and they produce strong fruity (banana) odor, on artificial media. Likewise, they produce ascomata with spiny bases and plate-like structures at the bases of the ascomatal necks as well as hat-shaped ascospores typical of species in the *C. moniliformis s.l.* complex. Most strains of *C. salinaria* sporulated readily on artificial media, producing ascomata and ascospore drops, whereas only one strain of *C. decipiens* was found to sporulate on artificial media, and this strain stopped sporulating after a single transfer to new media. *C. salinaria* and *C. decipiens* differed in their growth rates, the length of their ostiolar hyphae and the widths of their necks. Minor morphological differences were also found between these newly described species and *C. oblonga* and *C. savannae*, which are their closest phylogenetic relatives. The ascomatal neck lengths of both *C. salinaria* and *C. decipiens* as well as their bacilliform conidia were much shorter than those of *C. oblonga* and *C. savannae*. In addition, *C. decipiens* has necks with wider bases than those found in *C. oblonga* and *C. savannae*.

This study expands the host and geographic ranges of *C. savannae*, to now include *Eucalyptus* trees. *C. savannae* was first described from native trees in South Africa in the absence of disease (Kamgan Nkuekam *et al.* 2008). In this study, *C. savannae* was isolated from wounds on two *Eucalyptus* spp. grown in the Soutpansberg area of South Africa, as well as from a staphylinid beetle and from two nitidulid beetles collected in this area. Previous reports of *C. savannae* were from the Kruger National Park and Leeuwfontein Collaborative Nature Reserve, both in the

savanna regions in the eastern part of South Africa, similar to the Soutpansberg region. The discovery of *C. savannae* on eucalypts brings the host range of the fungus to seven tree species, spanning six genera and four families. The origin of *C. savannae* is unknown and it is not known whether it is a native fungus that has spread from native trees to infect non-native *Eucalyptus* trees, most probably mediated by insect dispersal, or whether it is an introduced pathogen that has adapted (Slippers *et al.* 2005) to other tree species.

C. oblonga was recently described from South Africa, associated with three nitidulid species, *Brachypepplus depressus*, *Carpophilus bisignatus* and *C. hemipterus* collected from both native savanna regions and from plantations of non-native *Acacia mearnsii* (Heath *et al.* 2009b). It was, therefore, not surprising to find *C. oblonga* on eucalypts associated with two nitidulid beetles, *B. depressus* and an unidentified *Carpophilus* sp. Results of this study expand the host range of the fungus in South Africa to include *Eucalyptus* trees and confirm the findings of Heath *et al.* (2009b) that nitidulid beetles are vectors of this fungus in South Africa.

Isolating *C. moniliformis* from *Eucalyptus* trees in this study was not surprising. The fungus has previously been reported from wounds on *E. grandis* trees in South Africa (Roux *et al.* 2004, Heath *et al.* 2009a) and Tanzania (Heath *et al.* 2009a). In this study, *C. moniliformis* was isolated from *E. maculata* grown in the Soutpansberg area and from *E. grandis* grown in the Sabie area. The fungus was also isolated from a staphylinid beetle infesting *E. grandis* trees in Sabie. This represents the first report of *C. moniliformis* from an insect and indicates that, like other *Ceratocystis* spp., the fungus is also probably vectored by a wide variety of insects, including staphylinid beetles.

Similar to previous studies involving eucalypt trees, *C. pirilliformis* was commonly found during the current study. The fungus was first described from wounds made on eucalypts in Australia (Barnes *et al.* 2003a) and was later discovered occurring on the same niche in South Africa (Roux *et al.* 2004, Kamgan Nkuekam *et al.* 2009). The current study expands the geographic and species host range of *C. pirilliformis* and it is thus now known from four Provinces in South Africa, spanning areas more than 2000km distant from each other. This study represents the first report of *C. pirilliformis* from an insect as a single strain of the fungus was isolated from a *B. depressus* collected in Lotebeni (Eastern Cape Province). It indicates that, like other *Ceratocystis* spp., the fungus is probably vectored by nitidulid insects. In recent population genetic studies of the fungus from South African collections, it was found that *C. pirilliformis* was probably introduced into South Africa, possibly from Australia, due to the low gene diversity and allelic richness of the fungus in South Africa (Kamgan Nkuekam *et al*. 2009).

C. eucalypticola, a recently described species in the *C. fimbriata s.l.* clade, has a broad geographic distribution on eucalypts, including South America, Africa and Asia (Van Wyk *et al.* 2011). Collections arising from this study confirm its association with insects for the first time. The fungus was isolated from two *Eucalyptus* spp., and from insects including staphylinid beetles, *Lithargus* sp., *Xyleborus affinis*, and three nitidulid species, *C. bisignatus*, *C. dimidiatus* and *B. depressus*.

Isolation of *T. basicola* from two nitidulid species, *B. depressus* and *Carpophilus* sp. was interesting. This fungus is known as a pathogen of several vegetable crops world-wide, including South Africa where it causes black pod rot of groundnuts (*Arachis hypogaea* L.) and black root rot of chicory (*Cichorium intybus* L.) (Prinsloo 1980, Prinsloo *et al.* 1991, Labuschagne & Kotze 1991, Geldenhuis *et al.* 2006). Population genetic studies using microsatellite markers have shown that two genotypes of *T. basicola* occur in South Africa, and that the fungus was most likely introduced into the country from Europe, probably through the distributions of root crops to the rest of the world (Geldenhuis *et al.* 2006). Nitidulid beetles in general pupate in soil or in wood infested by fungi, and also feed on a wide variety of substrates such as flowers, stored crop products and fungi (Hinton 1945, Habeck 2002). These insects could have acquired the fungus from one of these substrates before flying to cut stumps where they were collected. Yet the results suggest that the fungus is able to leave the soil environment and given its pathogenicity, it could contribute to disease in some cases.

T. thielavoidea was isolated from nitidulids in this study and this is the first report of this fungus from South Africa. The fungus was isolated from *B. depressus* and from an unknown *Carpophilus* sp. collected in the KwaZulu Natal Province. *T. thielavoidea* like other species in the genus *Thielaviopsis* is a soil borne fungal pathogen mainly of agronomic crops. It produces dark and thick-walled aleurioconidia also known as chlamydospores that enable it to survive in the soil for many years (Paulin-Mahady *et al.* 2002). Finding the fungus from insects is a possible indication that nitidulid insects spread the fungus in South Africa. Future studies considering the epidemiology of *T. thielavoidea* in concert with nitidulid insects is required to confirm this hypothesis.

This study represents the first report of *Ceratocystis* spp. from Staphylinidae. Four *Ceratocystis* spp., *C. eucalypticola*, *C. moniliformis*, *C. decipiens* and *C. savannae* were isolated from these insects. The Staphylinidae is one of the larger families of the Coleoptera (Lawrence & Newton 1995), which includes many predaceous and mycophagous species feeding mainly on macrofungi (Lawrence & Milner 1996). These insects are likely casual vectors of *Ceratocystis* spp., with no

fixed association given the number of *Ceratocystis* spp. that was isolated from them in a relatively limited study.

This study represents the most comprehensive consideration of *Ceratocystis* spp. and insects on *Eucalyptus* trees in South Africa. The number of species identified as well as insects from which they were isolated shows that the diversity of *Ceratocystis* spp. on *Eucalyptus* is still poorly understood in South Africa and even more so in other parts of the world. Future studies should explore the diversity of *Ceratocystis* spp. on native trees as well as on *Eucalyptus* trees in South Africa and in other African countries and will likely reveal numerous species of the fungi, some of which could be important tree pathogens.

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Table 1. Best fit models of evolution for each gene region used in distance analyses (Bayesian and Maximum Likelihood).

	Type of Analyses	ITS	BT	TEF	Combined tree
C. fimbriata s.l.	Bayesian	GTR+G	HKY+I	HKY	GTR+I+G
	Maximum likelihood	TVM+G (Rates=gamma, Shape=0.3029, Pinvar=0)	TrN+I (Rates=equal, Pinvar=0.5812)	TrN (Rates=equal, Pinvar=0)	TVM+I+G (Rates=gamma, Shape=0.5904, Pinvar=0.3169)
C. moniliformis s l.	Bayesian	HKY+I	HKY+I	K80+I	(HKY+I)
	Maximum likelihood	HKY+I (Rates=equal, Pinvar=0.9261)	TrN+I (Rates=equal, Pinvar=0.8006)	K80+I (Rates=equal, Pinvar=0.4669)	HKY+I (Rates=equal, Pinvar=0.8017)

Table 2. Fungi isolated from *Eucalyptus* trees during surveys in South Africa.

Provinces	locations	Climatic Types	CMW	Nber of	ID	Morpho-group	Hosts
Eastern Cape	Lotobeni	temperate	28204, 28205, 28206	3	*C. pirilliformis*	A2	*E. grandis*
"	Kumbo	temperate	27181, 27182	2	*C. pirilliformis*	A2	*E. grandis*
KwaZulu Natal	KwaMbonambi	subtropical	24984, 24975, 24976, 24979, 24980, 24974, 24952	7	*C. eucalypticola*	A1	*Eucalyptus* spp.
"	Pietermaritzburg	subtropical	24955, 24957, 24958, 24960, 24961, 24962, 24963, 24965, 24967, 24969, 24970, 24972	12	*C. eucalypticola*	A1	*Eucalyptus* spp.
Limpopo	Goedehoop	subtropical	26472, 26466	2	*C. pirilliformis*	A2	*E. cloeziana*
Limpopo	Soutpansberg	subtropical	30888, 30860, 30889, 30861, 30890, 30891	6	*C. eucalypticola*	A1	*E. saligna*
Limpopo	Soutpansberg	subtropical	30702, 30703, 30704	3	*C. salinaria*	B	*E. saligna*
"	Soutpansberg	subtropical	30701	1	*C. decipiens*	B	*E. saligna*
Limpopo	Goedehoop	subtropical	25920	1	*C. savannae*	B	*E. cloeziana*
"	Soutpansberg	subtropical	25909, 25915, 25916	3	*C. savannae*	B	*E. maculata*
"	Soutpansberg	subtropical	25910, 25911, 25913	3	*C. salinaria*	B	*E. maculata*
"	Goedehoop	subtropical	25917	1	*C. salinaria*	B	*E. cloeziana*
"	Soutpansberg	subtropical	25912	1	*C. moniliformis*	B	*E. maculata*
"	"	"	25914	1	*C. decipiens*	B	*E. maculata*
"	Goedehoop	subtropical	25918, 25919	2	*C. decipiens*	B	*E. cloeziana*
Mpumalanga	Sabie	subtropical	30892, 30893, 30894, 30895, 30896, 30897, 30898	7	*C. eucalypticola*	A1	*E. grandis*
"	Sabie	subtropical	30698	1	*C. oblonga*	B	*E. grandis*
"	Sabie	subtropical	30699, 30700	2	*C. moniliformis*	B	*E. grandis*
Tzaneen	Tzaneen	subtropical	25001, 24991, 24989, 24998, 24994, 25012, 25008, 25025, 25021, 25019, 25017, 25015,	13	*C. eucalypticola*	A1	*E. grandis*
Western Cape	Cape Town	mediteranean	28200, 29822	2	*C. pirilliformis*	A2	*Eucalyptus*
"	Cape Town	mediteranean	27162, 27163	2	*C. pirilliformis*	A2	*E. diversicolor*
"	Cape Town	mediteranean	27183, 27184, 27185, 27186, 27187, 27188	6	*C. pirilliformis*	A2	*E. saligna*
"	George	mediteranean	27047, 27155, 27048, 27157, 27049, 27050, 27051, 27052, 27053, 27054, 27259, 27055,	17	*C. pirilliformis*	A2	*Eucalyptus* spp.
Western Cape	George	mediteranean	27006, 27007	2	*C. salinaria*	B	*Eucalyptus* spp.
Total				**100**			

Table 3. Fungi isolated from insects infesting *Eucalyptus* stumps during surveys in South Africa.

Provinces	Locations	Climatic types	CMW nbers.	Nber of	ID	Morpho-group	Insect hosts	Tree Hosts
Eastern Cape	Lotobeni	temperate	29728, 29729, 29730	3	*T. basicola*	C	*B. depressus*	*E. grandis*
Eastern Cape	Lotebeni	temperate	29825	1	*C. pirilliformis*	A2	*B. depressus*	*E. grandis*
KwaZulu Natal	KwaMbonambi	subtropical	25037, 25040, 25041, 25045, 25046	5	*C. eucalypticola*	A1	*B. depressus*	*Eucalyptus* spp.
"	Pietermaritzburg	subtropical	25073	1	*C. eucalypticola*	A1	*B. depressus*	*Eucalyptus* spp.
KwaZulu Natal	Kwa-Mbonambi	subtropical	25052, 25053, 25054, 25056, 25057, 25062,	10	*C. eucalypticola*	A1	*Carpophilus* spp.	*Eucalyptus* spp.
KwaZulu Natal	Kwa-Mbonambi	subtropical	25039, 25043, 25044	3	*T. thielavioides*	C	*B. depressus*	*Eucalyptus* spp.
"	"	subtropical	25063, 25047, 25049	3	*T. thielavioides*	C	*Carpophilus* spp.	*Eucalyptus* spp.
Limpopo	Soutpansberg	subtropical	26360	1	*C. eucalypticola*	A1	*Xyleboris affinis*	*E. maculata*
"	"	subtropical	26355, 26356, 26357, 26358, 26359	5	*C. eucalypticola*	A1	*Litargus* sp.	*E. maculata*
"	"	subtropical	31211, 31222, 31212, 31215, 31216, 31219,	13	*C. eucalypticola*	A1	*B. depressus*	*E. saligna*
"	"	subtropical	31220, 31213, 31197, 26341, 26332, 26339, 26337, 26340, 26338,	14	*C. eucalypticola*	A1	*B. depressus*	*E. maculata*
Limpopo	Soutpansberg	subtropical	26335, 26336, 26326, 26333, 26330, 26334, 31228, 31231, 31232	3	*C. eucalypticola*	A1	*C. bisignatus*	*E. saligna*
		subtropical	31230, 31234, 31227,	3	*C. eucalypticola*	A1	*C. dimidiatus*	
		subtropical	31224, 31223, 31200, 31229, 31198, 31225,	9	*C. eucalypticola*	A1	*Carpophilus* spp.	
"	"	subtropical	31233, 31199, 31226 26235, 26236, 26237, 26238, 26351	5	*C. eucalypticola*	A1	*Carpophilus* spp.	*E. maculata*
Limpopo	Soutpansberg	subtropical	26244, 26246, 26241, 26243, 26245, 26239,	8	*C. eucalypticola*	A1	*C. bisignatus*	*E. maculata*
"	"	subtropical	26352, 26353, 26354,	8	*C. eucalypticola*	A1	*C. dimidiatus*	*E. maculata*
"	"	subtropical	26247, 26248, 26249, 31241, 31204, 31203, 31247, 31240, 31252, 31246, 31239, 31251,	23	*C. eucalypticola*	A1	*Staphylinid* spp.	*E. saligna*
Limpopo	Soutpansberg	subtropical	31245, 31238, 31202, 30846, 30836	2	*C. savannae*	B	*C. dimidiatus*	*E. saligna*
"	"	subtropical	30839, 30847, 30848,	4	"	B	*C. bisignatus*	"
"	"	subtropical	30850, 30844, 30842,	6	"	B	*Carpophilus* spp.	"
"	"	subtropical	30841, 30851, 30852, 30824, 30825, 30828,	6	"	B	*B. depressus*	*E. saligna*
"	"	subtropical	30831, 30832, 30833 30857	1	"	B	*Staphylinid sp.*	*E. saligna*
"	"	subtropical	30835, 30837	2	*C. oblonga*	B	*C. dimidiatus*	*E. saligna*
"		subtropical	30838, 30840	2	"	B	*C. bisignatus*	"
"		subtropical	30845	1	"	B	*Carpophilus spp.*	"
"	"	subtropical	30827	1	"	B	*B. depressus*	*E. saligna*
"	"	subtropical	30834, 30829, 30830	3	*C. decipiens*	B	"	*E. saligna*
"	"	subtropical	30855, 30853	2	"	B	*Staphylinid.* sp.	*E. saligna*

Mpumalanga	Sabie	subtropical	31196	1	*C. eucalypticola*	A1	*B. depressus*	*E. grandis*
"	"	subtropical	31207, 31208	2	*C. eucalypticola*	A1	*C. bisignatus*	*E. grandis*
"	"	subtropical	31259	1	*C. eucalypticola*	A1	*Carpophilus* spp.	
Mpumalanga	Sabie	subtropical	31206, 31258, 31257,	7	*C. eucalypticola*	A1	*Staphylinid* spp.	*E. grandis*
Mpumalanga	Sabie	subtropical	31205, 31256, 31255, 30856, 30858, 30859	3	*C. moniliformis*	B	*Staphylinid*	*E. grandis*
Total				**160**				

Table 4. List of *Ceratocystis* isolates from GenBank used in comparative studies and representative isolates sequenced in this study (*) and deposited in GenBank.

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
C. acacivora	CMW22562	EU588655	ITS	NA	*Acacia mangium*	M. Tarigan	Indonesia
		EU588635	BT	"	"	"	"
		EU588645	EF	"	"	"	"
	CMW22563	EU588656	ITS	NA	"	"	"
		EU588636	BT	"	"	"	"
		EU588646	EF	"	"	"	"
C. albifundus	CMW5329	AF388947	ITS	NA	*A. mearnsii*	J. Roux	Uganda
		DQ371649	BT	"	"	"	"
		EF070401	EF	"	"	"	"
	CMW4068	DQ520638	ITS	NA	*A. mearnsii*	J. Roux	South Africa
		EF070429	BT	"	"	"	"
		EF070400	EF	"	"	"	"
C. atrox	CMW19383	EF070414	ITS	CBS120517	*E. grandis*	MJ. Wingfield	Australia
		EF070430	BT	"	"	"	"
		EF070402	EF	"	"	"	"
	CMW19385	EF070415	ITS	CBS120518	*E. grandis*	MJ. Wingfield	Australia
		EF070431	BT	"	"	"	"
		EF070403	EF	"	"	"	"
C. bhutanensis	CMW8399	AY528959	ITS	CBS115772, BH 8/8	*Picea spinulosa*	T. Kirisits & DB.	Bhutan
		AY528964	BT	"	"	"	"
		AY528954	EF	"	"	"	"
	CMW8215	AY528958	ITS	CBS114290, PREM57805	*P. spinulosa*	T. Kirisits & DB. Chhetri	Bhutan
		AY528963	BT	"	"	"	"
		AY528953	EF	"	"	"	"
C. caryae	CMW14793	EF070424	ITS	CBS114716	*Carya cordiformis*	J. Johnson	USA
		EF070439	BT	"	"	"	"
		EF070412	EF	"	"	"	"
	CMW14808	EF070423	ITS	CBS115168	*C. ovata*	"	USA
		EF070440	BT	"	"	"	"
		EF070411	EF	"	"	"	"
C. colombiana	CMW5751	AY177233	ITS	CBS121792	*Coffea arabica*	M. Marin	Colombia
		AY177225	BT	"	"	"	"
		EU241493	EF	"	"	"	"
	CMW5761	AY177234	ITS	CBS121791	"	B. Castro	"
		AY177224	BT	"	"	"	"
		EU241492	EF	"	"	"	"
C. decipiens	*CMW30855	HQ203216	ITS	NA	*Staphylinid.* sp.	GNK. Kamgan & J.	South Africa
		HQ203233	BT	"	"	"	"
		HQ236435	EF	"	"	"	"
	*CMW25918	HQ203218	ITS	"	*E. cloeziana*	"	"
		HQ203235	BT	"	"	"	"
		HQ236437	EF	"	"	"	"
	*CMW25914	HQ203219	ITS	"	*E. maculata*	"	"
		HQ203236	BT	"	"	"	"

		HQ236438	EF	"	"	"	"
	*CMW30830	HQ203217	ITS	"	*B. depressus*	"	"
		HQ203234	BT	"	"	"	"
		HQ236436	EF	"	"	"	"
C. eucalypticola	CMW11536	FJ236723	ITS	CBS124016	*Eucalyptus* spp.	M. van Wyk	South Africa
		FJ236783	BT	"	"	"	"
		FJ236753	EF	"	"	"	"
	CMW10000	FJ236722	ITS	CBS124019	*Eucalyptus* spp.	M. van Wyk	"
		FJ236782	BT	"	"	"	"
		FJ236752	EF	"	"	"	"
	*CMW25015	HQ203224	ITS	NA	*E. grandis*	GNK. Kamgan & J.	"
		HQ203241	BT	"	"	"	"
		HQ236443	EF	"	"	"	"
	*CMW24984	HQ203225	ITS	NA	*Eucalyptus* sp.	"	"
		HQ203242	BT	"	"	"	"
		HQ236444	EF	"	"	"	"
C. fimbriata	CMW1547	AF264904	ITS	NA	*Ipomoea batatas*	NA	Papua N.
		EF070443	BT	"	"	"	"
		EF070395	EF	"	"	"	"
	CMW15049	DQ520629	ITS	CBS141.37	*I. batatas*	CF. Andrus	USA
		EF070442	BT	"	"	"	"
		EF070394	EF	"	"	"	"
C. fimbriatomima	CMW24174	EF190963	ITS	CBS121786	*Eucalyptus* sp.	MJ. Wingfield	Venezuela
		EF190951	BT	"	"	"	"
		EF190957	EF	"	"	"	"
	CMW24176	EF190964	ITS	CBS121787	*Eucalyptus* sp.	"	"
		EF190952	BT	"	"	"	"
		EF190958	EF	"	"	"	"
C. inquinans	CMW21106	EU588587	ITS	NA	*A.mangium*	M. Tarigan	Indonesia
		EU588666	BT	"	"	"	"
		EU588674	EF	"	"	"	"
	CMW21107	EU588588	ITS	"	"	"	"
		EU588667	BT	"	"	"	"
		EU588675	EF	"	"	"	"
C. larium	CMW25436	EU881908	ITS	CBS122607	*Styrax benzoin*	MJ. Wingfield	Indonesia
		EU881896	BT	"	"	"	"
		EU881902	EF	"	"	"	"
	CMW25437	EU881909	ITS	NA	"	"	"
		EU881897	BT	"	"	"	"
		EU881903	EF	"	"	"	"
C. manginecans	CMW13851	AY953383	ITS	"	*Mangifera indica*	M. Deadman	Oman
		EF433308	BT	"	"	"	"
		EF433317	EF	"	"	"	"
	CMW13852	AY953384	ITS	"	*Hypocryphalus*	"	"
		EF433309	BT	"	"	"	"
		EF433318	EF	"	"	"	"
C. microbasis	CMW21115	EU588592	ITS	"	*A.mangium*	M. Tarigan	Indonesia
		EU588671	BT	"	"	"	"
		EU588679	EF	"	"	"	"
	CMW21117	EU588593	ITS	"	"	"	"

		EU588672	BT	"	"	"	"
		EU588680	EF	"	"	"	"
C. moniliformis	CMW9590	AY431101	ITS	CBS116452	*Eucalyptus grandis*	J. Roux	South Africa
		AY528985	BT	"	"	"	"
		AY529006	EF	"	"	"	"
	CMW8379	AY528995	ITS	NA	*Cassia fistula*	MJ. Wingfield	Bhutan
		AY529005	BT	"	"	"	"
		AY529016	EF	"	"	"	"
	*CMW30856	HQ203211	ITS	"	*Staphylinid* sp.	GNK. Kamgan & J. Roux	South Africa
		HW203228	BT	"	"	"	"
		HQ236430	EF	"	"	"	"
	*CMW30700	HQ203212	ITS	"	*E. grandis*	"	"
		HQ203229	BT	"	"	"	"
		HQ236431	EF	"	"	"	"
C. moniliformopsis	CMW10214	AY528999	ITS	CBS115792, ORB33	*E. sieberi*	MJ. Dudzinski	Australia
		AY528988	BT	"	"	"	"
		AY529009	EF	"	"	"	"
	CMW9986	AY528998	ITS	CBS109441	*E. obliqua*	ZQ. Yuan	Australia
		AY528987	BT	"	"	"	"
		AY529008	EF	"	"	"	"
C. neglecta	CMW17808	EF127990	ITS	CBS121789	*Eucalyptus* sp.	MJ. Wingfield	Colombia
		EU881898	BT	"	"	"	"
		EU881904	EF	"	"	"	"
	CMW18194	EF127991	ITS	CBS121017	*Eucalyptus* sp.	"	"
		EU881899	BT	"	"	"	"
		EU881905	EF	"	"	"	"
C. oblonga	CMW23802	EU245020	ITS	CBS122820	*A. mearnsii*	RN. Heath	South Africa
		EU244992	BT	"	"	"	"
		EU244952	EF	"	"	"	"
	CMW23803	EU245019	ITS	CBS122291	*A. mearnsii*	"	"
		EU244991	BT	"	"	"	"
		EU244951	EF	"	"	"	"
	*CMW30698	HQ203220	ITS	NA	*E. nitens*	GNK. Kamgan & J. Roux	South Africa
		HQ203237	BT	"	"	"	"
		HQ236439	EF	"	"	"	"
	*CMW30835	HQ203221	ITS	"	*C. dimidiatus*	"	"
		HQ203238	BT	"	"	"	"
		HQ236440	EF	"	"	"	"
C. obpyriformis	CMW23807	EU245004	ITS	CBS122608	*A. mearnsii*	RN. Heath	South Africa
		EU244976	BT	"	"	"	"
		EU244936	EF	"	"	"	"
	CMW23808	EU245003	ITS	CBS122511	"	"	"
		EU244975	BT	"	"	"	"
		EU244935	EF	"	"	"	"
C. omanensis	CMW11048	DQ074742	ITS	CBS115780, PREM57815	*Mangifera indica*	AO. Al-Adawi	Oman
		DQ074732	BT	"	"	"	
		DQ074737	EF	"	"	"	
	CMW3777	DQ074740	ITS	NA	*M. indica*	AO. Al-Adawi	Oman

		DQ074730	BT	"	"	"	
		DQ074735	EF	"	"	"	
	CMW11046	DQ074739	ITS	CBS118112, PREM57814	*M. indica*	AO. Al-Adawi	Oman
		DQ074729	BT	"	"	"	
		DQ074734	EF	"	"	"	
C. papillata	CMW8850	AY233866	ITS	CBS121794	*Citrus x tangelo*	MJ. Wingfield	Colombia
		AY233875	BT	"	"	"	"
		EU241485	EF	"	"	"	"
	CMW8856	AY233867	ITS	CBS121793	*Citrus lemon*	"	"
		AY233874	BT	"	"	"	"
		EU241484	EF	"	"	"	"
C. pirilliformis	CMW6569	AF427104	ITS	PREM57322, DAR75993	*E. nitens*	MJ. Wingfield	Australia
		DQ371652	BT	"	"	"	
		AY528982	EF	"	"	"	
	CMW6579	AF427105	ITS	PREM57323, DAR75996	*E. nitens*	MJ. Wingfield	Australia
		DQ371653	BT	"	"	"	"
		AY528983	EF	"	"	"	"
	*CMW29822	HQ203227	ITS	NA	*Eucalyptus* log	GNK. Kamgan & J. Roux	South Africa
		HQ203244	BT	"	"	"	"
		HQ236446	EF	"	"	"	"
	*CMW29825	HQ203226	ITS	"	*B. depressus*	"	"
		HQ203243	BT	"	"	"	"
		HQ236445	EF	"	"	"	"
C. platani	CMW14802	DQ520630	ITS	CBS115162	*Platanus occidentalis*	TC. Harrington	USA
		EF070425	BT	"	"	"	"
		EF070396	EF	"	"	"	"
	CMW23918	EU426554	ITS	NA	"	MJ. Wingfield	Greece
		EU426555	BT	"	"	"	"
		EU426556	EF	"	"	"	"
C. polychroma	CMW11455	AY528973	ITS	CBS115774, PREM57822	*Syzygium aromaticum*	ECY. Liew & MJ. Wingfield	Indonesia
		AY528969	BT	"	"	"	
		AY528981	EF	"	"	"	
	CMW11436	AY528971	ITS	CBS115777, PREM57819	*S. aromaticum*	ECY. Liew & MJ. Wingfield	Indonesia
		AY528967	BT	"	"	"	
		AY528979	EF	"	"	"	
	CMW11449	AY528972	ITS	CBS115775, PREM57821	*S. aromaticum*	ECY. Liew & MJ. Wingfield	Indonesia
		AY528968	BT	"	"	"	
		AY528980	EF	"	"	"	
C. polyconidia	CMW23809	EU245006	ITS	CBS122289	*A. mearnsii*	RN. Heath	South Africa
		EU244978	BT	"	"	"	"
		EU244938	EF	"	"	"	"
	CMW23818	EU245007	ITS	CBS122290	"	"	"
		EU244979	BT	"	"	"	"
		EU244939	EF	"	"	"	"
C. populicola	CMW14789	EF070418	ITS	CBS119.78	*Populus* sp	J. Gremmen	Poland
		EF070434	BT	"	"	"	"
		EF070406	EF	"	"	"	"

	CMW14819	EF070419	ITS	CBS114725	*Populus* sp.	T. Hinds	USA
		EF070435	BT	"	"	"	"
		EF070407	EF	"	"	"	"
C. salinaria	*CMW25911	HQ203213	ITS	NA	*E. maculata*	GNK. Kamgan & J. Roux	South Africa
		HQ203230	BT	"	"	"	"
		HQ236432	EF	"	"	"	"
	*CMW30702	HQ203215	ITS	"	*E. saligna*	"	"
		HQ203232	BT	"	"	"	"
		HQ236434	EF	"	"	"	"
	*CMW30703	HQ203214	ITS	"	*E. saligna*	"	"
		HQ203231	BT	"	"	"	"
		HQ236433	EF	"	"	"	"
C. savannae	CMW17300	EF408551	ITS	PREM59423	*Acacia nigrescens*	GNK. Kamgan & J. Roux	South Africa
		EF408565	BT	"	"	"	"
		EF408572	EF	"	"	"	"
	CMW17297	EF408552	ITS	NA	*Combretum zeyheri*	GNK. Kamgan & J. Roux	South Africa
		EF408566	BT	"	"	"	"
		EF408573	EF	"	"	"	"
	*CMW30828	HQ203223	ITS	"	*B. depressus*	GNK. Kamgan & J. Roux	South Africa
		HQ203240	BT	"	"	"	"
		HQ236442	EF	"	"	"	"
	*CMW30846	HQ203222	ITS	"	*C. dimidiatus*	"	"
		HQ203239	BT	"	"	"	"
		HQ236441	EF	"	"	"	"
C. smalleyi	CMW14800	EF070420	ITS	CBS114724	*Carya cordiformis*	G. Smalley	USA
		EF070436	BT	"	"	"	"
		EF070408	EF	"	"	"	"
C. sumatrana	CMW21109	EU588589	ITS	NA	*A.mangium*	M. Tarigan	Indonesia
		EU588668	BT	"	"	"	"
		EU588676	EF	"	"	"	"
	CMW21111	EU588590	ITS	"	"	"	"
		EU588669	BT	"	"	"	"
		EU588677	EF	"	"	"	"
C. tanganyicensis	CMW15992	EU244999	ITS	CBS122293	*A. mearnsii*	RN. Heath & J. Roux	Tanzania
		EU244971	BT	"	"	"	"
		EU244931	EF	"	"	"	"
	CMW15999	EU244998	ITS	CBS122294	"	"	"
		EU244970	BT	"	"	"	"
		EU244939	EF	"	"	"	"
C. tribiliformis	CMW13015	AY529004	ITS	CBS115949	*Quercus* sp.	MJ. Wingfield	Indonesia
		AY528994	BT	"	"	"	"
		AY529015	EF	"	"	"	"
	CMW13013	AY529003	ITS	CBS115866	*Quercus* sp.	MJ. Wingfield	Indonesia
		AY528993	BT	"	"	"	"
		AY529014	EF	"	"	"	"
C. tsitsikammensis	CMW14276	EF408555	ITS	PREM59424	*Rapanea melanophloeos*	GNK. Kamgan & J. Roux	South Africa
		EF408569	BT	"	"	"	"

		EF408576	EF	"	"	"	"
	CMW14278	EF408556	ITS	NA	"	"	"
		EF408570	BT	"	"	"	"
		EF408577	EF	"	"	"	"
C. variospora	CMW20935	EF070421	ITS	CBS114715	*Quercus alba*	J. Johnson	USA
		EF070437	BT	"	"	"	"
		EF070409	EF	"	"	"	"
	CMW20936	EF070422	ITS	CBS114714	*Q. robur*	D. Houston	USA
		EF070438	BT	"	"	"	"
		EF070410	EF	"	"	"	"
C. virescens	CMW3276	DQ061281	ITS	NA	*Quercus* sp.	T. Hinds	USA
		AY528990	BT	"	"	"	
		AY529011	EF	"	"	"	
C. zombamontana	CMW15235	EU245002	ITS	CBS122297	*Eucalyptus* spp.	RN. Heath & J. Roux	Malawi
		EU244974	BT	"	"	"	"
		EU244934	EF	"	"	"	"
	CMW15236	EU245000	ITS	CBS122296	"	"	"
		EU244972	BT	"	"	"	"
		EU244932	EF	"	"	"	"

Table 5. Tree statistics for Maximum Parsimony analysis.

		ITS	BT	TEF	Concatenated tree
C. fimbriata s.l.	Total Characters	533	541	197	1251
	Parsimony informative characters	189	98	50	337
	Parsimony uninformative characters	28	35	27	90
	Constant characters	316	388	120	824
	Tree length	449	192	99	766
	CI	0.686	0.812	0.899	0.722
	RI	0.895	0.938	0.960	0.902
C. moniliformis s.l.	Total Characters	448	442	296	1186
	Parsimony informative characters	16	40	97	153
	Parsimony uninformative characters	0	4	6	10
	Constant characters	432	398	193	1023
	Tree length	20	62	150	245
	CI	0.900	0.887	0.807	0.792
	RI	0.985	0.980	0.956	0.955

Table 6. Summary of polymorphic nucleotides found within the BT gene region generated from phylogenetic analyses and showing differences between *C. salinaria*, *C. decipiens* strains and closest related taxa.

Isolates	BT																	
	14	116	126	127	131	134	138	139	140	141	143	150	158	167	168	254	323	335
CMW17300-*C. savannae*_T	C	T	C	T	T	C	A	T	C	A	A	G	C	T	C	C	T	T
CMW17297-*C. savannae*_T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30824-*C. savannae*	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.
CMW30846-*C. savannae*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30839-*C. savannae*	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW23803-*C. oblonga*_T	.	C	T	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.
CMW23802-*C. oblonga*_T	.	C	T	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.
CMW30845-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	A	.	.	.
CMW30835-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	A	.	.	.
CMW30698-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	.	.	.	.
CMW25918-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW25914-*C. decipiens*	.	C	.	C	.	A	.	C	.	.	.	A	.	.	.	.	C	C
CMW30701-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30855-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30830-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30704-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	C	C
CMW30703-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C
CMW30702-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C
CMW25911-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C

Table 7. Summary of polymorphic nucleotides found within the TEF gene region generated from phylogenetic analyses and showing differences between *C. salinaria*, *C. decipiens* strains and closest related taxa.

Isolates	TEF																														
	4	6	9	10	16	18	29	32	34	35	36	46	50	55	61	65	71	75	83	123	130	139	168	186	233	256	258	259	262	275	284
CMW17300-*C. savannae*	C	T	A	T	C	-	G	T	A	T	G	G	G	T	A	G	C	T	G	C	C	G	T	A	G	A	A	G	T	T	A
CMW17297-*C. savannae*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30824-*C. savannae*	.	.	.	.	.	-	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
CMW30846-*C. savannae*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30839-*C. savannae*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
CMW23803-*C. oblonga*	T	C	G	C	T	-	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.
CMW23802-*C. oblonga*	T	C	G	C	T	-	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.
CMW30845-*C. oblonga*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30835-*C. oblonga*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
CMW30698-*C. oblonga*	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW25918-*C. decipiens*	.	.	.	.	.	-	C	.	G	G	.	A	A	.	G	A	.	C	.	G	T	A	A	.	C	.	.	.	C	.	.
CMW25914-*C. decipiens*	.	.	.	.	.	-	C	.	G	G	.	A	A	.	G	A	.	C	.	G	T	A	A	.	C	.	.	.	C	.	.
CMW30701-*C. decipiens*	.	.	.	.	.	-	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30855-*C. decipiens*	.	.	.	.	.	-	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30830-*C. decipiens*	.	.	.	.	.	-	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30704-*C. salinaria*	.	.	.	.	.	T	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW30703-*C. salinaria*	.	.	.	.	.	-	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW30702-*C. salinaria*	.	.	.	.	.	-	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW25911-*C. salinaria*	.	.	.	.	.	-	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.

Table 8. Measures of relevant fruiting structures comparing *C. salinaria*, *C. decipiens* and closest related taxa.

Relevant structures and growth rate	***C. salinaria***	***C. decipiens***	***C. oblonga***	***C. savannae***
Growth rate	43 mm/3d at 30ºC	39 mm/3d at 30ºC	83 mm/7d at 25ºC	58 mm/4d at 30ºC
Ostiolar hyphae	19.5 – 123.5 µm	13 – 35.5 µm	22 – 31 µm	17 – 46 µm
Tip of necks	11.5 – 23 µm	9.5 – 16 µm	12 – 23 µm	13 – 23.5 µm
Middle of necks	19 – 31.5 µm	17.5 – 27.5 µm	NA	24 – 39 µm
Neck length	297.5 – 592 µm	355 – 596.5 µm	405 – 881 µm	359.5 – 755 µm
Neck bases	43 – 88 µm	47 – 102.5 µm	30 – 76 µm	37 – 62 µm
Ascomatal bases	138 – 272 x 124 – 232 µm	132.5 – 258.5 x 108 – 244 µm	149 – 372 x 130 – 315 µm	155 – 248 x 155 – 248 µm
Conidiophores	18.5 – 39.5 x 2 – 3.5 µm	15.5 – 35 x 2 – 4 µm	19 – 41 x 2 – 4 µm	15.9 – 52.2 x 2.4 – 5 µm
Ascospores	4.5 – 6 x 2.5 – 4 µm	4 – 5.5 x 2 – 3.5 µm	3 – 4 x 4 – 6 µm	4.5 – 5.8 x 2.2 – 3.7 µm
Conidia	Oblong: 5 – 9 x 1.5 – 3.5 µm	4.5 – 7.5 x 1.5 – 3.5 µm	5 – 9 x 3 – 6 µm	3.84 – 6.16 x 2.2 – 3.7 µm
	Bacilliform: 6.5 – 10.5 x 1 – 3 µm	5 – 10.5 x 1 – 2.5 µm	12 – 23 x 3 – 5 µm	5.35 – 10.27 x 1.71 – 3.94 µm

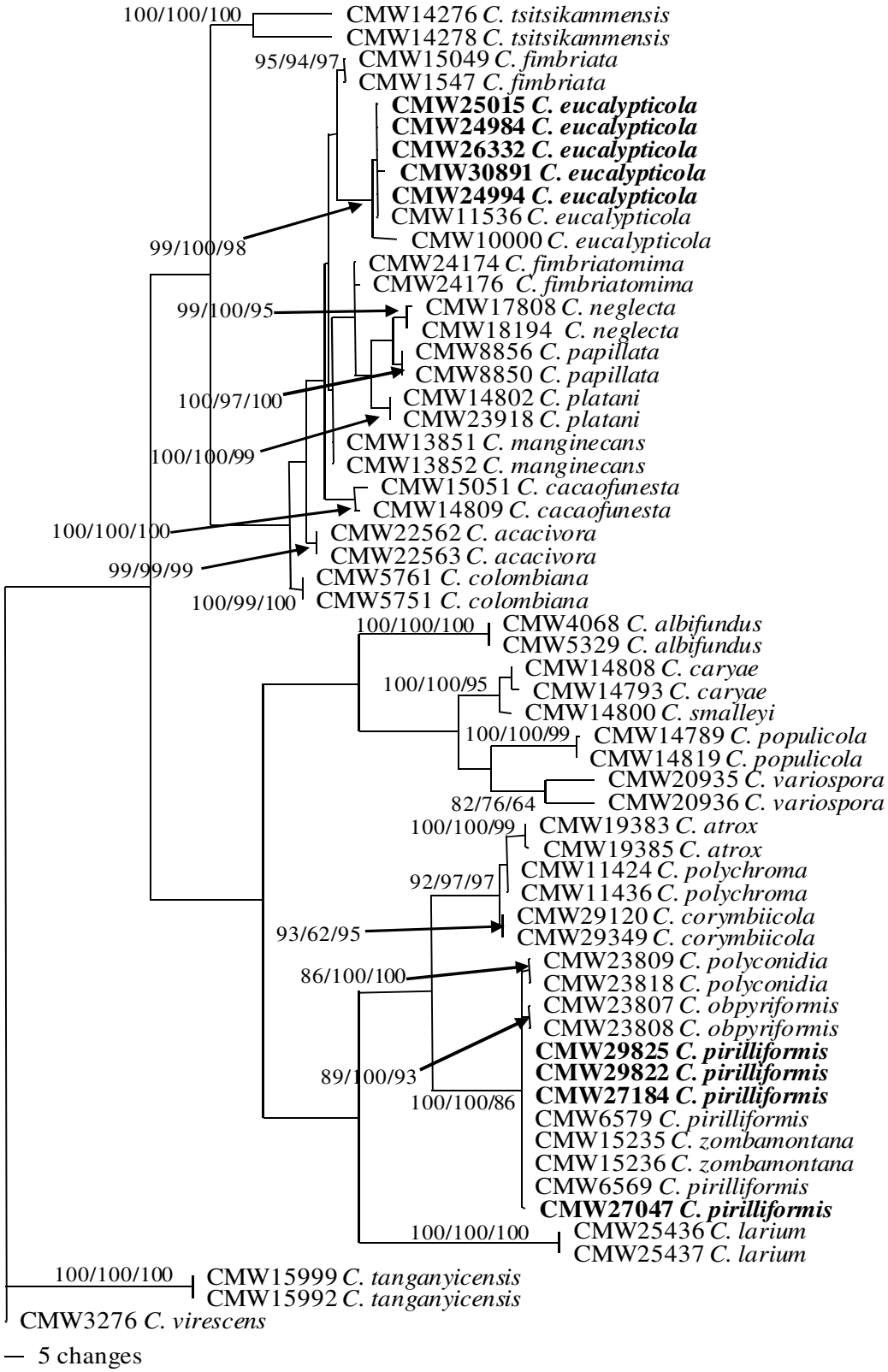

Figure 1: Phylogenetic tree produced from a heuristic search of the ITS sequence data, showing the relationship between members of *C. fimbriata s.l.* Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

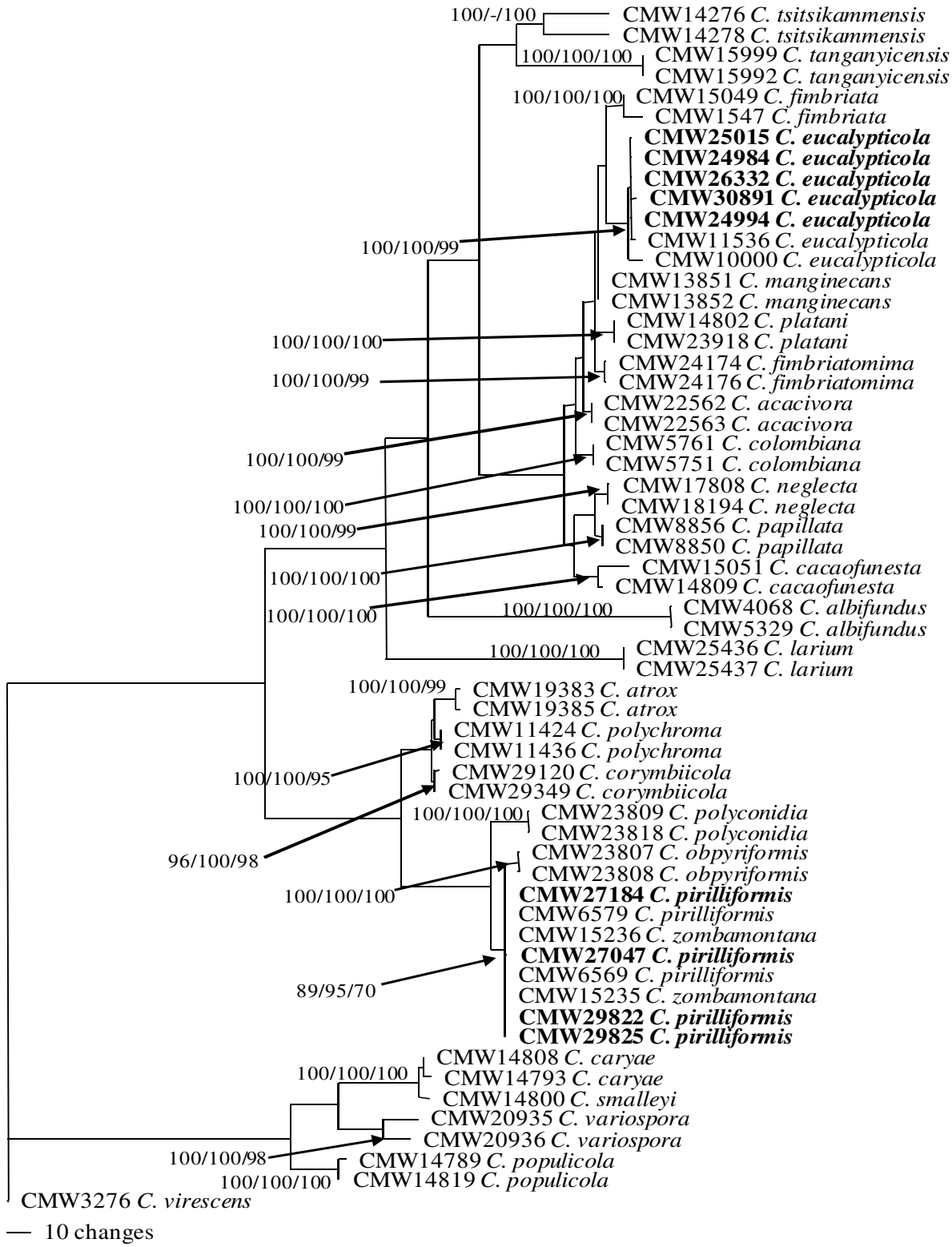

Figure 2: Concatenated tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between members of *C. fimbriata s.l.* Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

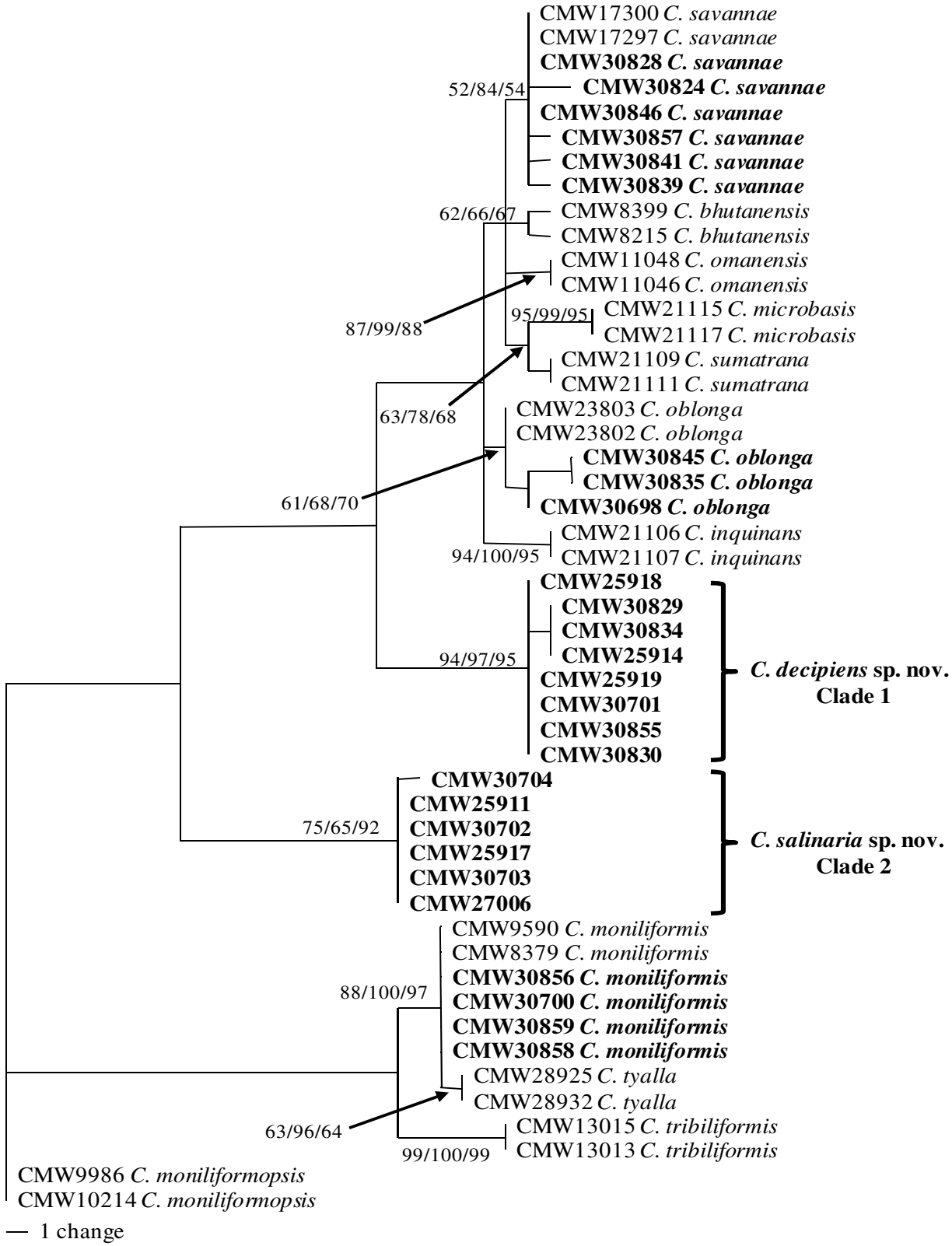

Figure 3: Phylogenetic tree produced from a heuristic search of the BT sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

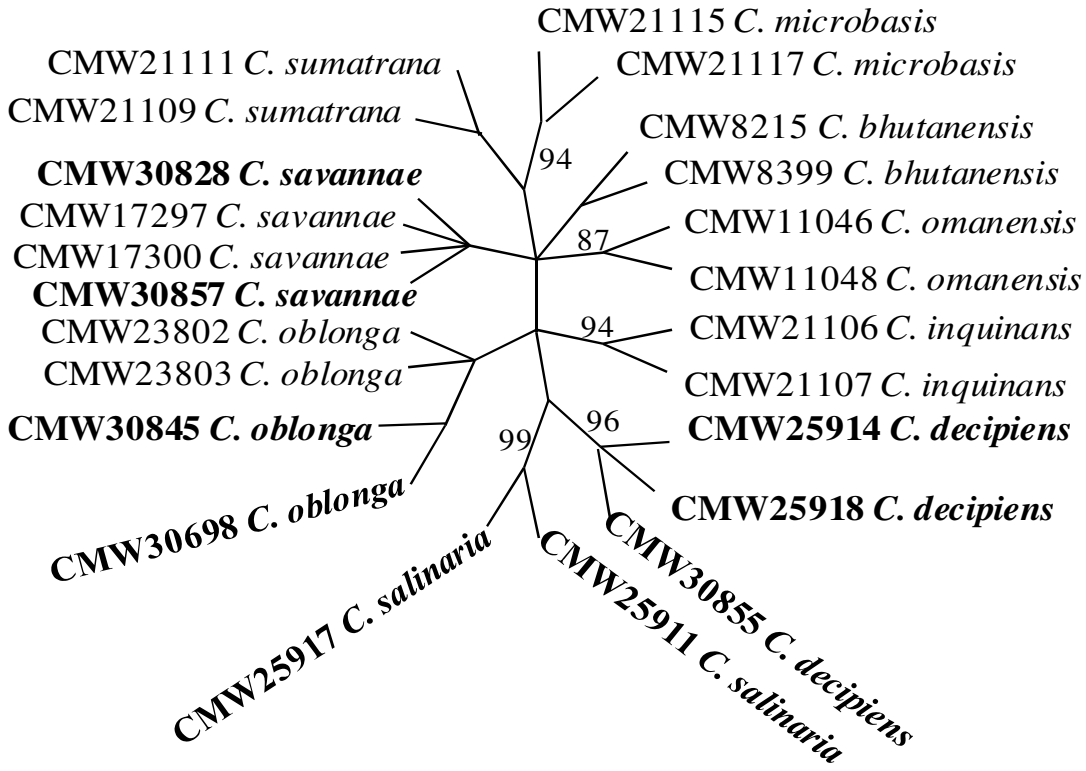

Figure 4: Unrooted maximum parsimony produced from a heuristic search of the BT sequence data, showing the relationship between members of *C. salinaria* sp. nov, *C. decipiens* sp. nov. from *Eucalyptus* trees in South Africa and their most closely related neighbor in the *C. moniliformis sensu lato* species complex.

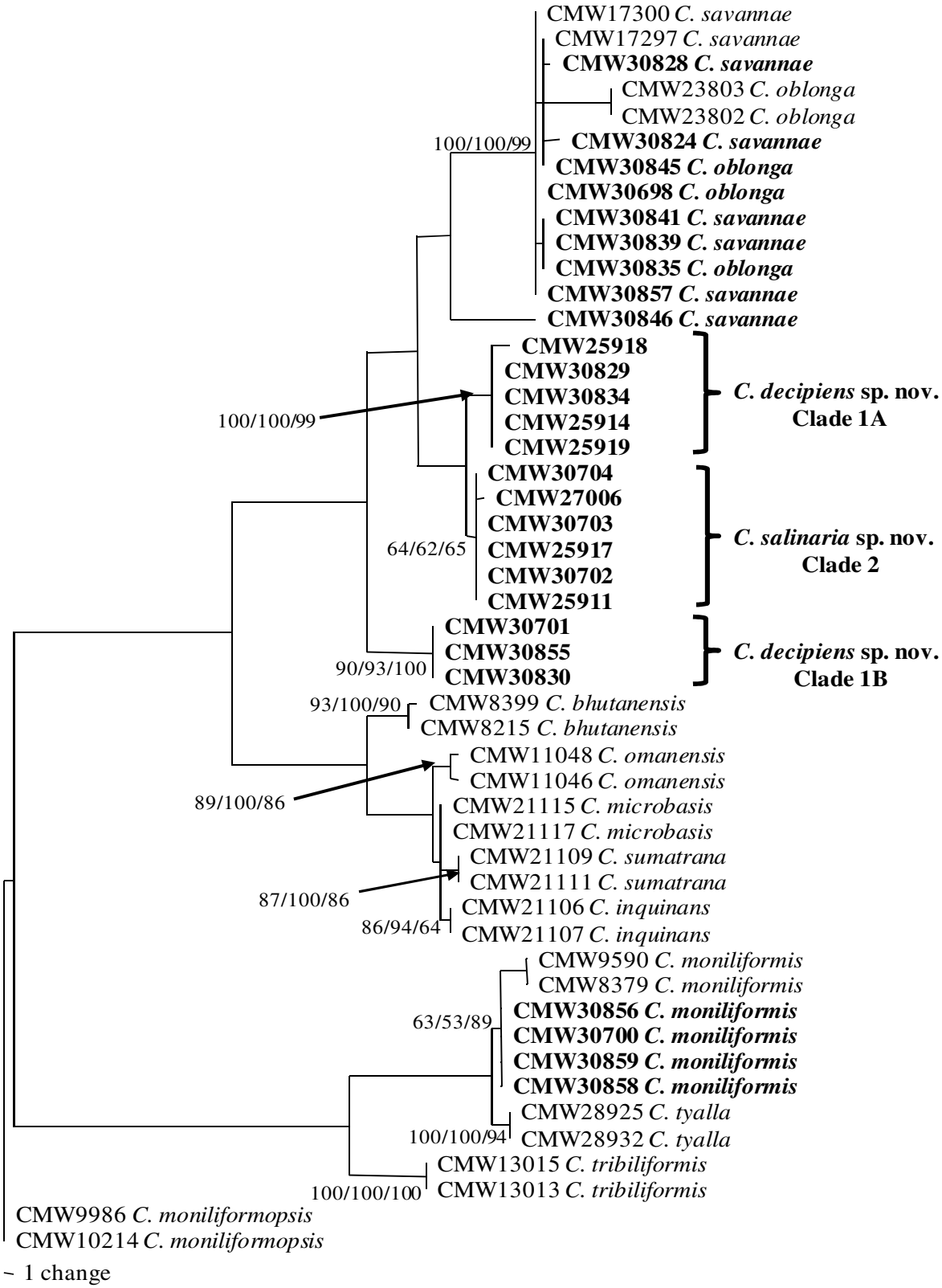

Figure 5: Phylogenetic tree produced from a heuristic search of the TEF sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

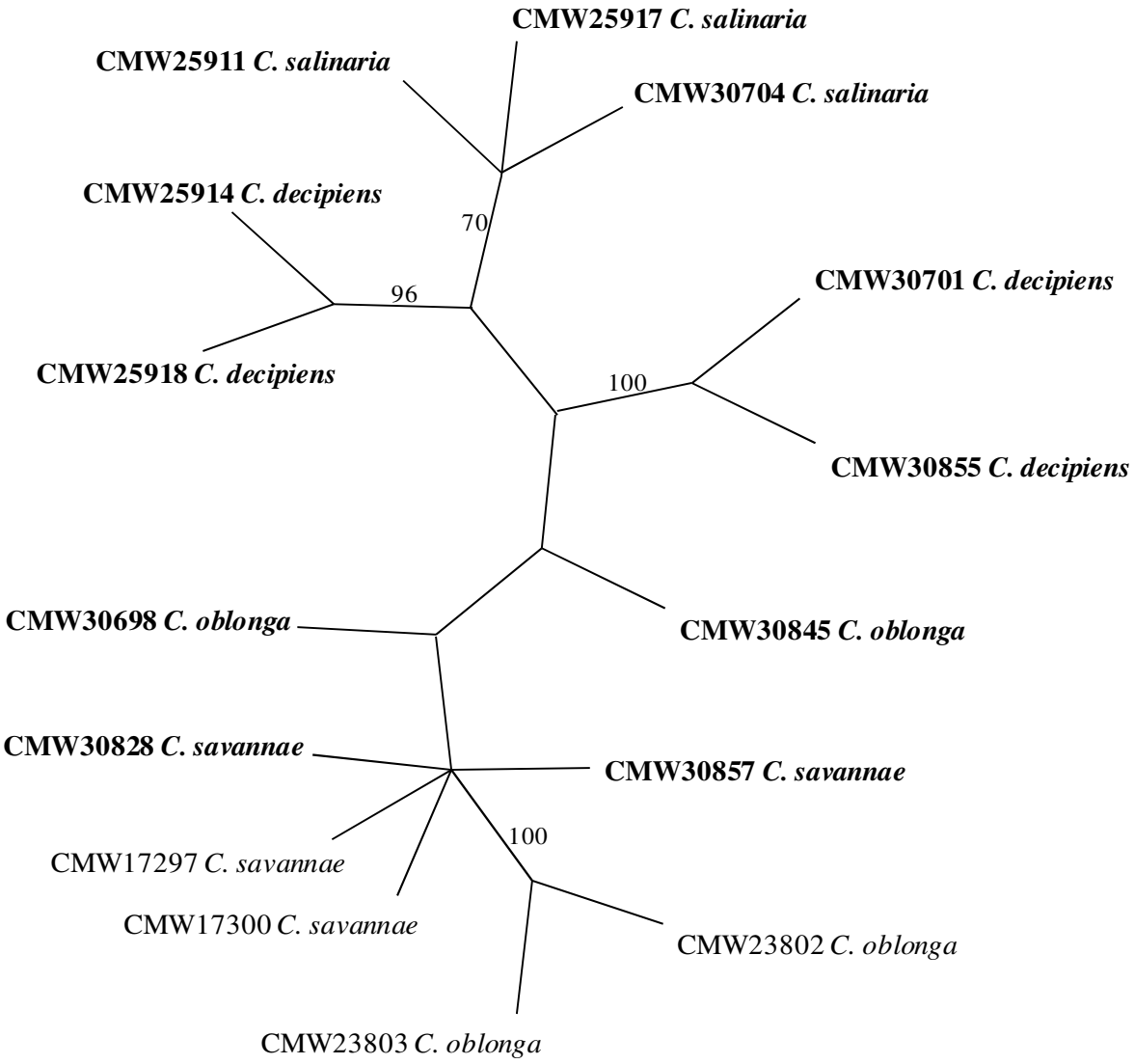

Figure 6: Unrooted maximum parsimony produced from a heuristic search of the TEF sequence data, showing the relationship between members of *C. salinaria* sp. nov, *C. decipiens* sp. nov. from *Eucalyptus* trees in South Africa and their most closely related neighbor in the *C. moniliformis s.l.* species complex.

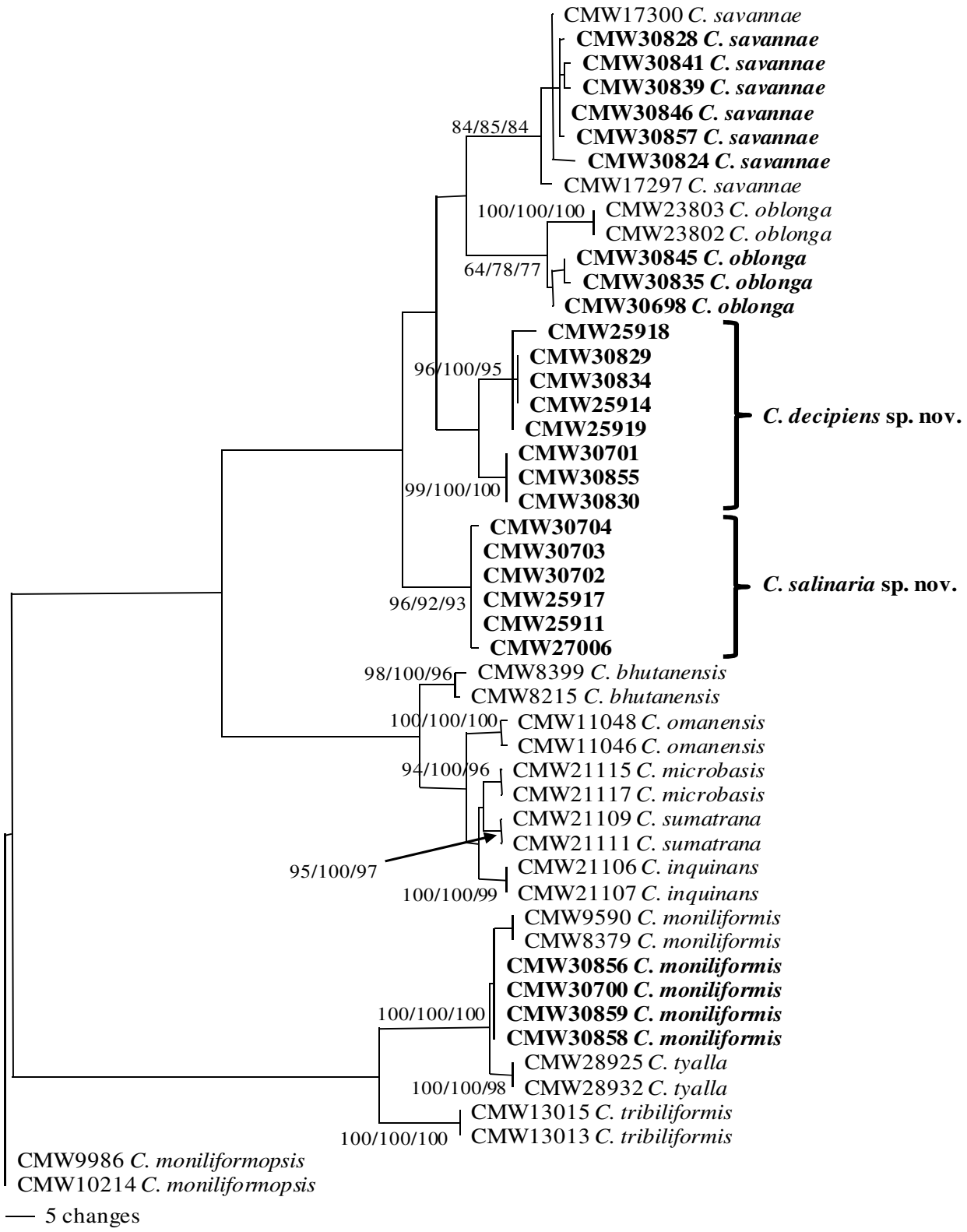

Figure 7: Concatenated tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

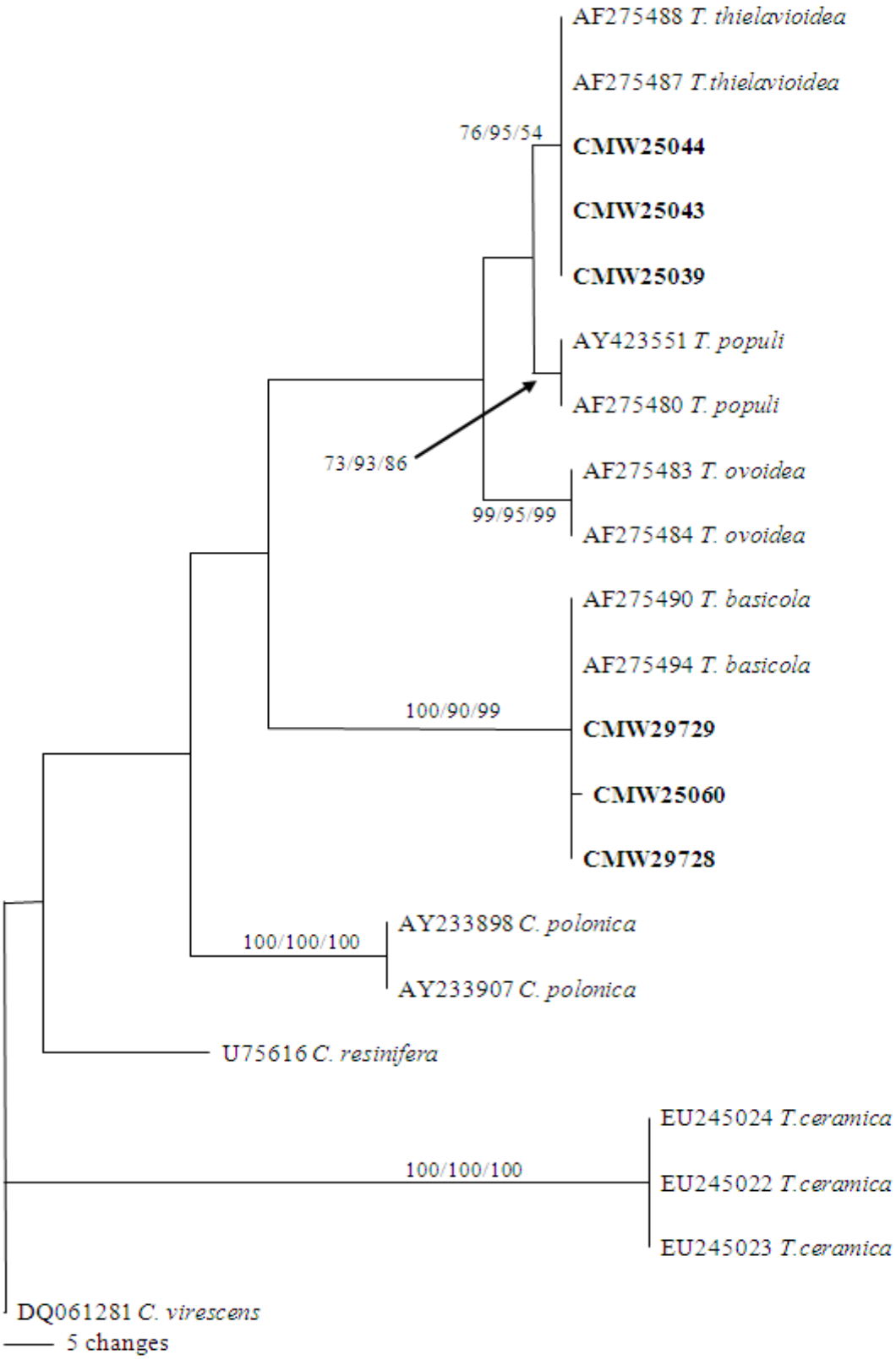

Figure 8: Phylogenetic tree produced from a heuristic search of the ITS sequence data, showing the phylogenetic identity of *Thielaviopsis* strains collected in South Africa amongt other known *Thielaviopsis* spp. Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values are indicated at each relevant node.

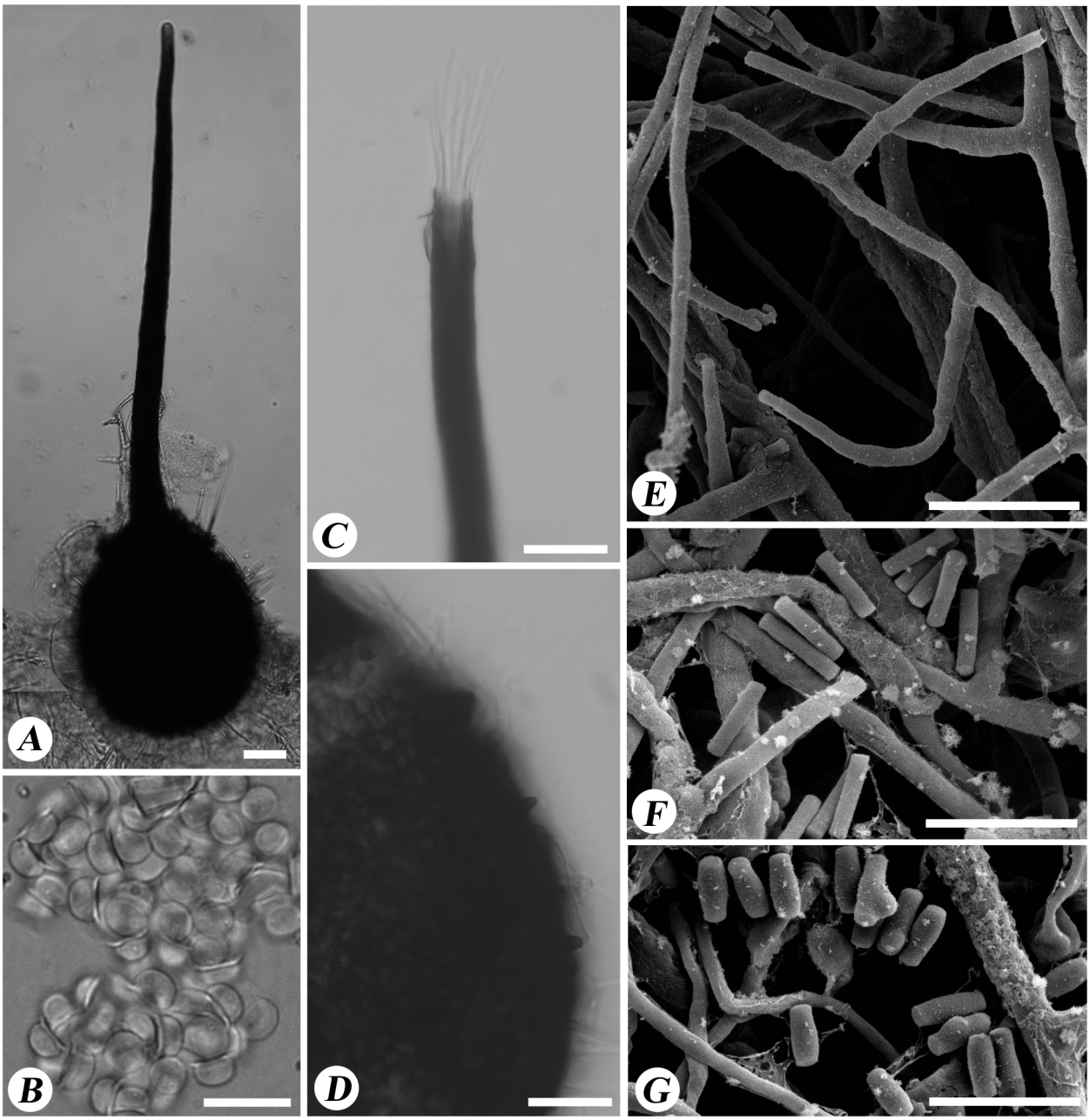

Figure 9: Morphological characteristics of *Ceratocystis salinaria* sp. nov. A) Globose ascomatal base (scale bar = 50 µm), B) Hat-shaped ascospores (scale bar = 10 µm), C) Divergent ostiolar hyphae (scale bar = 100 µm), D) Ascomatal base with conical spines (scale bar = 100 µm), E) Phialidic conidiogenous cell with emerging conidia (scale bar = 10 µm), F) Bacilliform shaped conidia (scale bar = 10 µm), G) Oblong shaped conidia (scale bar = 10 µm).

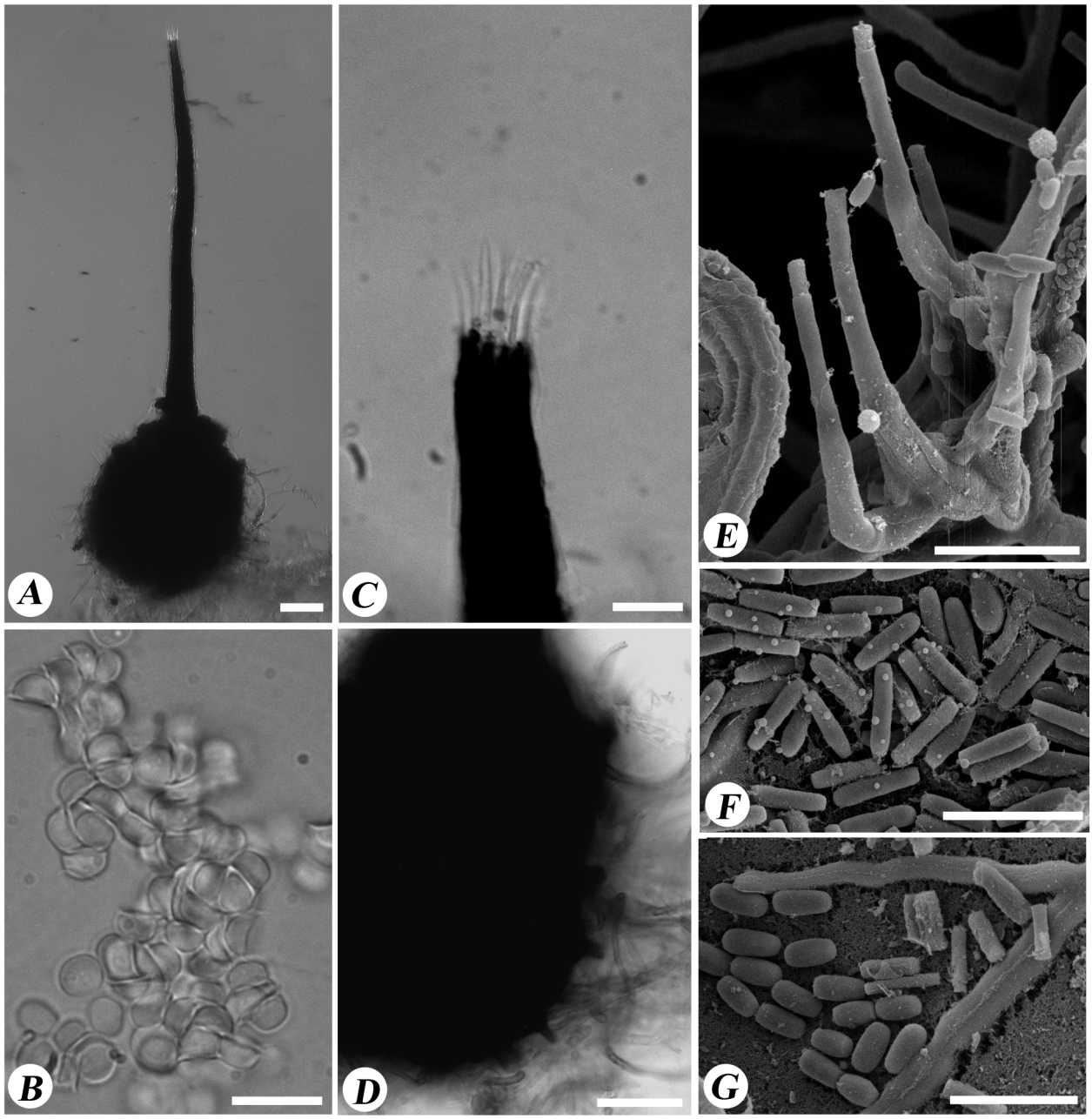

Figure 10: Morphological characteristics of *Ceratocystis decipiens sp. nov*. A) Globose ascomatal base (scale bar = 50 µm), B) Hat-shaped ascospores (scale bar = 10 µm), C) Divergent ostiolar hyphae (scale bar = 10 µm), D) Ascomatal base with conical spines (scale bar = 100 µm), E) Phialidic conidiogenous cell with emerging conidia (scale bar = 10 µm), F) Bacilliform shaped conidia (scale bar = 10 µm), G) Oblong shaped conidia (scale bar = 10 µm).

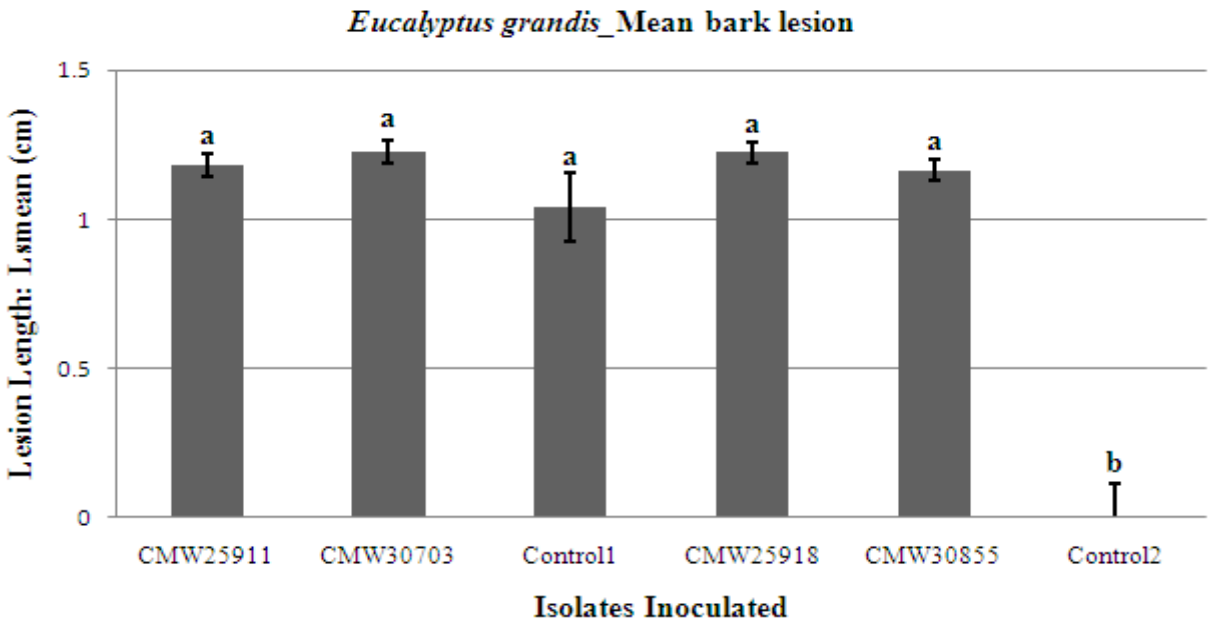

Figure 11: Vertical bar chart showing results of inoculation trial (bark lesion) with *C. salinaria* isolates (CMW25911, 30703) on *E. grandis* trees. Lsmean = 1.15, R = 0.75, CV = 9.9, P<0.0001, Confidence limit = 95%; and with *C. decipiens* isolates (CMW25918, 30855) on *E. grandis* trees. Lsmean = 0.8, R = 0.75, CV = 9.9, P<0.0001, Confidence limit = 95%. Columns with same letters are not statistically significant while those with different letters are statistically significant.

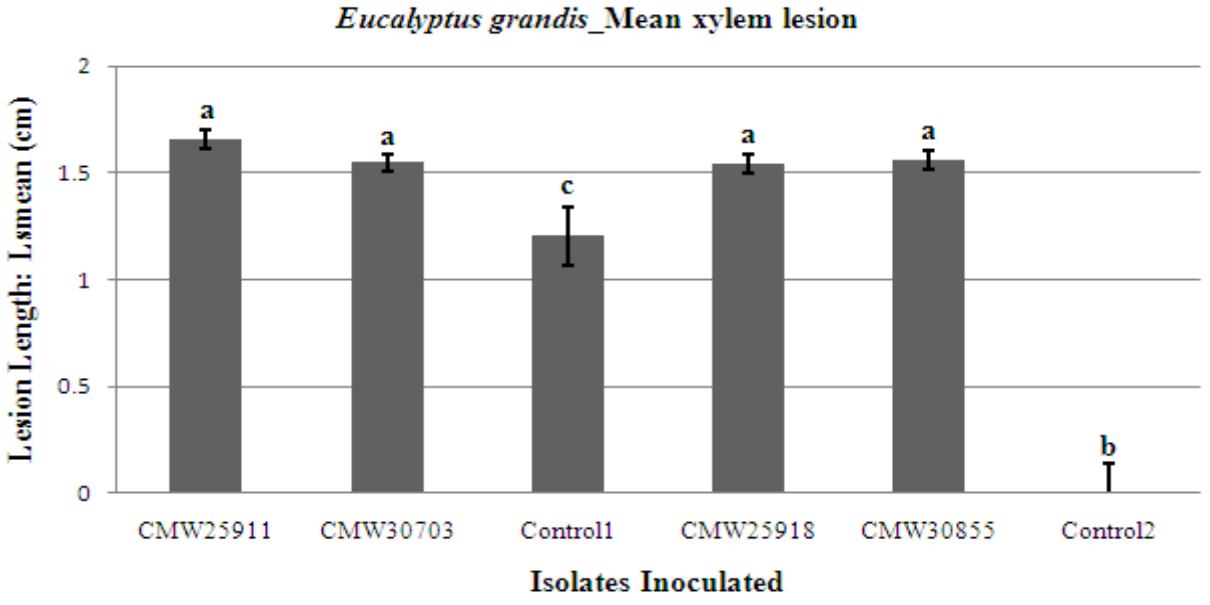

Figure 12: Vertical bar chart showing results of inoculation trial (xylem lesion) with *C. salinaria* isolates (CMW25911, 30703) on *E. grandis* trees. Lsmean = 1.47, R = 0.8, CV = 8.9, P<0.0001, Confidence limit = 95%; and with *C. decipiens* isolates (CMW25918, 30855) on *E. grandis* trees. Lsmean = 1.035, R = 0.8, CV = 8.9, P<0.0001, Confidence limit = 95%. Columns with same letters are not statistically significant while those with different letters are statistically significant.

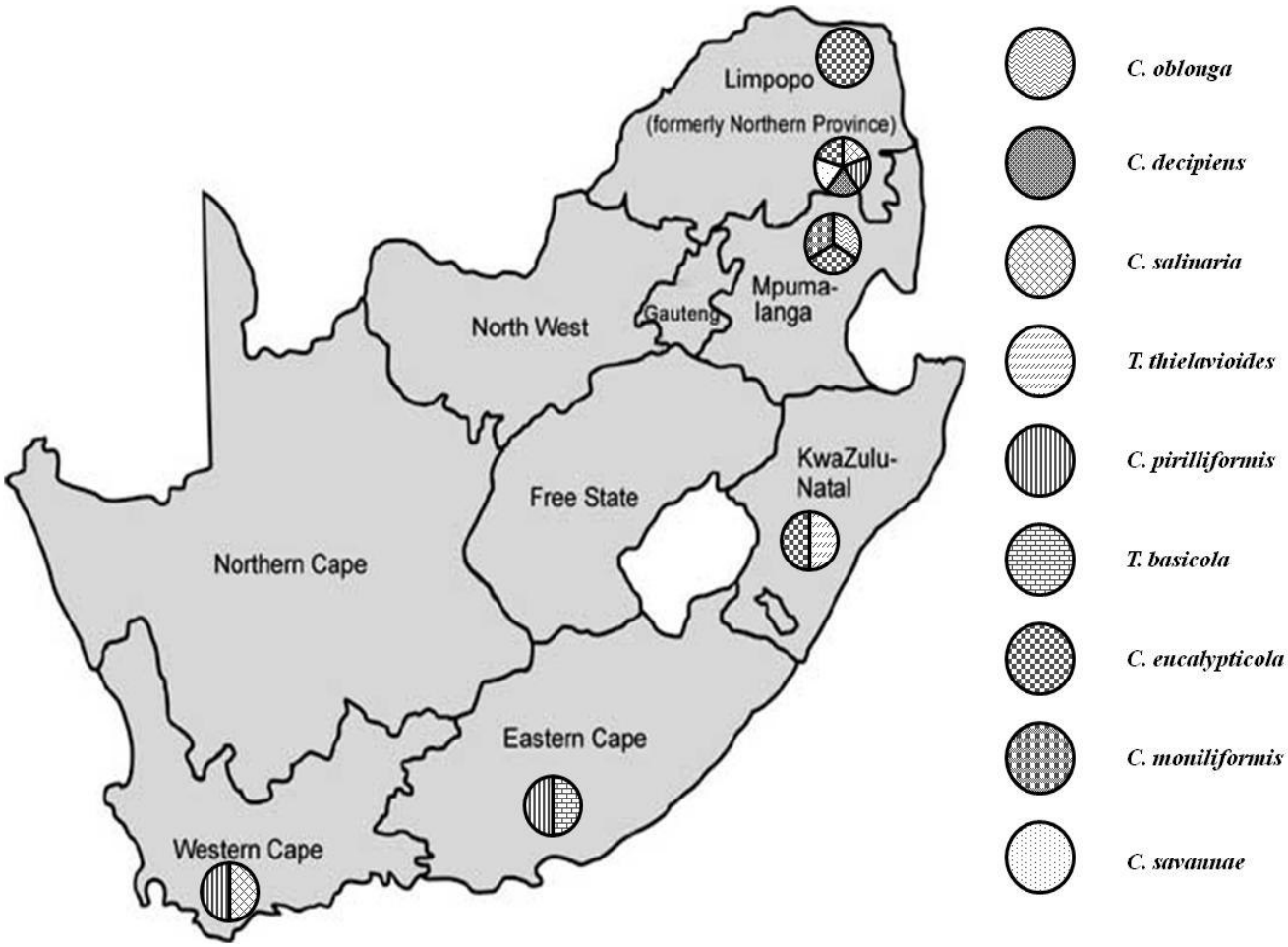

Figure 13: Map of South Africa showing the current geographic distribution of *Ceratocystis* spp. and *Thielaviopsis* spp. identified in this study from *Eucalyptus* spp. and insects in the country.

CHAPTER V

A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa

ABSTRACT

Fungi in the Ophiostomatales include some important pathogens of trees as well as agents of wood stain, reducing the economic value of timber. They rely on insects, such as bark beetles, for dispersal and are commonly associated with wounds on trees. Although *Ophiostoma* spp. have been reported from eucalypt wood chips in South Africa, very little is known about the diversity of the Ophiostomatales, or their insect associates, on plantation grown *Eucalyptus* spp. The aim of this study was to consider the diversity and distribution of the Ophiostomatales infecting fresh wounds on *Eucalyptus* trees in the country. Additionally, knowledge regarding their association with nitidulid beetles, which have previously been shown to carry *Ophiostoma* spp. was sought. Surveys were conducted in five provinces where *Eucalyptus* spp. are commonly grown and the fungi collected were identified using morphological comparisons and multigene sequence phylogenies. Of the 139 isolates collected, five *Ophiostoma* spp. were identified including *O. quercus*, *O. tsotsi* and *O. tasmaniense*. These were from cut stumps as well as the nitidulid beetles *Brachypeplus depressus* and *Carpophilus* spp. In addition, two new taxa in the *O. stenoceras* – *Sporothrix schenkii* complex were identified from *Eucalyptus* trees infested by *Phoracantha semipunctata*. The two new taxa are described as *O. candidum* sp. nov., and *O. fumeum* sp. nov., respectively. Results of this study clearly show that the diversity and ecology of *Ophiostoma* spp. on *Eucalyptus* trees in South Africa is poorly understood and further studies are required to determine the possible economic relevance of these fungi.

5.1. INTRODUCTION

The Ophiostomatales (Ascomycetes) includes the three fungal genera *Ceratocystiopsis* Upadhyay & Kendrick with *Hyalorhynocladiella* Upadhyay & Kendrick anamorph, *Grosmannia* Goidanich with *Leptographium* Lagerberg & Melin anamorph and *Ophiostoma* H. & P. Sydow with *Pesotum* Crane and *Sporothrix* Hektoen & Perkins anamorph (Zipfel *et al.* 2006). The sexual states of these fungi are characterized by mostly black ascomata with long erect necks giving rise to sticky spore drops that facilitate dispersal by insects (Malloch & Blackwell 1993). In the anamorph states, fruiting structures including synnemata, such as found in *Pesotum* spp. and mononematous conidiophores in *Leptographium* spp., *Hyalorhinocladiella* spp. and *Sporothrix* spp. are characteristic of the Ophiostomatales (Crane & Schoknecht 1973, Wingfield *et al.* 1991). Although more clearly defined than a decade ago, the taxonomy of fungi in the Ophiostomatales remains in a state of flux. For example, there is emerging evidence that the genus *Ophiostoma*, which comprises the largest number of species, is a generic aggregate including groups of species that represent distinct monophyletic lineages (De Beer & Wingfield 2006).

Species in the Ophiostomatales often are extremely variable in their culture morphology on artificial media, making their identification very difficult. Species in the *O. piceae* complex, for example, have been found in numerous studies to have variable culture morphology on artificial media, making morphological delineation of species in the complex subjective (Przybyl & De Hoog 1989, Brasier & Kirk 1993, Halmschlager *et al.* 1994, Kamgan Nkuekam *et al.* 2008a, b). Furthermore, there is substantial evidence that DNA-based studies using the ITS gene region is not sufficient to separate closely related species in the *O. piceae* complex (Chung *et al.* 2006, Kamgan Nkuekam *et al.* 2008a). Therefore, the trend in recent years has been to use sequence data for protein coding genes, such as the beta tubulin gene, which resolve species in the Ophiostomatales more appropriately (Chung *et al.* 2006, Kamgan Nkuekam *et al.* 2008a). In a recent study, Grobbelaar *et al.* (2009) used a multigene phylogeny including four gene regions to separate species of the *O. piceae* complex. The study led to the discovery and description of *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf., a cryptic species that was treated for many years collectively with *O. quercus* (Georgev.) Nannf. (Grobbelaar *et al.* 2010a).

The Ophiostomatales includes several important tree pathogens and number of species causing sapstain in wood. Important pathogens include the Dutch elm disease fungi, *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier (Gibbs 1978, Brasier 2000) and *Leptographium wageneri* (Goheen & F.W. Cobb) T.C. Harr. (Cobb 1988, Harrington 1993). Most species of *Ophiostoma*, however,

result in staining of the sapwood, particularly in conifers, that can reduce its commercial value (Seifert 1993). Amongst these fungi, *O. minus* (Hedgcock) H. & P. Sydow, *O. pluriannulatum* (Hedgcock) H. & P. Sydow, *O. piceae* (Munch) Syd. & P. Syd., *O. setosum* Uzunovic, Seifert, S. H. Kim & C. Breuil and *O. floccosum* Mathiesen are amongst the most important sapstain species of lumber in the Northern Hemisphere (Seifert 1993) while number of species of *Grosmannia* with *Leptographium* anamorph such as *L. wingfieldii* Morelet, *L. truncatum* (Wingfield & Marasas) Wingfield, *L. longiclavatum* S. Lee, J.-J. Kim & C. Breuil and *G. clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. are causing deep penetrating bluestain in logs (Uzunovic *et al.* 1999, Massoumi *et al.* 2007, Kim *et al.* 2007).

Fungi in the Ophiostomatales require wounds to infect their hosts and are commonly vectored by arthropods that either visit wounds on trees or produce these wounds themselves. Of these insects, bark beetles are by far the best known for their mutualistic relationship with these fungi (Whitney 1982, Paine *et al.* 1997, Six 2003, Kirisits 2004). However, a wide variety of insects, such as cerambycid beetles (Mathiesen-Käärik 1953, Jacobs & Wingfield 2001, Jacobs & Kirisits 2003), weevils (Mathiesen-Käärik 1953, Jacobs & Wingfield 2001, Kirisits 2004) and phoretic mites carried by bark beetles (Bridges & Moser 1986, Moser 1997, Malloch & Blackwell 1993) are also associated with these fungi.

South Africa has limited natural timber resources. The country mostly has a grassland vegetation type with native forests covering about 0.56% of the total land area in the country (Lawes *et al.* 2004). This exerts great pressure on the available natural tree resources, as timber suitable for construction, fire wood and other wood products are limited in the country. Plantations of non-native trees were established in South Africa in the last century in an attempt to alleviate the demand for timber and wood products (Anonymous 2008). These plantation areas now comprise approximately 1.3 million ha (1.2% of the country surface area). The plantations include non-native *Eucalyptus* spp. that cover approximately 0.58 million ha, (39%) of the total area planted (Anonymous 2008).

Reports of Ophiostomatoid fungi occurring on *Eucalyptus* spp. are limited. *Ophiostoma quercus* (Georgev.) Nannf., has been reported from *E. grandis* trees in Uruguay (Harrington *et al.* 2001) and most recently *O. tsotsi* was reported from *Eucalyptus* in Africa (Grobbelaar *et al.* 2009) and China (Grobbelaar *et al.* 2010b). There have been relatively few reports of *Ophiostoma* spp. on *Eucalyptus* from Africa and these include *O. quercus* on *E. grandis* (De Beer *et al.* 2003a) and *O. stenoceras* (Robak) Nannf. on *Eucalyptus* spp. in South Africa and from soil in eucalypt plantations in Kenya

(De Beer *et al.* 2003b). *Ophiostoma tsotsi* was found on *Eucalyptus* spp. in South Africa as well as in Malawi (Grobbelaar *et al.* 2009) and *Leptographium eucalyptophilum* K. Jacobs, M.J. Wingf. and J. Roux was described from *Eucalyptus* tree in the Republic of Congo (Jacobs et al. 1999). Other *Ophiostoma* spp. known from Africa are confined to reports from South Africa where they are known either from non-native bark beetles infesting *Pinus* spp. (Zhou *et al.* 2001, 2006), or from indigenous trees (De Beer *et al.* 2003a, b, Kamgan Nkuekam *et al.* 2008a, Roets *et al.* 2008, 2010).

The aim of this study was to increase the base of knowledge pertaining to fungi in the Ophiostomatales on plantation-grown *Eucalyptus* spp. in South Africa. Collections were focused on freshly made wounds on trees and those from nitidulid beetles (Coleoptera: Nitidulidae) that visit these wounds. Morphological characteristics, mating studies and phylogenetic inference based on DNA sequence data were used to identify these fungi.

5.2. MATERIALS AND METHODS

5.2.1. Collection of samples

5.2.1.1. Fungal isolates from trees

To investigate the occurrence of Ophiostomatoid fungi on eucalypts in South Africa, collections were made from freshly cut stumps, since wounds are known to be infection sites for these fungi. Wounds are also commonly visited by nitidulid beetles, making it possible to collect both Ophiostomatoid fungi and their possible nitidulid vectors. Collections were made between February 2007 and November 2008 in most of the commercial eucalypt growing areas of the country. The overall aim was to collect material from as many different geographical and climatic areas of the country as possible. Samples were mainly collected from stumps of felled trees as well as from logs lying on the plantation floor. However, where damage to the stems of trees, caused by wood boring beetles was observed, samples were also collected. Areas sampled included the Soutpansberg and Tzaneen areas (Limpopo Province), Lothair and Sabie (Mpumalanga Province), George, Cape-Town and Stellenbosch (Western Cape Province), Kumbu and Lotebeni (Eastern Cape Province) and localities near KwaMbonambi and Pietermaritzburg (KwaZulu Natal Province).

Pieces of bark and wood were collected from wounds, especially those with bark flaps, and stored in brown paper bags after they had been inspected with a 20X magnifying hand lens for the presence of fruiting bodies (ascomata or conidiophores) resembling those of the Ophiostomatoid fungi. All the samples were transported to the laboratory in plastic bags to retain moisture, and

processed following the methods described by Kamgan Nkuekam *et al.* (2008a, 2010a). Replicates of each pure isolate obtained were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative specimens were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

5.2.1.2. Collection of insects and fungal isolation

Nitidulidae were collected from beneath bark flaps on cut stumps and wounds on *Eucalyptus* spp. This was done using an aspirator (Fergusson 1982). Living insects were transferred to cylindrical labeled glass containers containing a piece of tissue paper that served to reduce contact and conflict among insects. In the laboratory, collected insects were inactivated by cooling the glass containers in a box containing ice. The insects were grouped according to morphological characteristics using an Axiocam dissection microscope (München-Hallbergmoos, Germany). Representatives of each insect group were preserved in 70% ethanol prior to identification, by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food & Agriculture, USA.

Isolation of fungi from insects was done by crushing individual specimens between two slices of carrot and incubating them for five days at 25ºC (Moller & Devay 1968, Heath *et al.* 2009). Fungi were isolated by transferring fruiting bodies (mycelium, ascomata, ascospores) growing on the carrots to 2% malt extract agar (MEA: 20 g/L malt extract and 15 g/L agar, Biolab, Midrand, South Africa and 1000ml sterile deionised water) containing 0.05 g/L of the antibiotic streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany).

5.2.2. Morphological characterization

All isolates resembling species of Ophiostomatales were characterized based on commonly used characteristics for this group. Spore drops (conidia, ascospores) from single apices of conidiophores or ascomata in pure cultures were transferred to Oatmeal agar media (OMA: 30 g Oats, 20 g Biolab agar and 1000 mL deionised water) to promote sporulation. Cultures were incubated at 25ºC until sporulation and then grouped into morphotypes based on colour (Rayner 1970) and macro-morphology. Fruiting structures from selected isolates representing each morphotype were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and ten measurements were

made for additional isolates. The means were then calculated for relevant morphological structures. Measurements were noted as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum).

Scanning electron microscopy was used to observe fruiting bodies (conidia, conidiophores) of the asexual states of the fungi. For this purpose, specimens were prepared as described by Grobbelaar *et al.* (2009). The specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater (Emitech K550X, Ashford, England) and examined using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

5.2.3. Growth in culture

Three isolates of each new species identified in this study were used for growth studies in culture. A disk of agar (9 mm diam.) bearing mycelium of the test isolates was transferred from the actively growing margins of seven-day-old cultures and placed upside down at the centres of 90 mm Petri dishes containing 2% MEA. Five replicate plates were used for each isolate at each growth temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed.

5.2.4. DNA sequence comparisons

Representative isolates of each morpho-group were selected for DNA sequence comparisons. Single spore drops collected from pure cultures were grown on 2% MEA for 7-10 days. Mycelium was scraped from the surface of the actively growing cultures and then transferred to 1.5 mL Eppendorf tubes using a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified with an Eppendorf Mastercycler (Merck, Hamburg, Germany) using primers ITS1 and ITS4 (White *et al.* 1990). Part of the β-tubulin (BT) gene and the transcription elongation factor-1α (TEF) gene were amplified using the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995), and EF1F and EF2R (Jacobs *et al.* 2004), respectively.

The PCR reaction mixtures as well as the thermal cycling conditions were the same as those described previously (Kamgan Nkuekam *et al.* 2008a, b). An aliquot of 5 µl of the PCR products were stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA), separated on a 1%

agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich, Steinheim, Germany), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). Sequencing reactions were performed using the Big Dye cycle sequencing kit with Amplitaq DNA polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturer's protocols on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Californis, USA). Sequencing PCRs were prepared as described by Kamgan Nkuekam *et al.* (2008a) and both DNA strands were sequenced.

5.2.5. Compilation of sequence data sets

Contigs of both sequenced strands for each isolate and each gene region were assembled using Sequence Navigator 1.01 (ABI PRISM, Perkin Elmer, Warrington, UK). Additional sequences of related species in the Ophiostomatales were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) for comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Katoh & Toh 2008). To avoid the inclusion of large numbers of identical sequences in the phylogenetic analyses, isolates collected were sequenced and grouped based on their BT haplotypes using MEGA 4.0.1 (Tamura *et al.* 2007). For each BT haplotype, only a few representatives were included in the analyses of the ITS and TEF data sets.

5.2.6. Phylogenetic analyses

Phylogenetic analyses of the ITS, TEF and BT data sets were performed independently of each other. This was done to avoid masking important information and because reference sequences are not available for the same sets of isolates for all gene regions. For each data set, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done. However, since the sequence data for intron 5 of the BT gene varies greatly between different species groups, the dataset was treated in subsets in order to obtain improved alignments for species definition. MP analyses were performed in a similar manner for both BT data sets in PAUP 4.0b10 (Swofford 1998), using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1000 replications).

Bayesian analyses based on Markov Chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) as outlined previously (Kamgan Nkuekam *et al.* 2010). Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (http://www.abc.se/~nylander/). The model applied to both ITS and TEF was

(GTR+I+G), while for the two subsets of BT data it was (HKY+G) and (HKY+G) respectively. Burn-in values were determined using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) to discard trees that formed before the point of convergence, and the posterior probability in the majority rule consensus trees were calculated by MCMC sampling in MrBayes V3.1.2, using the best-fit model of evolution mentioned above.

Maximum likelihood (ML) analyses were conducted online using PhyML 3.0 (Guindon & Gascuel 2003). The AIC was used in Modeltest 3.7 (Posada & Crandall 1998) to select appropriate substitution models for the three data sets. For ITS, the selected model was GTR+I+G (gamma Shape parameter=1.0169; Pinvar=0.2617), for the TEF, it was GTR+I+G (gamma shape parameter=2.0444; Pinvar=0.1411), while for the two subsets of BT it was TVM+G (gamma Shape parameter=0.1978) and HKY+G (gamma Shape parameter=0.1612), respectively.

5.3. RESULTS

5.3.1. Collection of samples

5.3.1.1. Fungal isolates from trees

A total of 115 isolates resembling species in the Ophiostomatales were obtained from eucalypt trees in this study. More than 300 trees were sampled in the process (Table 1). Samples were obtained from the stumps of recently felled trees, fresh logs lying in the field and from the tunnels of *Phoracantha* spp., in standing trees. Isolates were obtained from all areas sampled and included *E. grandis* W. Hill ex Maiden (Tzaneen, Sabie, Kumbu and Lotebeni), *E. saligna* Sm. (Cape Town, Soutpansberg), *E. diversicolor* F. Muell (Cape Town), *E. cloeziana* F. Muell (Soutpansberg), *E. maculata* Hook. (Soutpansberg), stumps of an unknown *Eucalyptus* species (George), logs lying on the plantation floor (Stellenbosch) and at a harbor (Cape Town) (Table 1). The climatic regions where samples were collected included Mediterranean (Cape Town, Stellenbosch), Temperate (Lotebeni, Kumbu) and Subtropical (KwaMbonambi, Tzaneen, Soutpansberg) regions and included winter (Cape Town, Stellenbosch), year-round (George) and summer (KwaZulu Natal, Mpumalanga, Limpopo Province) rainfall areas.

5.3.1.2. Collection of insects and fungal isolation

Nitidulid beetles were found in most of the areas sampled. A total of 271 insects were collected from stumps of various *Eucalyptus* trees and they could be broadly divided into two groups based on morphology. These were *Brachypeplus* sp. (140 specimens), identified as *B. depressus* Erichson, and a *Carpophilus* sp. identified as *C. humeralis* Fabricius (120 specimens). Some insect specimens

(11 insects) were recognized as a *Carpophilus* sp. but could not be identified to species level. Of the 24 *Ophiostoma* isolates obtained from insects, 13 were from *B. depressus* while 11 were from *Carpophilus* spp. (Table 1).

5.3.2. Morphological characterization

Three morphological groups were found among the Ophiostomatoid fungi collected. Morpho-group A, comprising 123 isolates, produced *Pesotum* anamorphs and *Sporothrix* synanamorphs on OMA. They also produced limited numbers of ascomata with long erect necks exuding slimy masses of spores, scattered over the colony surface. Morpho-group B, consisted of nine isolates with white cultures and ascomata produced abundantly and embedded within the mycelium mat. Morpho-group C consisted of eight isolates with grey-coloured cultures and ascomata with very short necks deprived of ostiolar hyphae, produced in a thick mycelial mat by some strains.

5.3.3. Compilation of sequence data and phylogenetic analyses

All isolates sequenced produced fragments of approximately 400 bps for the BT gene region. Blast searches in the GenBank data base showed that all isolates of morpho-group A were members of the *O. piceae* complex while isolates of morpho-group B and morpho-group C belonged to the *S. schenckii-O. stenoceras* complex. Furthermore, the morpho-group A isolates all had intron 4 and lacked intron 5, while isolates from morpho-groups B and C lacked intron 4 but presented intron 5. The BT data for the three groups of isolates were thus analyzed separately in two subsets to obtain improved alignments for species definition.

Comparisons of the BT sequence data for the *O. piceae* subset of isolates from South Africa with those of related *Ophiostoma* spp. from GenBank, resulted in a total of 238 characters including gaps, with 142 constant characters, 11 parsimony uninformative characters and 85 parsimony informative characters. Comparisons of the BT sequence data for the *O. stenoceras* subset of isolates with those of related *Ophiostoma* spp. from GenBank resulted in a total of 269 characters including gaps, with 145 constant characters, 3 parsimony uninformative characters and 121 parsimony informative characters.

Maximum parsimony analyses of the BT data set of the *O. piceae* subset of isolates generated 308 trees. The consistency indices (CI) and retention index (RI) values were 0.617 and 0.912, respectively. Analyses of the BT data set of the *O. stenoceras* subset of isolates generated 648 trees, with CI and RI values of 0.494 and 0.874 respectively. For each data set, a 50% majority rule tree obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as from

maximum likelihood analyses (ML). For each data set, a ML tree is presented (Figures 1, 2) indicating at the relevant node, MP bootstrap values, Bayesian posterior probabilities, and ML bootstrap values respectively.

In the BT tree (Figure 1), isolates representing the *O. piceae* subset were resolved into three groups. The first set of isolates grouped with strains of *O. quercus*. The second set of isolates grouped with strains of *O. tsotsi*, while the third set of isolates grouped with strains of *O. tasmaniense* (Figure 1). In the BT tree representing the *O. stenoceras* data set (Figure 2), isolates from South Africa could be resolved into two clades, well supported at the nodes and clearly distinct from other *Ophiostoma* reference strains. Isolates representing morpho-groups B and C resided in well resolved clades with good statistical support.

Representatives of each haplotype found in the BT data set of the *O. piceae* subset of isolates were selected for phylogenetic analyses of the TEF data set. Comparisons of the TEF data set of the *O. piceae* subset of isolates with those of related *Ophiostoma* spp. from GenBank, resulted in a total of 520 characters including gaps, with 95 constant characters, 18 parsimony uninformative characters and 407 parsimony informative characters. Maximum parsimony analyses of the TEF data set generated 1122 best trees, with a CI and RI values of 0.661 and 0.932 respectively. For the TEF data set, a 50% majority rule tree was obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as a ML (Figure 3) analyses.

Results of analyses of the TEF data (Figure 3) were similar to those of the BT data set. Representatives of the *O. piceae* subset of isolates were again resolved into three clades. One set of isolates grouped with strains of *O. quercus*, the second set grouped with strains of *O. tsotsi*, while the third set of isolates grouped with strains of *O. tasmaniense*.

For the ITS data set, representatives of each morpho-group found in the survey were selected for phylogenetic analyses. Comparisons of the ITS data set with those of related *Ophiostoma* spp. from GenBank resulted in a total of 799 characters including gaps, with 216 constant characters, 80 parsimony uninformative characters and 503 parsimony informative characters. Maximum parsimony analyses of the ITS data set generated 2069 best trees, with CI and RI values of 0.566 and 0.865 respectively. For the ITS data set, a 50% majority rule tree obtained from Bayesian analyses and a bootstrap tree was obtained from MP as well as ML analyses (Figure 4).

The two subsets of *Ophiostoma* spp., identified based on BT sequences and the presence and/or absence of introns 4 and 5, could clearly be recognized within the ITS tree. The *O. piceae* complex subset resided in the top half of the tree, while the *S. schenckii-O. stenoceras* complex was confined to the lower part of the tree (Figure 4). Isolates representing morpho-group A clustered with those of *O. quercus*, *O. tsotsi* and *O. tasmaniense*, but the clades representing these species were not well resolved. Isolates representing morpho-group B and morpho-group C formed two different clades within the *S. schenckii-O. stenoceras* complex. These clades were clearly separated from other *Ophiostoma* reference isolates and they were statistically well supported (Figure 4).

5.3.4. Taxonomy

Based on morphological examination and phylogenetic analyses using the ITS and BT gene regions, two *Ophiostoma* spp. from South Africa emerged as distinct and previously undescribed species within the *S. schenckii-O. stenoceras* complex of the Ophiostomatales. The following descriptions are provided for them.

Ophiostoma candidum Kamgan-Nkuek., Jol. Roux & Z. W. de Beer sp. nov. (Fig. 5). **MB519314**

***Etymology*:** The name refers to the dazzling white colony color of the fungus on artificial media.

Coloniae albae tegetibus mycelii crassis, in MEA ut videtur madidae; usque ad 11.5 mm diametro in 10 diebus in MEA in 25°C. Crescit optime in 25°C; non crescit in 10°C nec 30°C nec 35°C. *Ascomata* in superficie coloniae dispersa et in mycelio inclusa, guttas sporarum albocremeas facientes. *Colla* ascomatum nigra *hyphis* ostiolaribus divergentibus. Bases ascomatum nigrae globosae pilis hypharum. Bases collorum laeves. *Ascosporae* reniformes hyalinae non septatae (3.5-) 4.0-4.5 (-5.0) x (1.0-) 1.0-1.5 (-2.0) µm. Anamorpha *Sporothrix* conidiophoris hyalinis cylindricis apicem versus contractis, cum denticulis prominentibus. Conidia non septata, hyalina oblonga interdum acerosa nonnulla valde curvata (1.5-) 1.5-2.0 (-2.5) x (0.5-) 0.5-0.5 (-1.0) µm.

Colonies white, with thick mycelial mats, with a wet appearance on MEA. Reverse Buff (19''d). Colony diameters reaching 11.5 mm in 10 days on MEA at 25˚C. Optimal growth at 25˚C. No growth at 10˚C and at 30˚C or 35˚C. *Ascomata* scattered over the colony surface and embedded within mycelium on agar plates, producing white, creamy spore drops at the apices of the necks. *Ascomatal* necks black (270.5-) 506.0-875.0 (-970.5) µm long. Ascomatal bases black, globose (84.5-) 124.5-184.5 (-206.0) µm long and (90-) 119-182 (-233) µm wide, with hyphal hairs. Neck bases smooth (24.0-) 29.5-41.0 (-51.0) µm wide, middle of necks (16.5-) 19.0-25.0 (-27.5) µm

wide, tips of necks (9.0-) 11.5-16.0 (-22.0) µm wide. *Ostiolar hyphae* divergent (16.0-) 22.5-34.0 (-39.0). *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* reniform, aseptate, hyaline (3.5-) 4.0-4.5 (-5) x (1.0-) 1.0-1.5 (-2.0) µm.

Anamorph: *Sporothrix,* conidiophores hyaline, cylindrical, tapering towards the apex, (12.5-) 17.5-43.5 (-61.0) x (1.5-) 1.9-2.5 (-3.0) µm, prominent denticles present. Conidia aseptate, hyaline, oblong, occasionally acerose, distinctly curved in some cases (1.5-) 1.5-2.0 (-2.5) x (0.5-) 0.5-0.5 (-1.0) µm.

Specimens examined: South Africa**,** Limpopo Province, Goedehoop area of the Soutpansberg, isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam and Jolanda Roux, holotype PREM60470, living culture CMW26484 = CBS129713

Additional specimens: South Africa, Limpopo Province, Goedehoop area of the Soutpansberg, isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam & J. Roux, holotype PREM60471, living culture CMW26483 = CBS129714, isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam & J. Roux, holotype PREM60472, living culture CMW26485 = CBS129711

Ophiostoma fumeum Kamgan-Nkuek., Jol. Roux & Z. W. de Beer sp. nov. (Fig. 6). **MB519315**

***Etymology*:** The name refers to the smoky grey colony color of the fungus on artificial media.

Coloniae fumeae tegetibus mycelii crassis in MEA; usque ad 16.5 mm diametro in 10 diebus in MEA in 25°C. Crescit optime in 30°C; non crescit in 10°C. *Ascomata* in cultura serotina in mycelio inclusa, guttas sporarum albocremeas facientes. *Colla* ascomatum nigra sine *hyphis* ostiolaribus. Bases ascomatum nigrae globosae pilis hypharum. Bases collorum laeves. *Ascosporae* allantoideae hyalinae non septatae (4.0-) 4.5-5.5 (-6.0) x (1.0-) 1.0-1.5 (-2.0) µm. Anamorpha *Sporothrix* conidiophoris hyalinis cylindricis cum denticulis prominentibus. Conidia non septata, hyalina oblonga vel cylindrica (1.0-) 1.5-2.0 (-2.5) x (0.5-) 0.5-0.5 (-1.0) µm.

Colonies smoke grey (21’’’’d), with thick mycelium mat on MEA. Reverse smoke grey (21’’’’d). Colony diameters reaching 16.5 mm in 10 days on MEA at 25˚C. Optimal growth at 30˚C. No growth at 10˚C. *Ascomata* produced late in culture and embedded within mycelium, producing white, creamy spore drops at the neck apices. *Ascomatal* necks black (50.5-) 77.0-119.7 (-145.0) µm long. Ascomatal bases black, globose (81.0-) 103.0-144.5 (-165.0) µm long and (97.0-)

116.0-164.0 (-186.5) µm wide, with hyphal hairs. Neck base smooth, (31-) 30-43 (-50) µm wide. *Ostiolar hyphae* absent. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* allantoid, aseptate, hyaline (4.0-) 4.5-5.5 (-6.0) x (1.0-) 1.0-1.5 (-2.0) µm.

Anamorph: *Sporothrix,* conidiophores hyaline, cylindrical, (2.5-) 4.0-7.0 (-10.5) x (0.5-) 0.5-0.5 (-1.0) µm, prominent denticles present. Conidia aseptate, hyaline, oblong, to cylindrical (1.0-) 1.5-2.0 (-2.5) x (0.5-) 0.5-0.5 (-1.0) µm.

Specimens examined: South Africa, Limpopo Province, Soutpansberg area, isolated from *Eucalyptus cloeziana* infested by *Phoracantha* beetles, 21/06/2007, G. Kamgan Nkuekam and J. Roux, holotype PREM60473, living culture CMW26813 = CBS129712

Additional specimens: South Africa, Limpopo province, Soutpansberg area, isolated from *Eucalyptus cloeziana* infested by *Phoracantha* beetles, 21/06/2007, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW26816 = PREM60474 = CBS129715, isolated from *Eucalyptus* spp. infested by *Phoracantha* beetles in Zambia, Copperbelt Province, J. Roux, paratype, living culture CMW26818 = PREM60475 = CBS129716.

The geographic distribution of the Ophiostomatales collected from eucalypts in this study is varied. *Ophiostoma quercus* is the most widely spread species, occurring in all five Provinces sampled (Figure 7). It was obtained from three *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*, *E. grandis*) and is found on two nitidulid species (Table 1). *Ophiostoma tsotsi* was collected in two of the five Provinces sampled (Figure 7), infecting three *Eucalyptus* spp. (*E. grandis*, *E. maculata*, *E. cloeziana*). It was also found on two nitidulid species (Table 1). The remaining *Ophiostoma* species found in this study are currently known from only one Province each. *Ophiostoma candidum* and *O. fumeum* was recovered only from the Limpopo Province on *E. cloeziana*, while *O. tasmaniense* was obtained from two *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*) and from two nitidulid species in the Western Cape Province (Figure 7, Table 1).

5.4. DISCUSSION

Results of this study have shown that *Ophiostoma* spp. occur widely on *Eucalyptus* trees and logs in South Africa. Five *Ophiostoma* spp. were identified. Two of these were previously undescribed species in the *S. schenckii-O. stenoceras* complex for which the names *O. candidum* and *O. fumeum* were provided. *O. tasmaniense* was found for the first time from outside Australia, while the geographic and host ranges for *O. quercus* and *O. tsotsi* were expanded substantially.

Ophiostoma fumeum and *O. candidum*, the two new taxa described in this study are phylogenetically related, and morphologically similar to species in the *S. schenckii-O. stenoceras* complex. Most members of this complex have white or grey cultures, *Sporothrix* anamorphs and allantoid to reniform to orange section shaped ascospores (De Hoog 1974, De Beer *et al.* 2003b). *O. candidum* and *O. fumeum* both share these morphological characteristics.

Ophiostoma candidum and *O. fumeum* described in this study formed distinct clades within the *S. schenckii-O. stenoceras* complex. The complex includes nine species described from arthropod-infested *Protea* infructescences (Roets *et al.* 2006, 2008, 2010), 11 species associated with soil (Zhou *et al.* 2006, Marimon *et al.* 2007, De Meyer *et al.* 2008, Madrid *et al.* 2010), five from hardwoods (De Beer *et al.* 2003b, Aghayeva *et al.* 2004, 2005, De Meyer *et al.* 2008), four from conifers (Aghayeva *et al.* 2004, Zhou *et al.* 2006, De Meyer *et al.* 2008), and four human pathogens (Marimon *et al.* 2007, 2008). Fifteen of these species, including all those from the Proteaceae are known only from Southern Africa. Four of the South African species have some form of association with *Eucalyptus* spp. *O. stenoceras* was previously isolated from *E. smithii* R.T. Baker, *E. grandis* and *E. fastigata* Deane & Maiden in South Africa and soil from a *Eucalyptus* plantation in Kenya (De Beer *et al.* 2003b). *Sporothrix lignivora* de Meyer, Z.W. de Beer & M.J. Wingf. was first described from *Eucalyptus* utility poles (De Meyer *et al.* 2008), *S. variecibatus* Roets, Z.W. de Beer & P.W. Crous from decaying *Eucalyptus* leaves and mites (Roets *et al.* 2008), and *S. schenckii* from *Eucalyptus* wood stacks used to strengthen tunnels in gold mines (Findlay 1970). *O. candidum* was isolated from an *E. cloeziana* tree struck by lightning in the Limpopo Province, and *O. fumeum* from *Eucalyptus* trees infested with the cerambycid beetle *Phoracantha semipunctata* in the Limpopo Province and Zambia.

It has been suggested that possibly all the species in the *S. schenckii-O. stenoceras* complex from *Protea* infructescences are vectored by, and have some form of association with, arthropods, especially mites (Roets *et al.* 2007, 2009). The only other species in this complex that was isolated from an arthropod, in this case a root-infesting bark beetle, is *O. aurorae* X.D. Zhou & M.J. Wingf. (Zhou *et al.* 2006). However, this species was found with the beetle only in a single location during an extensive survey (Zhou *et al.* 2001). Another species, *S. variecibatus*, was isolated from a mite from *Protea* (Roets *et al.* 2008). The fact that *O. fumeum* was in the present study was isolated from the same host and *Phoracantha* galleries a few thousand kilometers apart, suggests that there could be some form of association between the host tree, fungus and insect. Given their their close association with Ophiostomatoid fungi (Malloch & Blackwell 1993, Roets *et al.* 2008), it is probable that mites are involved in this association. Considering the geographical distribution, hosts

and possible arthropod associations, it appears that Southern Africa could represent a centre of diversity for species in the *S. schenckii-O. stenoceras* complex.

The three known species of *Ophiostoma*, *O. quercus*, *O. tsotsi* and *O tasmaniense*, found in the present study are members of the *O. piceae* complex. The so-called *O. piceae* complex was first recognized to comprise nine species of fungi infecting either hardwood or coniferous trees (Harrrington *et al.* 2001). In recent years, the number of species in the complex has expanded substantially, and it is now recognized to consist of at least two major lineages, one of which includes only hardwood-infesting species (Harrington *et al.* 2001, Grobbelaar *et al.* 2009, 2010a, Linnakoski *et al.* 2009, Kamgan Nkuekam *et al.* 2008b, 2010a). All three species found on *Eucalyptus* in this study form part of this hardwood lineage.

It was not surprising to find *O. quercus*, on *Eucalyptus* trees in this study. The fungus was first reported from South Africa on native *Olinia* sp. and on *E. grandis* and *Quercus robur* L. (De Beer *et al.* 2003a). This was followed by reports of the fungus from non-native bark beetles infesting *Pinus* trees (Zhou *et al.* 2006) and more recently from wounds on native South African trees (Kamgan Nkuekam *et al.* 2008a). It has also been reported from Uganda (Kamgan Nkuekam *et al.* 2008b) and Tanzania (Grobbelaar *et al.* 2009). In this study, we collected *O. quercus* from three *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*, *E. grandis*) and from logs of various *Eucalyptus* trees grown in the Soutpansberg and Tzaneen areas (Limpopo Province), Cape Town, George, Stellenbosch (Western Cape Province), Kumbu and Lotebeni (Eastern Cape Province) (Table 1). This finding has expanded the host and geographic ranges of *O. quercus* in South Africa substantially. It further supports the view that *O. quercus* has a wide host and geographic distribution (Brasier & Kirk 1993, Harrington *et al.* 2001, De Beer *et al.* 2003a, Kamgan Nkuekam *et al.* 2008b). It has, however, been suggested that it represents a species complex (Grobbelaar *et al.* 2008), a fact that was also clear in the phylogenetic analyses conducted for the current study. This could change views regarding the host and geographic range of the fungus in the future.

Isolates of *O. quercus* collected from eucalypt trees in this study were found to be genetically diverse, especially in the BT sequence data, where nine haplotypes of the fungus were identified. The ITS and TEF data were more conserved. This trend has emerged in the past from numerous studies considering *O. quercus* (Grobbelaar *et al.* 2008, 2009, Kamgan Nkuekam *et al.* 2010). It provides further evidence of the fact that this heterothallic fungus reproduces sexually in Southern Africa, and if it is not native to the area it has been present in the country for a very long period of time.

O. quercus was collected from a wide variety of sites in this study. The fungus was found in the Western Cape Province, which has a Mediterranean climate with hot sunny summers and cold, rainy winters, in Limpopo with a tropical climate, Tzaneen and Mpumalanga with sub-tropical climates, and in the Eastern Cape Province with a temperate climate with year-round rainfall (Figure 7). The fungus thus seems to be highly adaptive to various climatic conditions, which explains why it has a worldwide distribution (Morelet 1992, Brasier & Kirk 1993, Harrrington *et al.* 2001, De Beer *et al.* 2003a, Kamgan Nkuekam *et al.* 2008a, b, Paciura *et al.* 2010).

Ophiostoma tsotsi is a recently erected member of the *O. piceae* complex, closely related to *O. quercus*. It occurs throughout southern and eastern Africa on hardwood trees in countries such as Malawi, Uganda and South Africa (Grobbelaar *et al.* 2009). In Malawi, *O. tsotsi* occurs on *E. grandis* and native *Julbenardia globiflora* (Benth.) Troupin, in Uganda it is known from non-native *Acacia mearnsii* while in South Africa it occurs on various *Eucalyptus* species (Grobbelaar *et al.* 2009). It has recently also been reported from *Eucalyptus* pulpwood chips in China (Grobbelaar *et al.* 2010b). In this study, *O. tsotsi* was found on three *Eucalyptus* spp. (*E. grandis*, *E. maculata*, *E. cloeziana*) and is reported for the first time from nitidulid beetles. *O. tsotsi* was for many years confused with *O. quercus* based on their cultural resemblance and similar morphology. However, it was recently recognized as a distinct taxon based on genetic studies using polymorphic sequence repeats (Grobbelaar *et al.* 2008) and multigene DNA sequence phylogenies (Grobbelaar *et al.* 2009). The presence of many haplotypes within the BT and TEF data sets shows that the species is highly variable.

Similar to *O. quercus*, *O. tsotsi* was collected from a wide variety of sites in this study. It was found in most areas where *O. quercus*, a closely related species, was collected (Figure 7). Recent reports of *O. tsotsi* from *Eucalyptus* trees in Australia and China, and from agar wood in Vietnam, clearly show that the distribution and host range of the fungus is poorly understood (Paciura *et al.* 2010, Kamgan Nkuekam *et al.* 2010, Grobbelaar *et al.* 2010b). It has also been shown that, like *O. quercus*, *O. tsotsi* could have a global distribution and can survive under wide varieties of climatic conditions.

This study represents the first report of *O. tasmaniense* on *Eucalyptus* trees in South Africa. The fungus was first described from eucalypts in Australia (Kamgan Nkuekam *et al.* 2011). *O. tasmaniense* appears to prefer a Mediterranean climate. It was found exclusively in the Western Cape Province (Cape Town and Stellenbosch) in South Africa, while in Australia, it originated from

numerous localities in Tasmania, which resides in a temperate climatic zone. In Australia, *O. tasmaniense* occurs on *E. nitens*, *E. globulus* and *E. saligna* (Kamgan Nkuekam *et al.* 2011), while in South Africa it was found on *E. saligna*, *E. diversicola* and it was isolated from nitidulid beetles. It is possible that this fungus is native to Australia where eucalypts are endemic.

This study represents the first reports of *O. quercus*, *O. tsotsi* and *O. tasmaniense* from nitidulid beetles. The three fungi were isolated from *Brachypeplus depressus*, from an unknown *Carpophilus* species and from *Carpophilus humeralis*. *O. quercu*s in the northern Hemisphere is vectored by bark beetles in what is considered to be a loose association (Kirisits 2004, Romón *et al.* 2007). Most recently it was also reported from a *Scolytus* sp. infesting birch trees in Norway (Linnakoski *et al.* 2009). The only other report of an *Ophiostoma* spp. associated with nitidulid beetles is *O. piceae* that was isolated from the surfaces of seven free-flying nitidulid beetles in the United States of America (Juzwik & French 1983). In the southern Hemisphere, the only report of *O. quercus* from insects is from non-native bark beetles infesting pine trees in South Africa (Zhou *et al.* 2001). Our study strongly suggest that *O. quercus* and other closely related *Ophiostoma* spp. can be vectored by nitidulid beetles and that these insects spread these fungi in this country.

This study represents the most comprehensive consideration of *Ophiostoma* and nitidulid beetles infecting *Eucalyptus* trees in South Africa ever to have been undertaken. The results show that *Ophiostoma* spp. are diverse on wounds on *Eucalyptus* spp. in South Africa and they highlight the need for additional studies of this type where other *Ophiostoma* species are likely to be discovered. Isolating *O. quercus*, *O. tasmaniense* and *O. tsotsi* from nitidulid beetles collected during the surveys suggests that nitidulid beetles are probably the vectors of these fungi, and it emphasizes the value of monitoring these insects and their fungal associates. Of the fungi collected in this study, *O. quercus*, *O. tasmaniense* and *O. tsotsi* have been reported from trees in countries other than South Africa. This suggests intercontinental movement of these fungi, most likely through infected plant material and insects often associated with them.

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Table 1: Geographic distribution, host range and Genbank accession numbers for *Ophiostoma* isolates collected in this study, including BT haplotypes of isolates from the *O. piceae* complex.

Species	CMW	Host/Insect	Origin	ITS	BT		EF
				Acc. no.	Haplotypes	Acc. no.	Acc. no.
O. candidum sp. nov.	26482	*E. cloeziana*	Soutpansberg, LIM	NA	X	NA	NA
	26483	"	"	HM051408	X	HM041873	"
	26484	"	"	HM051409	X	HM041874	"
	26485	"	"	HM051410	X	HM041871	"
	26486	"	"	HM051411	X	HM041872	"
	26487	"	"	NA	X	NA	"
	26488	"	"	"	X	"	"
	26489	"	"	"	X	"	"
	26817	"	"	"	X	"	"
O. fumeum sp. nov.	26813	*Eucalyptus* sp.	"	HM051412	Y	HM041878	"
	26814	"	"	HM051413	Y	HM041875	"
	26815	"	"	NA	Y	NA	"
	26816	"	"	HM051414	Y	HM041876	"
	26818	"	Zambia	HM051415	Y	HM041877	"
	26819	"	"	NA	Y	NA	"
	26820	"	"	NA	Y	NA	"
	26822	"	"	NA	Y	NA	"
O. quercus	25020	*E. grandis*	Tzaneen, LIM	NA	A	NA	"
	27057	*Eucalyptus* sp.	George, WC	NA	F	NA	"
	27058	"	"	HM051398	J	HM041859	"
	27059	"	"	NA	B	NA	"
	27060	"	"	NA	H	NA	"
	27062	"	"	NA	J	NA	"

27064	"	"	NA	F	NA	"
27071	"	"	NA	F	NA	"
27072	"	"	NA	H	NA	"
27073	"	"	NA	G	NA	"
27074	"	"	NA	H	NA	"
27075	"	"	NA	F	NA	"
27076	"	"	NA	I	NA	"
27077	"	"	NA	I	NA	"
27078	*E. diversicolor*	Cape Town, WC	NA	A	NA	"
27089	"	"	NA	A	NA	"
27145	*E. saligna*	"	NA	B	NA	"
27147	*Eucalyptus* sp.	George, WC	NA	F	NA	"
27150	"	"	HM051405	G	HM041865	"
27152	"	"	HM051404	C	HM041860	HM041895
27164	*Eucalyptus* logs	Stellenbosch, WC	HM051403	F	HM041864	HM041894
27165	"	"	NA	A	NA	NA
27168	*E. grandis*	Kumbu, EC	"	A	"	"
27169	"	"	"	B	"	"
27170	"	"	"	A	"	"
27171	"	"	"	B	"	"
27172	"	"	"	B	"	"
27173	"	"	HM051397	H	HM041856	HM041897
27174	"	"	NA	B	NA	NA
27191	*E. diversicolor*	Cape Town, WC	"	A	"	"
27192	"	"	HM051399	A	HM041858	HM041893
28196	*Eucalyptus* sp.	George, WC	NA	H	NA	NA

	28201	*Eucalyptus* logs	Cape Town, WC	HM051400	H	HM041857	HM041892
	28212	*E. grandis*	Lotebeni, EC	NA	B	NA	NA
	28213	"	"	"	B	"	"
	28214	"	"	"	A	"	"
	28215	"	"	HM051402	E	HM041863	HM041891
	28216	"	"	NA	B	NA	NA
	29693	*Carpophilus* sp.	Cape Town, WC	"	A	"	"
	29717	*B. depressus*	"	"	A	"	"
	29734	"	Kumbu, EC	HM051407	D	HM041862	HM041896
	29736	"	"	NA	A	NA	NA
	29738	"	"	"	A	"	"
	29824	"	"	"	B	"	"
	29827	"	Stellenbosch, WC	"	F	"	"
	29832	"	"	"	A	"	"
	30863	*E. saligna*	Soutpansberg, LIM	"	B	"	"
	30864	"	"	HM051406	B	HM041861	HM041890
	30865	"	"	NA	B	NA	NA
	30868	*E. grandis*	Sabie, MP	"	A	"	"
	30869	"	"	"	A	"	"
O. tasmaniense	27090	*E. diversicolor*	Cape Town, WC	"	L	"	"
	27146	*E. saligna*	"	HM051391	M	HM041849	"
	27161	*E. diversicolor*	"	HM051392	K	HM041850	HM041886
	27189	"	"	NA	K	NA	NA
	27190	"	"	"	K	"	"
	27193	"	"	HM051396	L	HM041853	"
	27195	"	"	HM051393	K	HM041851	HM041887

	29685	*Carpophilus* sp.	"	NA	K	NA	NA
	29688	"	"	HM051395	K	HM041852	HM041888
	29691	"	"	NA	K	NA	NA
	29712	*B. depressus*	"	HM051401	L	HM041855	HM041889
	29831	"	Stellenbosch, WC	HM051394	L	HM041854	HM041885
O. tsotsi	24954	*Eucalyptus* sp.	Pietermaritzburg, KZN	NA	2	NA	NA
	24956	"	"	"	1	"	"
	24959	"	"	"	1	"	"
	24964	"	"	"	5	"	"
	24966	"	"	"	1	"	"
	24968	"	"	"	1	"	"
	24971	"	"	"	1	"	"
	24977	"	KwaMbonambi, KZN	"	1	"	"
	24978	"	"	"	5	"	"
	24981	"	"	"	6	"	"
	24982	"	"	HM051390	3	HM041845	HM041882
	24983	"	"	HM051388	5	HM041847	HM041880
	24985	"	"	NA	1	NA	NA
	24986	*E. grandis*	Tzaneen, LIM	HM051385	4	HM041848	HM041879
	24987	"	"	NA	2	NA	NA
	24988	"	"	HM051387	2	HM041846	HM041883
	24990	"	"	NA	5	NA	NA
	24992	"	"	"	1	"	"
	24993	"	"	"	1	"	"
	24996	"	"	"	1	"	"

24997	"	"	"	5	"	"
24999	"	"	"	1	"	"
25000	"	"	"	2	"	"
25002	"	"	"	1	"	"
25003	"	"	"	1	"	"
25004	"	"	"	1	"	"
25005	"	"	"	2	"	"
25006	"	"	"	1	"	"
25007	"	"	"	1	"	"
25009	"	"	"	1	"	"
25010	"	"	"	2	"	"
25011	"	"	"	1	"	"
25013	"	"	"	1	"	"
25014	"	"	"	1	"	"
25016	"	"	"	5	"	"
25018	"	"	"	1	"	"
25022	"	"	"	1	"	"
25024	"	"	"	1	"	"
25036	*B. depressus*	KwaMbonambi, KZN	"	1	"	"
25038	"	"	HM051389	1	HM041843	HM041881
25042	"	"	NA	5	NA	NA
25048	*C. humeralis*	"	"	5	"	"
25055	"	"	"	2	"	"
25058	"	"	"	5	"	"
25066	"	"	"	1	"	"
25068	"	"	"	1	"	"

25071	"	"	"	1	"	"
25072	"	"	HM051386	6	HM041844	HM041884
26328	*B. depressus*	Soutpansberg, LIM	NA	2	NA	NA
26459	*E. cloeziana*	"	"	1	"	"
26460	"	"	"	1	"	"
26461	"	"	"	1	"	"
26462	"	"	"	1	"	"
26463	"	"	"	1	"	"
26464	"	"	"	1	"	"
26465	"	"	"	5	"	"
26467	*E. maculata*	"	"	1	"	"
26468	"	"	"	1	"	"
26469	"	"	"	1	"	"

EC = Eastern Cape

WC = Western Cape

LIM = Limpopo

KZN = KwaZulu Natal

MP = Mpumalanga

NA = not available

Figure 1: Phylogram obtained from maximum likelihood analyses of DNA sequences data from part of BT gene region, showing the relationship between members of *Ophiostoma piceae* complex from eucalypt trees in South Africa and other *Ophiostoma* reference strains. Isolates sequenced in this study are in bold font type. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

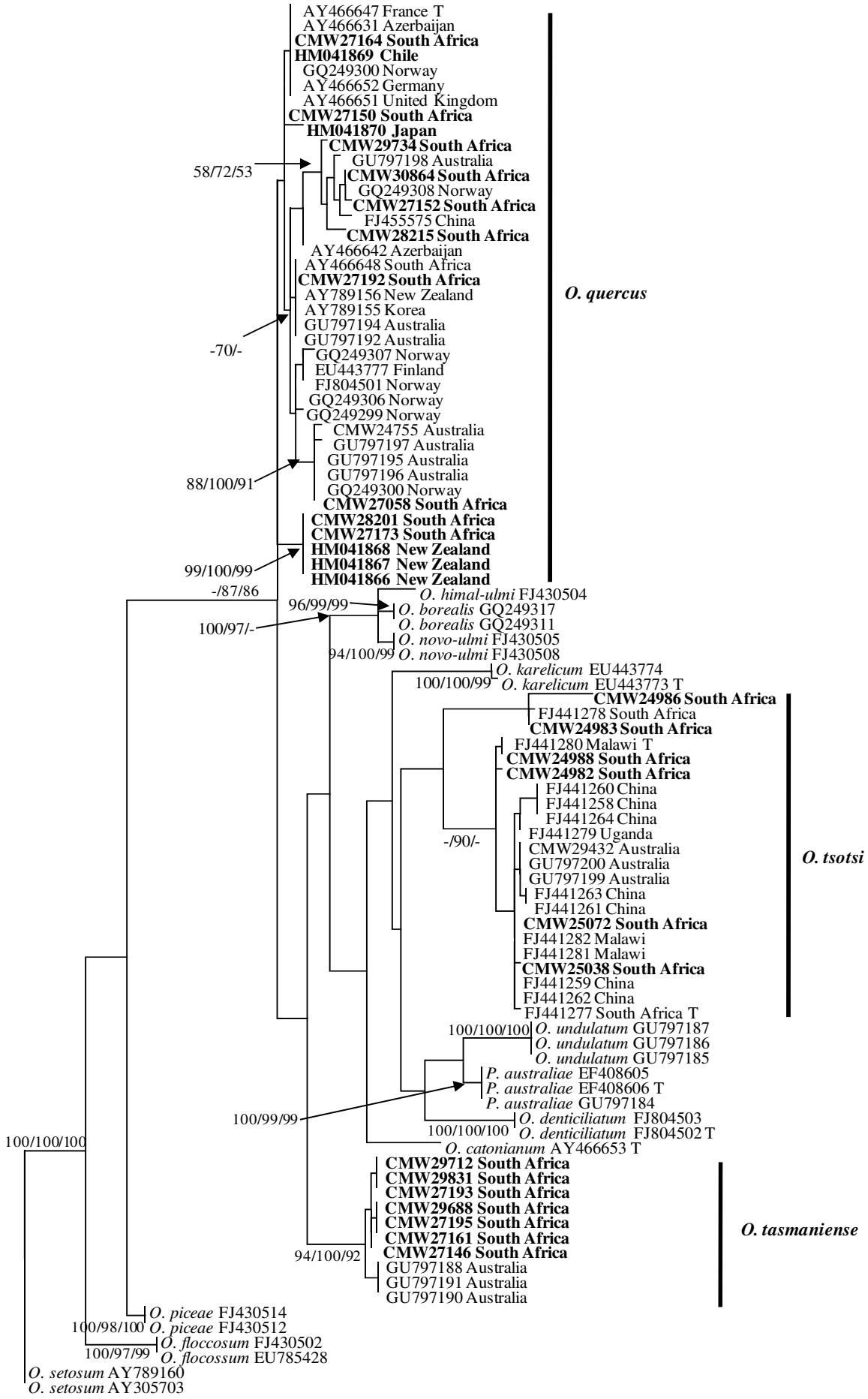


Figure 2: Phylogram obtained from maximum likelihood analyses of DNA sequences data from part of BT gene region, showing the relationship between *O. fumeum*, *O. candidum* from eucalypt trees in South Africa and other members of *Ophiostoma stenoceras* reference strains. Isolates sequenced in this study are in bold type. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

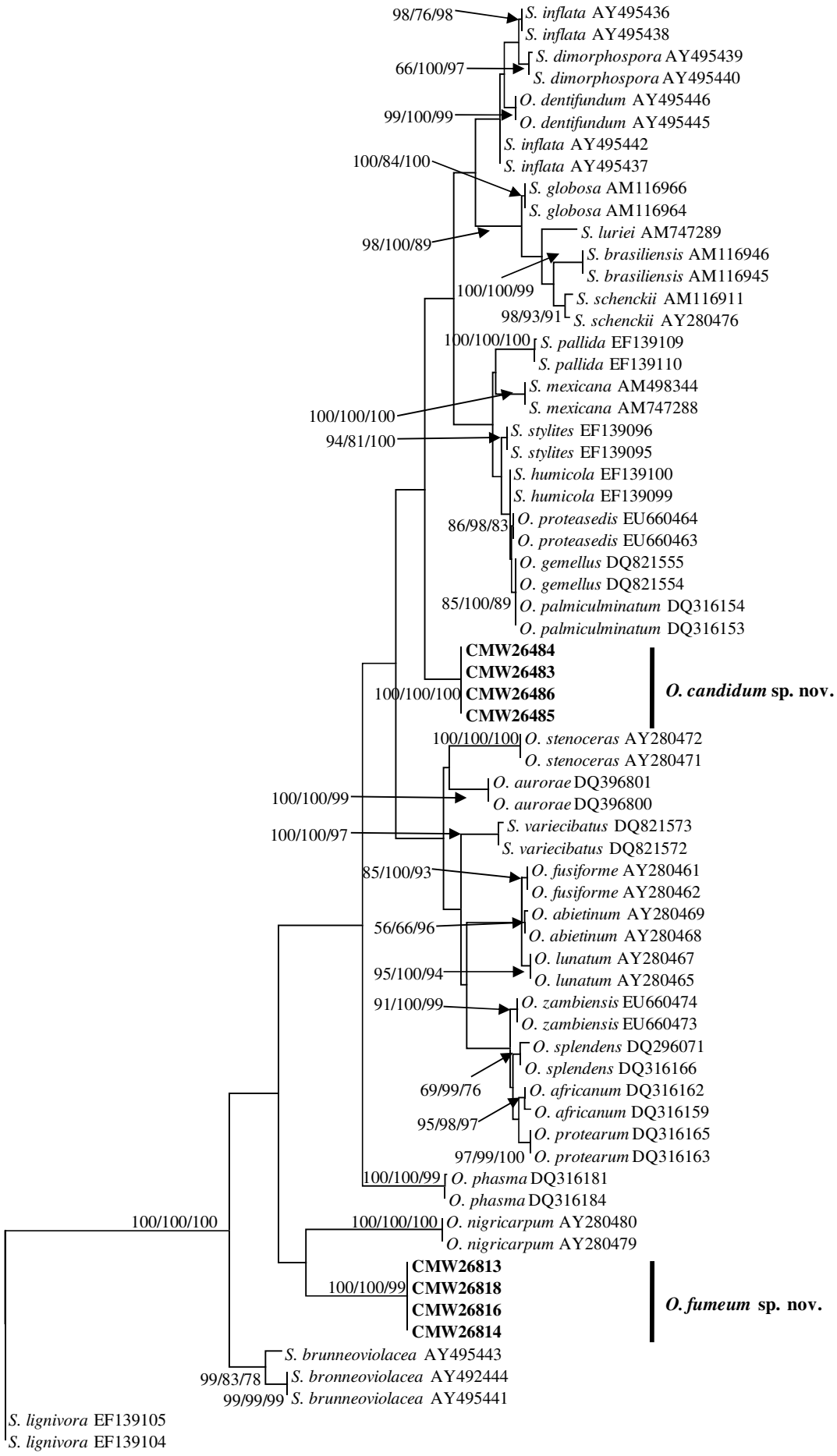


Figure 3: Phylogram obtained from maximum likelihood analyses of DNA sequences of the TEF gene region, showing the relationship between members of *Ophiostoma piceae* complex from eucalypt trees in South Africa and other *Ophiostoma* reference strains. Isolates sequenced in this study are in bold font type. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node.

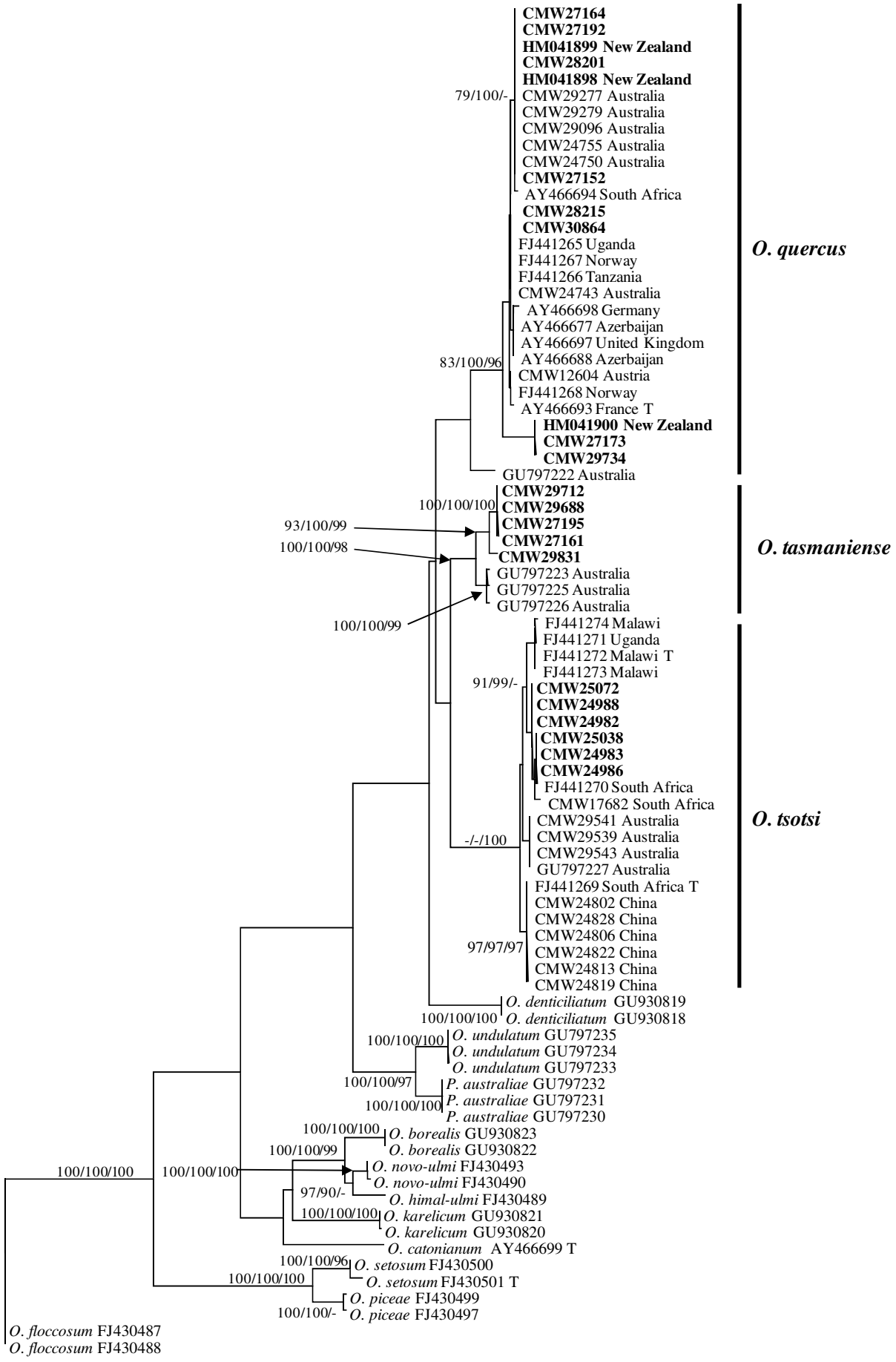
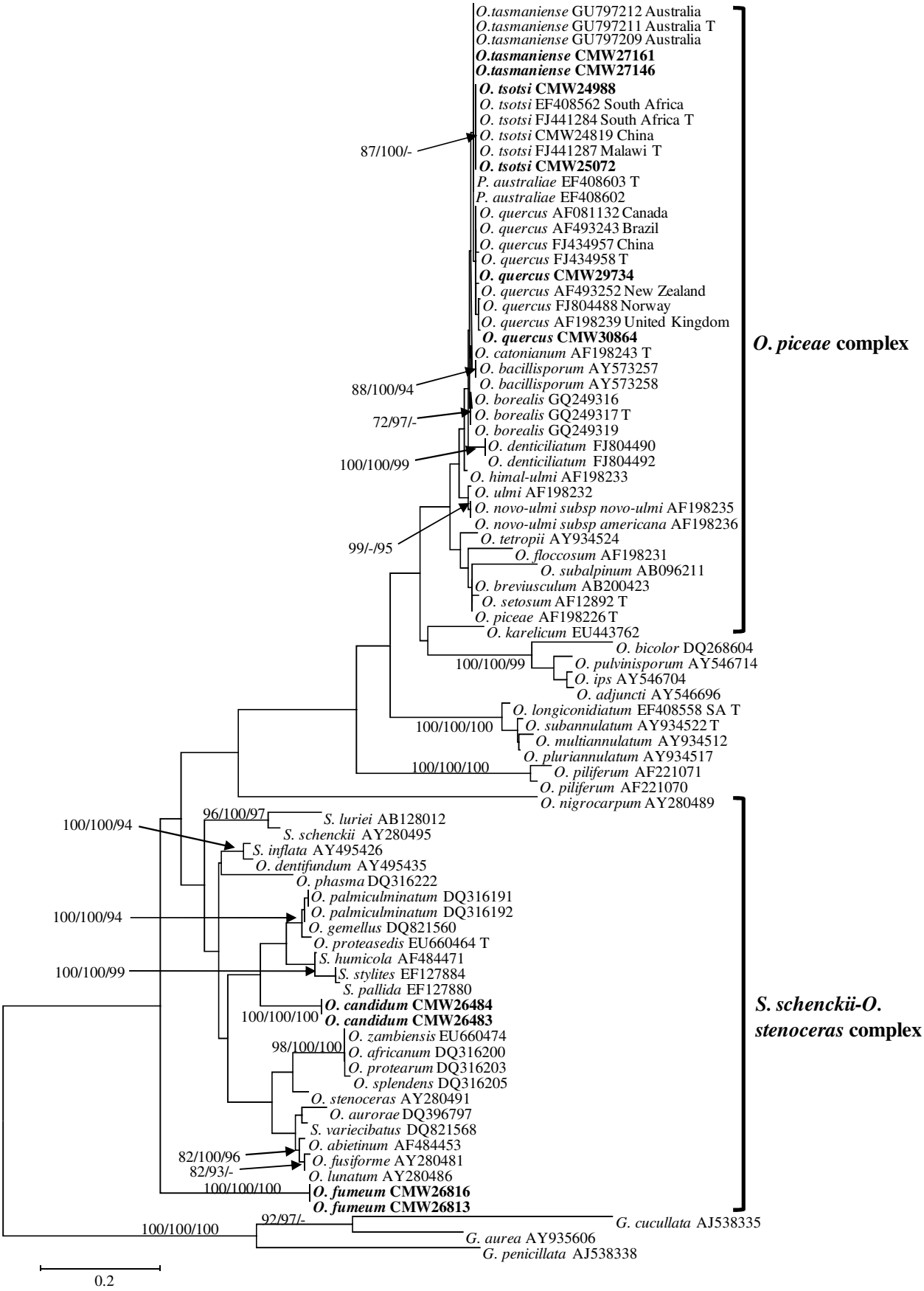


Figure 4: Phylogram obtained from maximum likelihood analyses of DNA sequences of the ITS gene region, showing the relationship between *Ophiostoma* species from *Eucalyptus* trees in South Africa and other *Ophiostoma* reference strains. Isolates sequenced in this study are in bold type. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node



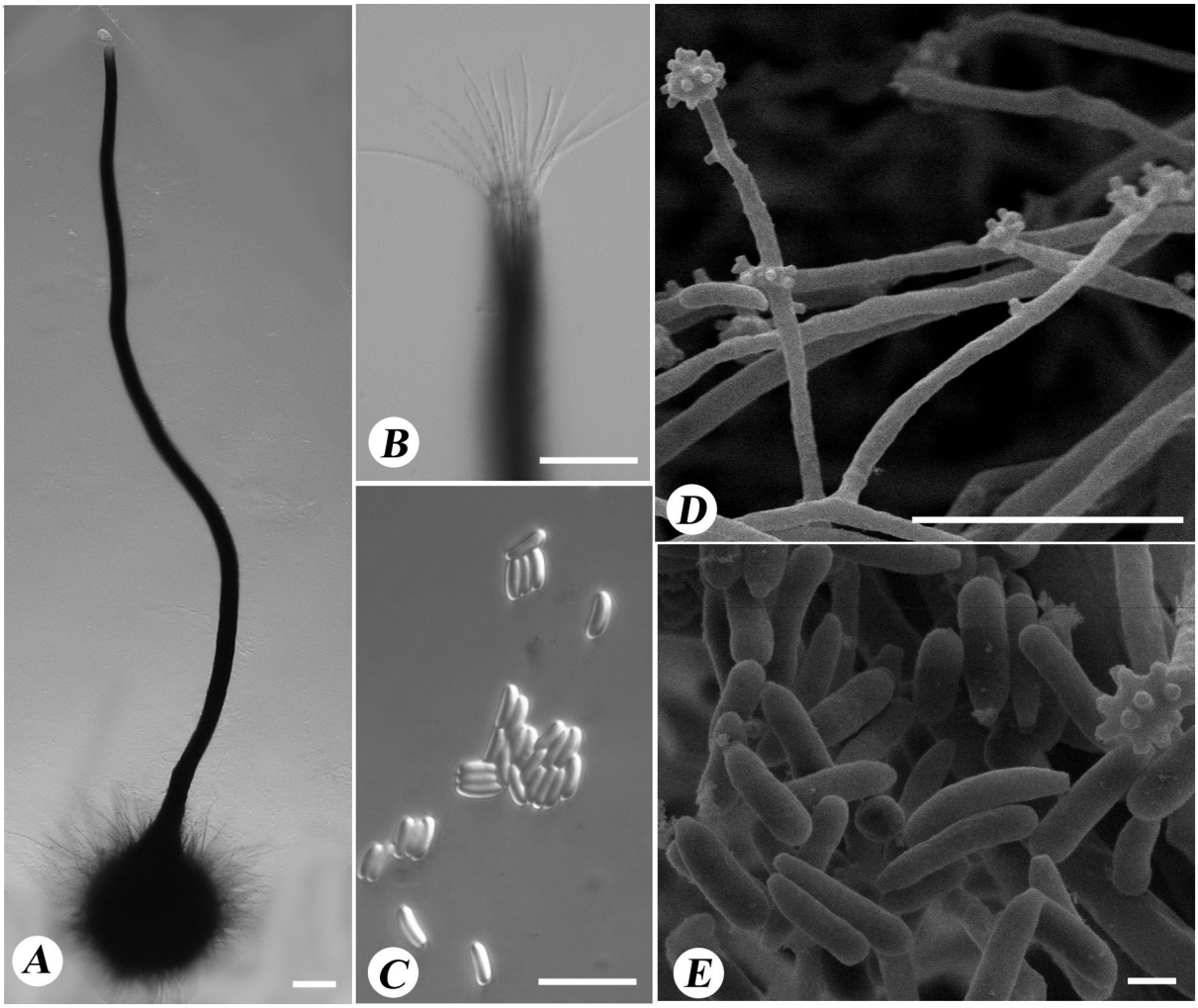


Figure 5: Morphological characteristics of *Ophiostoma candidum* sp. nov. A) Globose ascomatal base (scale bar = 50 µm), B) divergent ostiolar hyphae (scale bar = 20 µm), C) Reniform ascospores (scale bar = 10 µm), D) *Sporothrix* conidiogenous cell with denticles visible on tip of conidiogenous cell (scale bar = 10 µm), E) Oblong to curved conidia (scale bar = 1 µm).

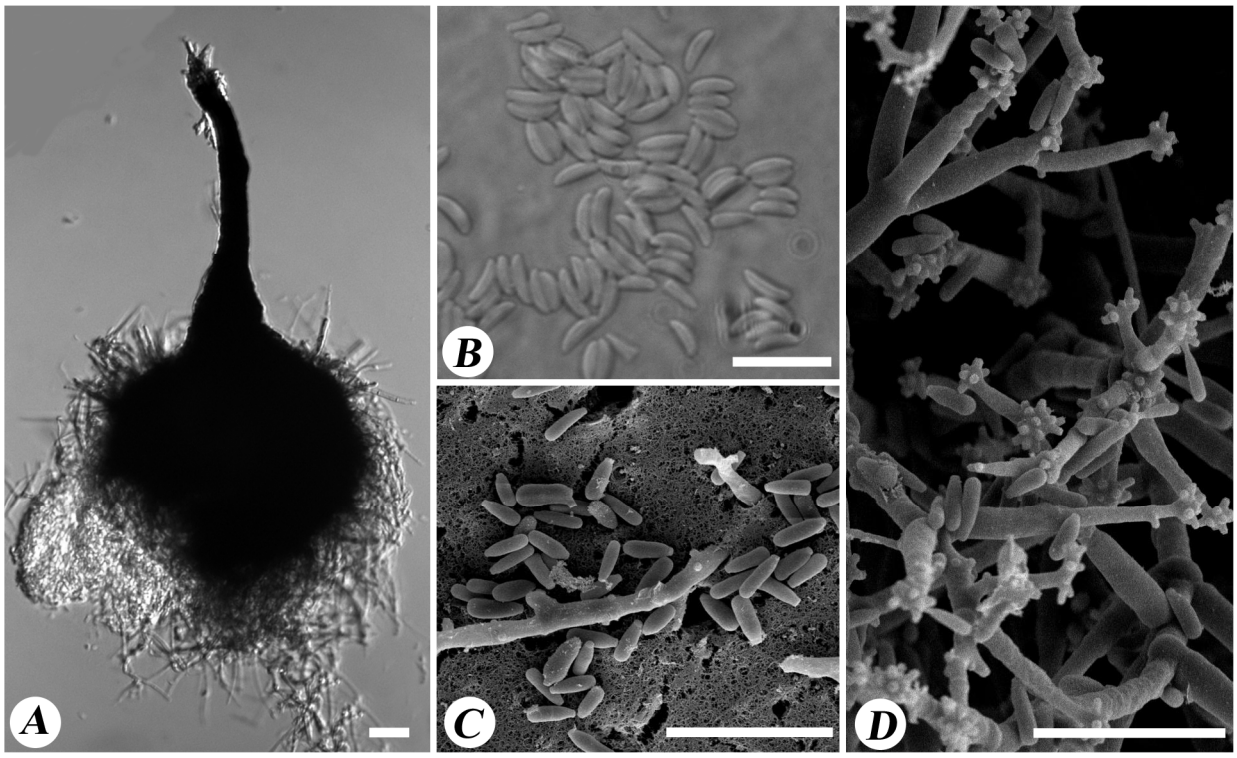


Figure 6: Morphological characteristics of *Ophiostoma fumeum* sp. nov. A) Globose ascomatal base (scale bar = 20 µm), B) Allantoid ascospores (scale bar = 10 µm), C) Oblong conidia (scale bar = 10 µm), D) *Sporothrix* conidiogenous cell with denticles visible on tip of conidiogenous cell (scale bar = 10 µm)

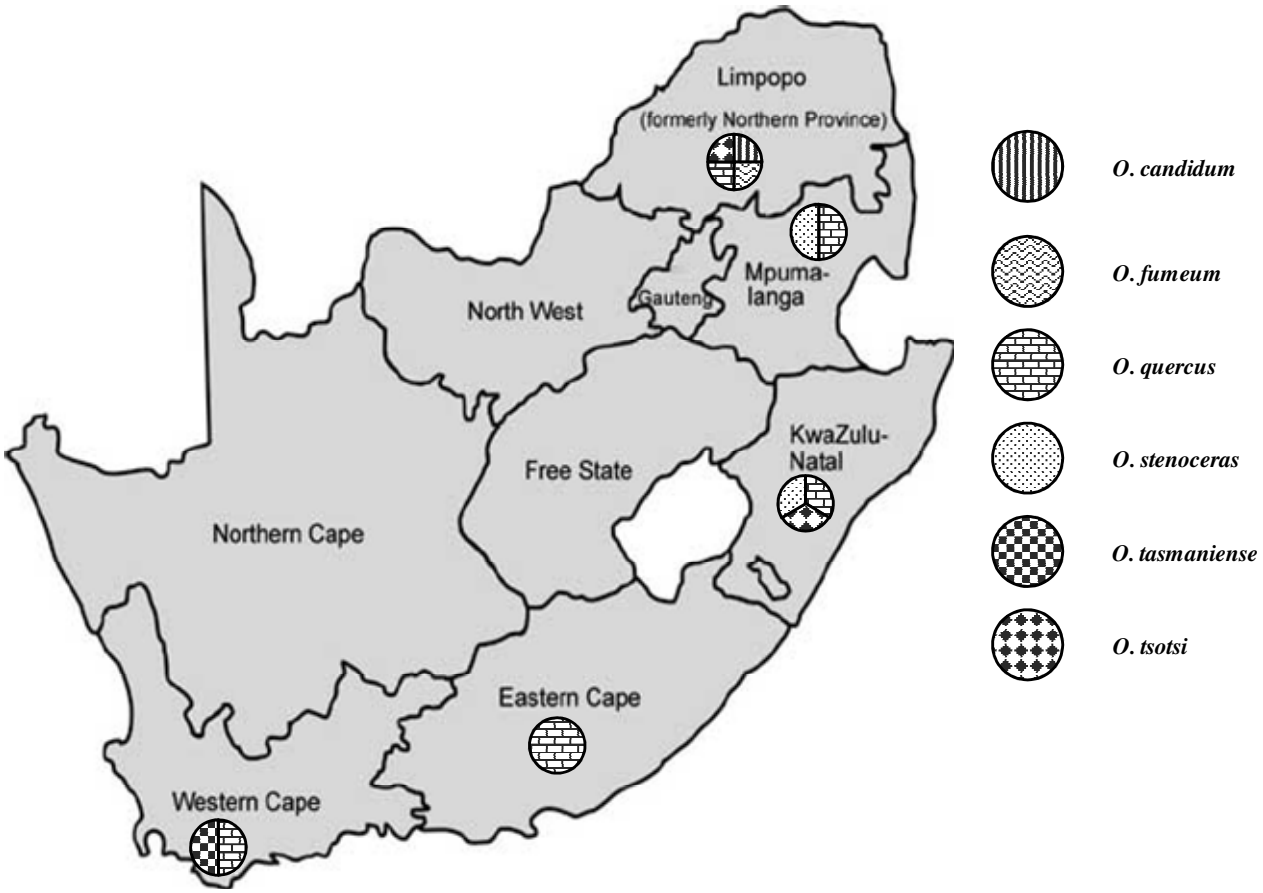


Figure 7: Map of South Africa showing the current geographic distribution of Ophiostomatalean fungi on eucalypt in the country.

Summary

The eucalypt plantation industry in South Africa is an important source of revenue for the country. Eucalypt trees have been planted in the country for more than 200 years, as ornamentals, sources of fuel and construction wood, and more recently also for the production of paper and rayon, amongst other uses. The planting of eucalypts in South Africa has contributed to the development of many forestry industries. It has also reduced the logging of indigenous forests. Eucalypts in South Africa are, however, affected by numerous pests and pathogens. These include native South African pests and pathogens, as well as those introduced from other countries. Of the pathogens, those commonly reffered to as pathogenic Ophiostomatoid fungi, particularly species of *Ceratocystis* and *Ophiostoma* are probably the best known. In recent years, some fungal species in the genera *Ceratocystis* and *Ophiostoma* have emerged as important threats to plantation forestry globally and it is critical to understand these fungi in order to reduce their future impact on eucalypts. In this dissertation, Ophiostomatoid fungi that infested eucalypt trees in Australia and South Africa, that were also identified from nitidulid beetles that visit these hosts, were identified using morphology and DNA sequence data. As a foundation for the studies in the dissertation chapter one provided a review of the literature pertaining to insects in the Nitidulidae and their association with fungi including *Ceratocystis* spp. In chapters two and three, four new taxa, *C. tyalla*, *C. corymbiicola*, *O. tasmaniense*, *O. undulatum* as well as four previously known species, *C. pirilliformis*, *Ophiostoma quercus*, *O. tsotsi*, *Pesotum australiae* were identified from eucalypts in Australia. The four new taxa were not pathogenic to *E. grandis* seedlings in greenhouse inoculation studies. In the last two chapters, seven *Ceratocystis* and five *Ophiostoma* spp. were identified from various *Eucalyptus* spp. and including some isolated from nitidulid beetles in South Africa. The *Ceratocystis* spp. included two new taxa (*C. salinaria*, *C. decipiens*) and five known species, *C. eucalypticola*, *C. pirilliformis*, *C. moniliformis*, *C. oblonga*, *C. savannae*. The *Ophiostoma* spp. included two new taxa of the *Ophiostoma stenoceras-Sporothrix schenkii* species complex (*O. candidum*, *O. fumeum*) and three known species (*O. quercus*, *O. tsotsi*, *O. tasmaniense*). The new taxa of *Ceratocystis* and *Ophiostoma* identified in South Africa were not pathogenic to *E. grandis* seedlings in a greenhouse inoculation studies. Most of the fungi identified were encountered on some nitidulid insects, and four of these fungi where found in both Australia and South Africa. This suggests an intercontinental movement of *Ceratocystis* and *Ophiostoma*, probably mediated by insects, in particular nitidulid beetles.