Ceratocystis and *Ophiostoma* species infecting wounds on hardwood trees, with particular reference to South Africa

By

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DECLARATION

I, the undersigned, hereby declare that this thesis submitted herewith for the degree Magister Scientiae 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

January 2007

I dedicate this thesis to my late grandmother Ngankam Pauline and my father Nkuekam Rene

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PREFACE

Africa is a continent with limited forest resources, with only 17% of the world's naturally forested areas occurring on the continent. The increasing demand for wood and wood products has lead to the over-exploitation of natural forests, worldwide. This is particularly true in Africa where natural forests have and continue to be cleared for agricultural land, fire and construction wood. During the course of the past few decades, planted forests of non-native tree species have been established in many African countries to reduce deforestation of native trees. This has also been to increase wood biomass production to respond to the world demand for wood and wood products. These planted forest areas have increased considerably on the continent, and currently cover approximately 8 million ha. As solutions to one problem often lead to others, the impact of pests and diseases has become a serious problem for the sustainability of these plantations. These infestations by pests and diseases also pose a threat to the remaining native forests on the continent.

Ceratocystis and *Ophiostoma* are ascomycete genera, commonly known collectively as the Ophiostomatoid fungi. Species of these genera are spread by insects with which they have symbiotic and in some cases mutualistic associations. *Ceratocystis* and *Ophiostoma* include well-known plant pathogens that cause diseases of crop plants and forest trees worldwide. Disease symptoms range from sap-stain and canker to wilt and in many cases tree death. Ophiostomatoid fungi require wounds to infect their host trees. These wounds are commonly caused by animals, humans, wind, hail and insects during the relatively long life cycles of the trees.

Very little is known regarding the biodiversity and pathogenicity of the Ophiostomatoid fungi on the African continent. Reports from Africa are restricted mostly to the southern part of the continent and from a limited number of studies. This lack of knowledge has prompted the investigations presented in this dissertation. It is also of great importance to have a global perspective of pathogens, therefore, this dissertation also includes two chapters dealing with Ophiostomatoid fungi on wounds in countries in Europe and in Australia, the latter a country of origin of a number of non-native plantation trees grown in Africa.

The first chapter of this dissertation presents a literature review and focuses on *Ceratocystis* spp. and *Ophiostoma* spp. that have been associated with broad-leaved tree species, especially on the African continent. Attention is given to wounds as infection sites for these fungi and on reports from Africa. The review also includes a brief introduction to the state of world forestry in general. Particular focus is placed on the current status of forestry on the African continent. A short summary on the Ophiostomatoid fungi and their confused taxonomy is included and this is followed by treatments on infection sites, insect associations, economic importance and occurrence of these fungi on the African continent.

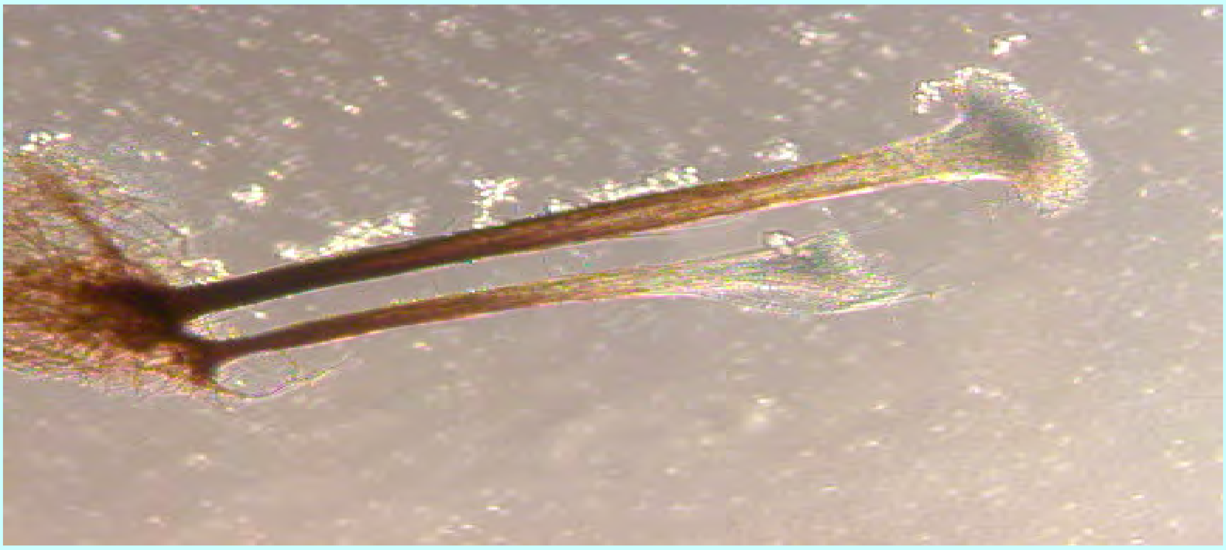
Chapter two of the dissertation deals with the population biology of *Ceratocystis pirilliformis* in South Africa. *C. pirilliformis* has been reported only from Australia and South Africa where it occurs on wounds on *Eucalyptus* spp. Very little is known regarding the epidemiology of this species. Therefore, studies presented in this chapter intend to provide clues about the possible distribution and origin of *C. pirilliformis* in South Africa. High levels of polymorphisms, abundant throughout the genome, are found in this fungus and closely related species contain similar repetitive regions. Thus, microsatellites markers developed for *Ceratocystis fimbriata* are used in this chapter to study the gene diversity in Australian and South African populations of *C. pirilliformis*.

Chapter three concerns the identity of *Pesotum* spp. associated with *Acacia mearnsii* trees in Uganda and Australia. *Pesotum* spp. are anamorphs of *Ophiostoma* species. In nature, many species of *Ophiostoma* produce their asexual form rather than their sexual form. This, in addition to great inter-species morphological and cultural variation makes their identification difficult. Very few species of *Ophiostoma* and their anamorphs have been recorded in the Southern Hemisphere. The work presented in this chapter was, therefore, planned to increase our knowledge regarding the distribution and identity of *Pesotum* spp. in the Southern Hemisphere, particularly in Africa. Use is made of both morphological characteristics and DNA sequence analysis.

Chapter four deals with the biodiversity of *Ceratocystis* spp. and *Ophiostoma* spp. associated with wounds on native hardwood tree species in South Africa. Very little research has been done with regard to these fungi on native tree species in Africa. However, recent reports from South Africa suggest strongly that a number of species, including potential pathogens, await reporting from Africa. The aim of this chapter was to collect *Ceratocystis* spp. and *Ophiostoma* spp. from native broad-leaved trees and to identify them.

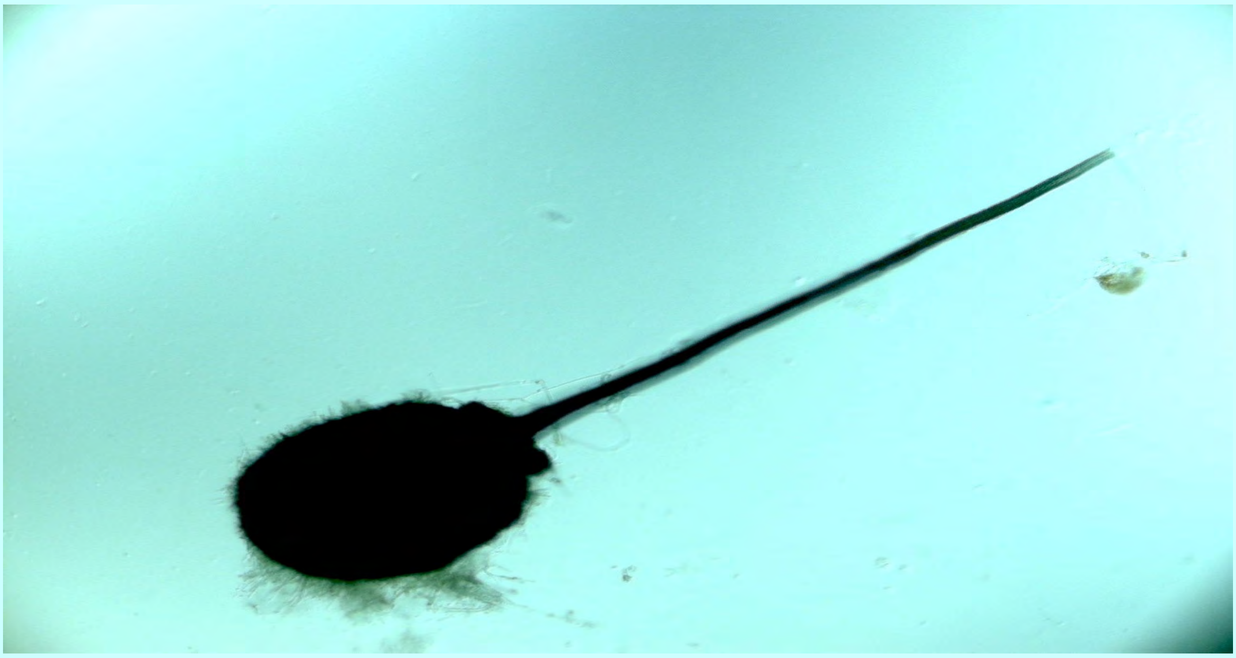
Chapter five of this dissertation concerns *Ophiostoma* spp. infecting wounds on native broad-leaved tree species in Norway. Some isolates obtained from Sweden and from researchers in Austria were also included for comparative purposes. Over the past decades, research on this group of fungi in Nordic countries and Europe has been focused on those species associated with conifer infesting bark beetles. However, very few species of *Ophiostoma* have been reported from native hardwood tree species. The aim of this study was thus to collect *Ophiostoma* spp. from wounds on native broad-leaved trees in Norway, Austria and Sweden and to identify them appropriately.

Studies in this dissertation serve to emphasize the lack of knowledge that scientists have in general, regarding fungal biodiversity on planet earth. This is especially true, since species of *Ceratocystis* and *Ophiostoma* are considered some of the more important fungi economically and thus most likely studied in more detail than other fungal groups. The work in this dissertation does not present a complete picture, but I hope to have exposed certain important lines of future research focus, which I trust will contribute to our understanding of the fungi and the diseases they cause on trees.



CHAPTER 1

Ceratocystis and *Ophiostoma* species, with particular reference to wound infection and those species reported from trees in Africa.



1.0 INTRODUCTION

The total forest area of the world is estimated at 3869 million hectares and this is made up of approximately 3682 million ha natural forests, and 187 million ha of plantation forests (Anonymous 2005, Carnus *et al*. 2003). On the African continent, natural forests cover 642 million ha, or 17% of the world's natural forested areas (Anonymous 2005, Carnus *et al*. 2003). These natural forests consist primarily of timber forests and woodland (thorn and brush) (Zon & Sparhawk 1923). Timber forests can be roughly divided into three large groups: (1) dense tropical forests, (2) open tropical forests and (3) subtropical and temperate forests consisting of conifers and hardwoods (Zon & Sparhawk 1923). Trees from these forests are mainly used for firewood, construction of huts and boats, handles for implements, weapons, and fencing material. In previous centuries, European settlers also brought with them a demand for construction timber for houses, furniture, vehicles, mining timber, railroad ties, fuel and many other uses. The commodity value of these forests has supported the economies of many countries on the continent. However, the continent also values forests for the provision of watersheds, wilderness areas, recreation, and habitats for threatened and endangered fish and wildlife species (Thawley & Meyer 2004).

Despite international efforts, deforestation in Africa continues today. This places severe strain on the available resources and trees suitable for timber production are becoming increasingly scarce on the continent (Anonymous 2003a, Hennig 2006, Mkoka 2005). Over the past few decades, many African countries have established plantations of non-native tree species including species of *Eucalyptus* L'Her. *Pinus* L., *Acacia* Mill. and *Cupressus* L. to name some of the most commonly planted genera (Kiwuso 1991, Negash 1997, Odera 1991, Persson 1995). For example, Tanzania has approximately 89 000 ha of industrial plantations (Nshubemuki *et al*. 1996) and Kenya has approximately 165 000 ha of plantation species which comprises trees such as *Pinus*, *Cupressus*, *Eucalyptus* and various indigenous hardwoods (Odera 1991). In Uganda, plantations of non-native trees are dominated by *C. lusitanica* and *Pinus* spp. (Kiwuso 1991). A wide variety of *Eucalyptus* spp., with *E. grandis* being the dominant species, are also planted in Uganda (Ruyooka 1999). In Ethiopia, plantations of non-native trees cover a total area of approximately 200 000 ha (Anonymous 1994, Vercoe 1995), while in South Africa, approximately 1.3 million ha of land is covered by plantations of non-native species (Anonymous 2003b). Products derived from these plantations include structural timber, wood for pulp and paper production, utility poles, fuel and mine props (Anonymous 2003b, Sutton 1995). In many cases, however, the non-native tree species are severely affected by fungal and bacterial diseases, resulting in death of trees, reduction of growth and loss of profit (Wingfield 1990, Wingfield & Roux 2000).

The success of plantation forestry using non-native tree species has largely been attributed to the fact that the trees planted have been separated from their natural enemies, mainly pests and pathogens (Bright 1998, Wingfield *et al*. 2001). This has, however, not meant that plantation forestry on the African continent has been without problems. Numerous tree diseases have been reported from African plantations. Examples of important pathogens of plantation forest species in Africa include *Diplodia pinea* (Desm.) Kickx, that infects and kills certain *Pinus* spp. after hail damage (Swart *et al*. 1985). Likewise, *Rhizina undulata* Fr. can cause significant losses to *Pinus* spp. after plantation fires (Germishuizen 1984). Diseases such as Dothistroma needle blight in East Africa, have lead to the discontinuation of the planting of *P. radiata* D. Don in that part of the continent (Gibson *et al*. 1964). Other examples of diseases that changed the species composition of plantations in Africa include Cypress canker of *Cupressus macrocarpa* Hartweg, caused by *Seiridium cupressi* (Guba) Boesewinkel (syn = *Rhynchosphaeria cupressi)* in eastern Africa (Natttrass *et al*. 1963, Wimbush 1944) and *Mycosphaerella nubilosa* (Cooke) Hansf., which lead to the discontinuation of the planting of *E. globulus* Labill. in South Africa (Purnell & Lundquist 1986). Other important pathogens of plantation forestry species in Africa include *Armillaria* spp. that cause root rot (Alemu *et al*. 2004a, Coetzee *et al*. 2001, Roux *et al*. 2005), *Chrysoporthe* spp. causing cankers on *Eucalyptus* spp. (Gibson 1981, Gryzenhout *et al.* 2004, Hodges *et al*. 1986, Myburg *et al.* 2003, Roux *et al.* 2000, 2005, Wingfield *et al*. 1989), *Botryosphaeria* spp. causing cankers (Alemu *et al*. 2004b, Smith *et al*. 1994) and the canker pathogen *Colletogloeopsis zuluense* (MJ. Wingf., Crous & TA. Cout.) MN Cortinas, MJ Wingf. & Crous (syn. *Coniothyrium zuluense*) (Alemu *et al*. 2005, Roux *et al*. 2005, Wingfield *et al*. 1997a), to name, but a few of the most common pathogens.

The so-called Ophiostomatoid fungi including *Ceratocystis* spp., *Ophiostoma* spp., *Ceratocystiopsis* spp. and *Grosmannia* spp. are responsible for significant economic losses to forests, especially in the Northern Hemisphere. Fungi belonging to the Ophiostomatoid group are characterized by mostly dark, globose ascomata with elongated necks. Asci generally disappear early in the development and are seldom seen (Upadhyay 1981). These genera include some well-known plant pathogens, which cause diseases of both agricultural and forestry crops, world-wide (Upadhyay 1981, Wingfield *et al*. 1993a). Examples of these diseases include rot of sweet potatoes and wilt of coffee, rubber and other trees caused by *C. fimbriata* Ellis & Halsted, Dutch elm disease caused by races of *Ophiostoma ulmi* (Buismann) Nannf, and *O. novo-ulmi* Brasier and oak wilt caused by *C. fagacearum* (Bretz) Hunt (Bretz 1952, Sinclair *et al*. 1987, Wingfield *et al*. 1993a). The majority of species in these genera are, however, saprophytic, and many cause blue-stain of timber (Münch 1907, Lagerberg *et al.* 1927, Seifert 1993, Uzunovic & Webber 1998). Furthermore, Ophiostomatoid fungi are known for their complex symbiotic relationships with insects, in particular bark beetles (Harrington 2005, Kirisits 2004, Paine *et al*. 1997, Six 2003).

Reports of *Ceratocystis* spp. causing diseases of non-native plantation tree species have increased greatly during the course of the last 10 years (Roux *et al*. 2004). In Africa, *C. fimbriata* has been reported to result in rapid wilting of *Eucalyptus* spp. in the Republic of Congo (Roux *et al*. 2000) and Uganda (Roux *et al*. 2001a). *Ceratocystis pirilliformis* Barnes & Wingfield has been shown to cause lesions on *Eucalyptus grandis* (Hill) Maiden in South Africa (Roux *et al*. 2004), while *C. albifundus* De Beer, Wingfield & Morris, is considered the most important pathogen of non-native *Acacia mearnsii* de Wild trees in South Africa (Morris *et al*. 1993, Roux & Wingfield 1997), and more recently in Uganda (Roux *et al*. 2001a) and Kenya (Roux, personal communication).

Very little is known regarding the biodiversity and impact of the Ophiostomatoid fungi on the African continent. Due to the nature of plantation forestry and use of native tree species on the continent, trees are commonly wounded, providing infection sites for these fungi. The aim of this review is to summarize the taxonomic history of the Ophiostomatoid fungi and to provide an overview of knowledge pertaining to wounds as infection sites, especially where these involve Ophiostomatoid fungi. In addition, particular focus is given to those Ophiostomatoid species reported from hardwood trees in Africa.

2.0 TAXONOMIC HISTORY OF THE OPHIOSTOMATOID FUNGI

Genera of the Ophiostomatoid fungi have been the subject of considerable taxonomic treatment and debate during the course of the last Century, resulting in a complex and often unclear history. At one stage only one genus was recognized within the Ophiostomatoid fungi (Halsted 1890, Sydow &

Sydow 1919), but today at least four genera are commonly recognized (Zipfel *et al*. 2006). The following two synonymies for *Ceratocystis* and *Ophiostoma* aptly illustrate the confusion and overlaping of generic names for these two keystone members of the Ophiostomatoid fungi.

The Genus *Ceratocystis*

Ceratocystis Ellis & Halsted N.J. Agr. Exp. Sta. Bull. 97:14, 1890 (nom. gen. cons. prop.)
=*Sphaeronaemella* Karsten, Hedwigia 23:17,1884 (nom. gen. rej. prop.)
=*Rostrella* Zimmerman, Meded. S'Lands plantentuin 37:24, 1900-non fabre (1878)
=*Endoconidiophora* Munch, Nat. Z. Forst. U. Landw. 6:34, 1907
=*Linostoma* Hohnel, Ann. Mycol. 16:91, 1918-non wallich (1831)
≡*Ophiostoma* H. & P. Sydow, Ann. Mycol. 17:43, 1919
=*Grosmannia* Goidanich, R. staz. Pat. Veg. Bol. Rome, n. s. 16:26, 1936
=*Europhium* Parker, can. J. bot. 35:175, 1957
Type species: *Ceratocystis fimbriata* Ellis & Halsted (Halsted 1890)

The Genus *Ophiostoma*

Ophiostoma H. & P. Sydow, Ann. Mycol. 17:43, 1919
=*Linostoma* Hönel, Ann. Mycol. 16:91, 1918-non Wallich? (1831)
≡*Ceratocystis* Bakshi (1951)
≡*Europhium* Upadhyay (1981)
Type species: *Ophiostoma piliferum* (Fries) Sydow & Sydow (1919)

The taxonomic histories of the Ophiostomatoid fungi date back more than a Century. Within the Ophiostomatoid fungi, the genus *Ceratocystis* (ceratos =horn and cyst = pouch or sac) was first described by Ellis and Halsted (Halsted 1890), for a fungal strain associated with black rot of sweet potato (*Ipomoea batatas* Lam) in the United States of America (Halsted 1890, Hunt 1956). Although no formal generic description was provided in the original paper, *Ceratocystis* was later described as a monotypic genus, with *Ceratocystis fimbriata* Ellis & Halsted as the type species (Halsted & Fairchild 1891). In the original description, the ascomata and the ascospores, produced in evanescent asci, were misinterpreted as pycnidia and conidia, leading to considerable confusion and renaming of *C. fimbriata.* On the basis of the supposed pycnidial stage, Saccardo (1892) discarded *Ceratocystis* and transferred the species to *Sphaeronaemella fimbriatum* Fr. (Saccardo

1892). In 1918, *S. fimbriatum* was transferred to *Linostoma* Von Hönel based on its observed carbonaceous perithecia with long necks and ovoid asci containing spores arranged in several rows (Von Hönel 1918). Unfortunately, the name *Linostoma* had already been given to a genus of flowering plants in the Thymeleaceae. Thus Sydow & Sydow (1919) transferred the genus to *Ophiostoma* Sydow & Sydow, describing *Ophiostoma piliferum* (Fries) Sydow & Sydow, as the type species and the second genus to be known in the Ophiostomatoid fungi. They included *C. fimbriata* and all related species previously in *Linostoma* in this new genus. This was the first occasion where *Ceratocystis* and *Ophiostoma* became intermingled and it was the start of considerable taxonomic debate surrounding the status of these two genera.

The taxonomic history of *C. fimbriata* after 1919 is complex and characterized by considerable confusion. Elliot (1923), established that the "pycnidia" of *C. fimbriata* (=*O. fimbriata*) were indeed perithecia and he transferred the species back to *Sphaeronaemella* as *S. fimbriata* (Elliot 1923). Subsequently the species was re-transferred back to *Ophiostoma* by Nannfeldt as *O. fimbriata* (in Melin & Nannfeldt 1934), and later to *Endoconidiophora* Münch (Davidson 1935), a monotypic genus established for species that formed conidia endogenously.

Nannfeldt (1932) established the family Ophiostomataceae for the genus *Ophiostoma*. In 1934, Melin and Nannfeldt divided *Ophiostoma* into two sections based on neck lengths. Those species accommodated in the section Brevirostrata had short, conical perithecial necks. The species assigned to section Longirostrata were further divided to accommodate species with endogenous conidia [*Thielaviopsis* Went (=*Chalara* (Corda) Rabenh.)] (Paulin-Mahady *et al*. 2002) and those with exogenous conidia, in *Sporothrix* Hektoen & Perkins, *Leptographium* Lagerberg & Melin, *Pesotum* Crane and *Hyalorhinocladiella* Upadhyay & Kendr (Melin & Nannfeldt 1934).

A number of other genera have been associated with the Ophiostomataceae. Goidanich (1936) proposed the genus *Grosmannia* to include species of *Ophiostoma* with *Leptographium* anamorphs. Parker (1957), established the genus *Europhium* Parker with the type species *E. trinacriforme* Parker, which he considered to be closely related to *Ceratocystis*, but differing from the latter genus in its neckless, closed ascocarps. Benny and Kimbrough (1980), and Upadhyay (1981) later reduced this genus to synonymy with *Ceratocystis*. However, Von Arx and Van der Walt (1988) resurrected the genus and placed it in the family Ophiostomataceae.

Numerous taxonomic revisions have been made in both the teleomorph and anamorph names of *Ceratocystis* and *Ophiostoma*, based on a number of characters. These include microscopic, ultrastructural, chemical, biochemical, immunological, and genetic analyses to study the species within *Ceratocystis* and *Ophiostoma*. Bakshi (1951) revived the generic name *Ceratocystis* for species with both endogenous and exogenous conidia. He reduced to synonymy six genera (*Ceratocystis*, *Rostrella* Zimmermann, *Endoconidiophora*, *Linostoma*, *Ophiostoma* and *Grosmania* G. Goidanich) with *Ceratocystis*. Hunt (1956) in his revision of the Ophiostomatoid fungi, accepted Bakshi's (1951) changes and classified species of *Ceratocystis* and *Ophiostoma* known at that time within *Ceratocystis.* He based his decision on the similarities of the shape and size of ascomata, the morphology of the ascospores and the presence or absence of ostiolar hyphae. Based on characters of the anamorph stages, Hunt (1956) established three Sections. Species with an endoconidial state (*Thielaviopsis*) were accommodated in Section 1, species with a *Leptographium* or *Graphium* state in Section 2 while species with mycelial conidia only (*Sporothrix*) resided in Section 3 (Hunt 1956).

2.1 Ascospore characteristics

A number of authors recognized the importance of ascospore characters to aid in the identification of species of *Ceratocystis sensu lato* (Griffin 1968, Hunt 1956). In particular, Olchowecki and Reid (1974) stressed the importance of these characters while surveying the *Ceratocystis* spp. in Manitoba, Canada. Based on ascospore characteristics, they established four groups in the genus: The Minuta-group, was thus characterized by elongated, usually curved ascospores with terminally attenuated sheaths. The Ips-group was characterized by cylindrical or dumbbell-shaped ascospores with gelatinous sheaths. The Pilifera-group was characterized by curved, ovoidal or cylindrical ascospores without sheaths, and a heterogenous "Fimbriata-group''was established to house the remaining *Ceratocystis* spp. Upadhyay and Kendrick (1975), proposed another division of *Ceratocystis* based on ascospore morphology. Species with falcate ascospores were classified in the newly established genus *Ceratocystiopsis* Upadhyay and Kendrick while the remaining species were treated in *Ceratocystis* regardless of their anamorphs (Upadhyay & Kendrick 1975, Upadhyay 1981). *Ceratocystiopsis* spp. corresponded more or less to the Minuta-group of Olchowecki and Reid (1974).

Species of *Ceratocystiopsis*, previously segregated from *Ophiostoma* purely on the basis of their falcate, sheathed ascospores (Upadhyay & Kendrick 1975), do not represent a monophyletic group based on DNA sequence comparison (Hausner *et al*. 1993a). Wingfield (1993), in his review on the status of *Ceratocystiopsis* suggested that the falcate sheaths in some *Ceratocystiopsis* species might represent vestiges of the ascomatal centrum and not distinct sheaths. In addition, the anamorphs of *Ceratocystiopsis* and *Ophiostoma* are extremely similar and species of the two genera are also similarly adapted to dispersal by bark beetles (Wingfield 1993). These facts suggest that *Ophiostoma* and *Ceratocystiopsis* form a homogenous group and that *Ceratocystiopsis* should be considered a synonym of *Ophiostoma* (Wingfield 1993). Subsequently, the genus *Ceratocystiopsis* was synonomised with *Ophiostoma* based on analyses of their DNA sequence data (Hausner *et al*. 1993a). Phylogenetic analysis of partial ribosomal DNA (rDNA) sequences from both the small subunit (ssrDNA) and large ribosomal subunit (lsrDNA) showed that *Ceratocystiopsis* spp. reside within the same clade as *Ophiostoma* spp. (Hausner *et al*. 1993a).

2.2 Biochemistry

The biochemistry of many species of *Ceratocystis* and *Ophiostoma* has been studied in some detail. Marked differences were found that more or less correlated with anamorph distribution. Species with *Thielaviopsis* anamorphs lack cellulose in their cell walls (Jewell 1974, Rosinski & Campana 1964, Smith *et al*. 1967), whereas this compound is present in the remaining species. In addition, all analyzed strains of the latter group contain rhamnose (Spencer & Gorin 1971, Weijman & De Hoog 1975), while this compound is absent in species with *Thielaviopsis* anamorphs (De Hoog & Scheffer 1984). Weijman & De Hoog (1975), divided the genus *Ceratocystis* into two groups, based on the results of cell wall cellulose analyses (Smith *et al.* 1967) and the presence of rhamnose (Spencer & Gorin 1971). The species with *Thielaviopsis* states (phialidic endogenous conidial form) were treated as *Ceratocystis sensu stricto* and those with *Graphium*-like states as *Ophiostoma* H. & P. Sydow (De Hoog & Scheffer 1984, Weijman & De Hoog 1975). Studies using selective media including cycloheximide have been used in the past to isolate *Ophiostoma* spp. (Hicks *et al*. 1980, Schneider 1956). Harrington (1981), expanded on these studies while trying to connect tolerance to cycloheximide and taxonomy of some selected *Ceratocystis* and *Ophiostoma* spp. He found that, in culture *Ceratocystis* spp. are extremely sensitive to the antibiotic cycloheximide, whereas species of *Ophiostoma* tolerate high concentrations of this compound (Harrington 1981).

2.3 Ultrastructure

During the late 1980's and 1990's, ultrastructural characters were found to be useful in distinguishing species in the Ophiostomatoid fungi. Centrum development, in particular was shown to be useful to separate species in *Ceratocystis* and *Ophiostoma*. Studies of the development of the teleomorph structures revealed that, in *Ceratocystis* spp. young asci are produced from ascigerous cells that line, or are adjacent to the inner ascomatal (centrum) wall. In *Ophiostoma* spp. the production of asci is restricted to the base of the ascomatum (Van Wyk *et al.* 1991, Van Wyk *et al*. 1993). However, hat-shaped ascospores in *Ceratocystis* and *Ophiostoma* are not the same. The ascospores of *C. moniliformis* and *C. fimbriata* for example are bowler-hat shaped in side view while ascospores in *Ophiostoma* species appear triangular in end view and therefore, would appear hat-shaped only in side view (Van Wyk *et al*. 1993).

A niche of Ophiostomatoid fungi, including five species has been described from the infructescences of *Protea* spp. in South Africa. Unlike other Ophiostomatoid fungi they do not require wounds for infection of their host trees and are unique in that they are confined to the infructescences of *Protea* spp. These fungi include, *Ceratocystiopsis protea* Wingfield, Van Wyk & Marassas (Wingfield *et al*. 1988), *O. capense* Wingfield & Van Wyk (Wingfield & Van Wyk 1993), *O. splendens* Marais & Wingfield (Marais & Wingfield 1994), *O. protearum* Marais & Wingfield (Marais & Wingfield 1997) and *O. africanum* Marais & Wingfield (Marais & Wingfield 2001). Of these, *C. protea* was the first species described. It shared characteristics with both *Ceratocystis* and *Ophiostoma* but due to its falcate ascospores, was placed in the genus *Ceratocystiopsis* (Wingfield *et al*. 1988). *O. capense* resembles *C. protea* in many aspects, differing only in ascospore morphology (Wingfield 1993). The two species have an unusual *Knoxdaviesia* Wingfield MJ., Van Wyk PS & Marasas anamorph and have been transferred to a new genus *Gondwanamyces* (Marais & Wingfield 1994, Marais 1996, Marais *et al*. 1998).

2.4 DNA sequence data

The long and protracted controversy regarding the significance of morphological (primarily in the anamorphs), biochemical and physiological distinctions between genera in the Ophiostomatoid fungi (Table 1) has been conclusively solved by analyses of rDNA sequences. These have clearly shown that the Ophiostomatoid fungi are polyphyletic (Hausner *et al*. 1992, 1993b, Okada *et al.* 1998, Spatafora & Blackwell 1993, 1994a, 1994b, Zipfel *et al*. 2006). Most recent multigene phylogenies based on nuclear large sub-unit (LSU), partial ribosomal DNA (rDNA), as well as β-tubulin genes have shown conclusively that *Ceratocystis* and *Ophiostoma* represent two distinct genera, residing in two separate orders. *Ceratocystis* spp. reside within the Microascales, while *Ophiostoma* spp. reside in the Ophiostomatales, closely related to the Diaporthales and very distant from *Ceratocystis* (Spatafora & Blackwell 1994b). Furthermore, the genera *Grosmannia* and *Ceratocystiopsis* also represent distinct genera and reside in the Ophiostomataceae with *Ophiostoma* (Zipfel *et al*. 2006). The genus *Ophiostoma* now comprises only species with *Sporothrix* and *Pesotum* anamorphs. Species with *Leptographium* anamorphs were placed in the re-instated genus *Grosmannia*, while species with *Hyalorhinocladiella* anamorphs were placed in the re-instated genus *Ceratocystiopsis* (Zipfel *et al*. 2006). Species with *Knoxdaviesia* anamorphs are placed in the genus *Gondwanamyces* in the order Microascales and unrelated to the Ophiostomatales (Viljoen *et al*. 1999).

2.5 Current Ophiostomatoid taxonomy

Despite the great progress that has been made in delimiting the Ophiostomatoid fungi, much work is still required to clearly define the species within these various genera. It has been shown with sequence data that the genus *Ceratocystis* is polyphyletic, possibly representing more than one genus (Witthuhn *et al*. 1999, Wingfield *et al*. 2006). This has become obvious in recent studies, which have shown that two main phylogenetic clades exist in the genus *Ceratocystis*. One of these contains species such as *C. moniliformis* (Hedgcock) Moreau, *C. fagacearum* (Bretz) J. Hunt and *C. virescens* (Davidson) Moreau, while the other consists of *C. fimbriata*, *C. albifundus* and *C. pirilliformis* (Witthuhn *et al*. 1999, Barnes *et al*. 2003a, Roux *et al*. 2004). At the species level, many *Ceratocystis* spp. have also been shown to represent species complexes and require further illucidation. Both within *C. fimbriata* and *C. moniliformis* for example, a number of cryptic species have recently been identified and described as separate species and many still remain to be illucidated (Barnes *et al*. 2003b, Johnson *et al*. 2005, Baker Engelbrecht & Harrington 2005, Van Wyk *et al* 2004a).

With new species of *Ceratocystis* and *Ophiostoma* still being described regularly, together with studies of known species, valuable information is being collected to clarify the best delineation of the Ophiostomatoid fungi and all the genera currently and previously associated with them. There are, however, also still many countries and even continents from which very little information is available regarding the Ophiostomatoid fungi. The inclusion of species from these continents, including Africa, could greatly impact on the taxonomy of these genera and needs carefull consideration.

3.0 OPHIOSTOMATOID FUNGI AS PLANT PATHOGENS

Several economically important species reside in the Ophiostomatoid fungi. These include species that are pathogens of crop plants (Baker & Harrington 2001, Halsted 1890), forest and shade trees (Harrington 1993a, Kile 1993), while some are associated with staining of forest products (Lagerberg *et al*. 1927, Seifert 1993). Others are known to be associated with insect infestation of wood (Grossmann 1931, 1932, Harrington 2005, Kirisits 2004, Six 2003). There are also some species to which no particular economic role has been attributed (Hunt 1956, Upahyay 1981, Wingfield *et al*. 1993a).

Numerous important plant pathogens, including tree pathogens in the genus *Ceratocystis* have been reported, especially from the American continent. In the United States of America (USA), for example, *C. fagacearum* causes Oak wilt, which annually results in the death of large patches of these trees (Griswold 1958, Gibbs & French 1980, Norris 1953, Sinclair *et al*. 1987). In South America, *C. fimbriata* causes wilt and canker-stain diseases of numerous woody plants including coffee (Marin *et al*. 2003), *Eucalyptus* (Roux *et al*. 1999, Barnes *et al*. 2003b) and *Acacia* (Ribeiro *et al*. 1988). *Ceratocystis* spp. are also known as pathogens in Europe and in recent years, they have been reported from a number of hosts in Asia. For example, *Ceratocystis polychroma* M. Van Wyk, M. J. Wingfield & E. C. Y. Liew, has been associated with dramatic dieback of clove trees (*Syzygium aromaticum* L. Merr. & Perry) in Sulawesi (Van Wyk *et al*. 2004a) while *C. platani* (Walter) Engelbrecht & Harrington causes disease of plane trees (*Platanus* spp.) in Europe (Panconesi 1999, Walter 1946, Walter *et al*. 1952). Yet, these are only examples and *Ceratocystis* spp. have a cosmopolitan distribution, and cause diseases of a wide range of hosts on all continents (Kile 1993, Wingfield *et al*. 1993a).

Well-known plant pathogens in *Ophiostoma* are *Ophiostoma ulmi* and *O. novo-ulmi*, the causal agents of Dutch Elm Disease (Brasier 2000, Hubbes 1999). A large component of the blue-stain in conifer timber is caused by species of *Ophiostoma* and *Grosmannia* and their anamorphs (Seifert

1993). The three varieties of *Leptograghium wageneri* (Goheen & F.W.Cobb) T.C.Harr., for example, are responsible for black-stain root disease of conifers in the Western United States of America (Cobb *et al*. 1987, Cobb 1988, Harrington 1993b, Wagener & Mielke 1961), while *L. serpens* (Goid.) M. J. Wingfield, has a much wider distribution including Europe and South Africa (Wingfield & Marasas, 1980, 1981, Wingfield *et al.* 1993a) and is thought to contribute to tree disease in some situations. Other *Leptographium* spp. known to be associated with diseases on trees include *L. procerum* (W. B. Kendr.) M. J. Wingf. that is associated with a root disease of pines (Jacobs & Wingfield 2001, Wingfield & Marasas 1980, 1981), *L. terebrantis* Barras & Perry consistently associated with extensive lesions on pines (Jacobs & Wingfield 2001, Wingfield 1986), and *L. calophylli* (Wiehe) JF. Webber, K. Jacobs & MJ. Wingfield causing vascular wilt disease of takamaka trees (*Calophyllum inophyllum* L.) (Ivory & Andre 1995, Kudela *et al*. 1976, Wiehe 1949, Crandall 1949, Webber *et al*. 1999). Additional information on Ophiostomatoid fungi causing disease and sapstain of hardwood trees in Africa is presented in more detail later in this review.

4.0 SPREAD AND INFECTION STRATEGIES OF OPHIOSTOMATOID FUNGI

Ophiostomatoid fungi rely on a number of mechanisms for spread. The majority of species, however, rely on wounds for infection of their host plants (Wingfield *et al*. 1993a). These wounds may be caused by natural phenomena or by specific insect vectors, as the Ophiostomatoid fungi are specifically adapted to dispersal by insects. Wounds and insects are important in forest pathology, as trees are commonly wounded during the course of their lives. Tree wounds may be caused by silvicultural practices, hail damage, frost, harvesting practices, animals and insects. Fresh wounds attract sap-feeding insects that carry fungi to these substrates. The adaptation of Ophiostomatoid fungi to insect dispersal, wound infection and the fact that trees are commonly wounded act together to make tree species very vulnerable to diseases caused by Ophiostomatoid fungi. In the following section, the role of wounds in the life cycle of the Ophiostomatoid fungi is discussed, including the importance of insects in this interaction.

4.1 Wounds

Wounds are very important and common sites of infection for Ophiostomatoid fungi, both in agriculture and forestry. In some cases, these wounds are created by insects that vector specific Ophiostomatoid species, but this association will be discussed in more detail under section 4.3.

Other types of wounds commonly infected by Ophiostomatoid fungi include wounds made during silvicultural and agricultural practices and also those caused by natural phenomena such as hail.

4.1.1 *Ceratocystis* species

Wounds, such as those caused by pruning or harvesting practices, hail damage etc., are common infection sites for many *Ceratocystis* spp. They reach these wounds in several ways. This can either be by being carried to the wounds by insects, such as by Nitidulid beetles (Coleoptera) (Crone & Bachelder 1961, Hinds 1972, Moller & DeVay 1968), by contaminated equipment (Teviotdale & Harper 1991, Walter 1946, Walter *et al.* 1952) or through wind blown frass (Iton 1960). Insects may also create their own wounds and thus introduce *Ceratocystis* spp. into trees (Harrington & Wingfield 1998, Wingfield *et al*. 1997b).

Bark injuries such as those produced by harvesting instruments provide important infection courts for *C. fimbriata* in deciduous fruit orchards (Moller & Devay 1968). On *Theobroma cacao* L. wounds made at harvesting when pods and stem sprouts are removed may become infected by *C. fimbriata* (Malaguti 1958). In Colombia, it has been reported that the principal factor associated with the dispersal of Ceratocystis canker on coffee trees, is infection through wounds on the stem made by the shoes of farmers as they try to secure themselves on the steep slopes on which coffee is cultivated (Castro 1991, Marin *et al*. 2003).

In South Africa, it has been shown that both hail wounds and pruning wounds are infection sites for *C. albifundus* (Morris *et al*. 1993, Roux *et al*. 2001b). The first report of *C. albifundus* in this country was from *Acacia mearnsii* trees that had been damaged by the removal of branches (Morris *et al*. 1993), while Roux *et al*. (2001b) reported the infection of *A. mearnsii* trees by *C. albifundus* after hail damage to the trees.

Recently, Barnes *et al*. (2003a) and Roux *et al*. (2004) have shown that artificially creating wounds represents a highly effective manner in which to collect *Ceratocystis* spp. By creating bark and xylem wounds these researchers have been able to isolate a previously undescribed species of *Ceratocystis*, *C. pirilliformis* (Barnes *et al*. 2003a). They also managed to expand the geographic range of *C. fimbriata* and *C. pirilliformis* to include *Eucalyptus* spp. in South Africa (Roux *et al*. 2004). This report of *C. fimbriata* in South Africa represents the first confirmed report of this important pathogen in the country, as previous reports have been shown to be incorrect.

4.1.2 *Ophiostoma* species

Similar to *Ceratocystis* spp., *Ophiostoma* spp. can also infect mechanical and other wounds, such as those created by insects and silvicultural practices. It has been found that many *Ophiostoma* spp. occur on logs that have been wounded in such a way as to create sapwood wounds that were protected by residual flaps of bark (Dowding 1970, 1973, Gibbs 1993). On the other hand, species such as *O. minus* (Hedgcock) Sydow & Sydow and *O. piliferum* frequently colonize mechanically injured or partially de-barked logs (Mathiesen-Käärik 1960, Gibbs 1993). Inoculum of these fungi is either wind borne and linked to the *Sporothrix* asexual states that have dry spores, or by casual insects that visit the freshly wounded wood.

4.1.3 Condition of wounds

Wounds are not always succesfully infected by the Ophiostomatoid fungi. The success of infection depends on a number of factors including the type of wound, environmental conditions, inoculum level and the presence of vectors.

The depth of the wound plays an important role in the initiation of infection. With the vascular wilt diseases such as Dutch elm disease and Oak wilt, the wound must reach the wood for the pathogen to penetrate and invade the xylem elements succesfully (Gibbs & French 1980). With a vascular stain disease such as *Ceratocystis* canker of fruit trees, a superficial wound exposing only live inner bark is all that is required for infection (Devay *et al*. 1968).

The age of wounds is an important factor in the initiation of infection by species of *Ceratocystis* and *Ophiostoma*. Kuntz & Drake (1957), showed that the wound must be fresh, as a lapse of only 24 hours from the time of the wounding to the first visit by insect vectors can significantly reduce the likelihood of infection. In the case of *C. fagacearum* for example, no germination of spores is possible in wounds older than 36 hours at the time of inoculation under laboratory conditions (Cole & Fergus 1956).

Other factors that could reduce or prevent infection include the accumulation of anti-fungal host metabolites such as phenolics (Cobb *et al.* 1965) and the rapid colonization of wounds by saprophytic fungi common to the host tree, which outcompete and exclude the pathogen (Gibbs

1980). Infection of wounds is most successful when there is a "bark flap" which retains some moisture and protects the fungus (Dowding 1970, 1973, Gibbs 1993).

Temperature and relative humidity during germination of spores influence the success of wound infection (Cole & Fergus 1956). Studies on *C. fagacearum* have shown that this fungus can survive temperatures as low as -10℃ for 83 days under laboratory experiments (Cole & Fergus 1956). Low humidity was found to favor germination and infection of wounds by *C. fagacearum*. However, ascospores are more resistant to heat and humidity than conidia, since they could survive for 48H at 50℃ and 20% relative humidity, but failed to germinate after 6H at 100% relative humidity. In contrast no conidia remained viable longer than 2H at 50℃ at any humidity level. Conidia survived at low humidity almost as long as ascospores at high humidities (Cole & Fergus 1956).

4.2 Root grafts

4.2.1 *Ceratocystis* species

Ceratocystis platani (*syn. C. fimbriata f. sp. platani*) has been reported to spread readily between adjacent *Platanus* trees via root grafts (Accordi 1986). As trees of the same species growing in close proximity tend to have their root systems grafted together, the fungus will move freely from the first tree in an ever-widening circle, killing the surrounding trees as it moves from one root system to another. This form of dispersal has also been demonstrated for *C. fagacearum* (Gibbs & French 1980, Kile 1993), and probably accounts for the clumping of diseased *Nothofagus cinninghamii* (Hook.) Oerst infected by *Platypus subgranosus* Schedl in Tasmanian rainforests (Helliot *et al.* 1987).

4.2.2 *Ophiostoma* and *Grosmannia* species

Ophiostoma and *Grosmannia* can spread via the roots from diseased to adjacent healthy trees (Gibbs 1974). This type of epidemiology is well documented for the Dutch Elm disease pathogens, especially in hedgerows where *Ulmus* trees arise as coppice shoots and are thus linked to each other via their root network (Gibbs 1974). Many *Leptographium* spp. causing root diseases on conifers, especially *L. wageneri* can also be vectored from diseased to healthy trees through root grafts and sometimes through root contacts (Goheen 1976, Hessburg 1984, Harrington & Cobb 1988).

4.3 Insects

Ceratocystis spp. and *Ophiostoma* spp. are common associates of insects (Griswold 1953, 1955, 1958, Harrington 1993a, 1993b, Moller & Devay 1968). The insects help the fungi in their dissemination and fertilization and open up new and suitable substrates for them. Sap feeding nitidulids, for example, act as agents of fertilization by carrying spores of *C. fagacearum* between different trees (Webber & Gibbs 1989). The fungi in turn may serve as food for the beetles (Bakshi 1950).

Ceratocystis and *Ophiostoma* spp. are specifically adapted for dispersal by insects. Their spores are embedded in sticky mucilages at the tops of the ascomatal necks, while conidia are similarly embedded at the heads of conidiophores. These "sticky spores'' are then conveniently carried away on the bodies of insects such as bark beetles (Bakshi 1950). The mucilage also prevents complete digestion of spores in the alimentary canal of the beetle and it is likely that some of them remain in a viable condition after they have passed through the bodies of the insects and line in the insect tunnels along with faeces (Bakshi 1950). Many species of *Ceratocystis* produce a characteristic banana oil or fruit odor, and in some of the other species, an acidic, or honey-like, aromatic odor is characteristic (Hunt 1956, Lanza *et al*. 1976). These aromas attract insects, which feed on them. The association between *C. fagacearum* and sap-feeding beetles (nitidulids and scolytids) is a well-known example. The fungus lures the beetles by an attractive odor, the beetle feeds on the fungus and becomes contaminated with ascospores and conidia, which is then carried to other trees (Beaver 1989). A wide range of insects is implicated in transmission of *Ceratocystis* and *Ophiostma* spp. However, only wound-making insects such as bark beetles and ambrosia beetles can probably act as significant vectors, the former breed in the phloem while the latter breed in the wood (Beaver 1989).

4.3.1 *Ceratocystis* species

Ceratocystis spp. have different types of interactions with insects. Some species have very specific associations, while those of others are non-specific and at random. *Ceratocystis* spp. are commonly vectored by sap-feeding nitidulids and flies in a non-specific association (Griswold 1953, 1955, 1958, Moller & Devay 1968). Bark injuries are an appropriate breeding place for these two insect groups and coincidentally, the insect provides an effective means to inoculate the fungus into these wounds (Moller & Devay 1968). For example, five members of the family drosophilidae, besides

Drosophila melanogaster Meig, have been shown to be capable of transmitting *C. fagacearum* to wounds on healthy *Quercus* trees under controlled laboratory conditions (Griswold 1953, 1955, 1958). *C. fimbriata sensu lato* on the other hand is well-known as associate of both a drosophylid fly (*Chymomyza procnemoides* Wheeler) and a nitidulid beetle (*Carpophilus freemani* Dobson) (Moller & Devay 1968). However, transmission of *Ceratocystis* spp. by flies and nitidulids, especially *C. fimbriata sensu lato* is circumstancial, because these insects have been observed to visit wounds on trees where Ceratocystis canker is not known to occur, thus the fungus is not essential to their existence (Juzwik & French 1983, Moller & Devay 1968).

Three species of *Ceratocystis* have been recorded as obligate associates of bark beetles (Harrington & Wingfield 1998, Wingfield *et al*. 1997b). These are *C. polonica* (Siemaszko) C. Moreau, adapted to *Picea* spp. and associated with *Ips typographus* L. (Furniss *et al*. 1990, Solheim 1992), *C. laricicola* Redfern & Minter, adapted to *Larix* spp. and associated with *Ips cembrae* Heer (Redfern *et al*. 1987, Harrington *et al*. 1996) and *C. rufipenni* Wingfield, Harrington & Solheim, adapted to *Picea* spp. and associated with *Dendroctonus rufipenni* Kirby (Wingfield *et al*. 1997b). These three species are well-documented, virulent pathogens of conifers in the Northern Hemisphere (Harrington & Wingfield 1998, Wingfield *et al*. 1997b, Solheim 1992, Redfern *et al*. 1987). Sexual fruiting structures in these fungi are mostly confined to the bark beetle galleries and their development is stimulated by wounds. They lack the fruity aroma characteristic of other *Ceratocystis* spp. (Harrington *et al*. 1996). It has been suggested that their lack of fruiting aroma derive from their consistent association with bark beetles (Harrington *et al*. 1996).

Recently *C. bhutanensis* M. Van Wyk, M. J. Wingfield & T. Kirisits, a species new to science has been described in association with a conifer-infesting bark beetle. The fungus was isolated from *Ips schmutzenhoferi* Holzschuh on *Picea spinulosa* (Griffith) A. Henry in Bhutan (Eastern Himalayas) (Van Wyk *et al*. 2004b). *C. bhutanensis* shares very few morphological and phylogenetic features with other *Ceratocystis* spp. associated with bark beetles and it resides in the *C. moniliformis* as opposed to the *C. coerulescens* group (Wingfield *et al*. 2006). In addition it produces a strong aroma in culture that the other above mentioned species lack (Van Wyk *et al*. 2004b).

4.3.2 *Ophiostoma* species

For many years, it has been known that *Ophiostoma* spp. depend on bark beetles for their dispersal, with which they often form specific relationship (Mathiesen-Käärik 1953, Whitney 1982, Paine *et al.* 1997). On the other hand, *Ophiostoma* spp. are also vectored by ambrosia beetles (Bakshi 1950, Mathiesen-Käärik 1953, Kirisits 2004). Some species of *Ophiostoma* serve as ambrosia fungi (Beaver 1989, Grosmann 1967) and appear to be selectively maintained in the mycangia of bark and ambrosia beetles (Barras & Perry 1972, Malloch & Blackwell 1993). Many *Ophiostoma* spp. have also been reported from a wide variety of other insects that act as their vectors. These include 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 1983, 1986, Levieux *et al*. 1989, Moser *et al*. 1989, Moser 1997, Malloch & Blackwell 1993).

The most effective means of fungal dissemination through insect association is the one that involves insect-fungus-mite interactions (Klepzig *et al*. 2001). A well-known example of this complex relationship involves the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), the most damaging of North American forest insects, and its associated mites (Drooz 1985, Klepzig *et al*. 2001, Price *et al*. 1992, Tatcher *et al*. 1980). At least fifty-seven species of mites (Moser & Roton 1971, Moser *et al*. 1974, Moser & Macias-Samano 2000) and forty species of fungi and bacteria (Bridges *et al*. 1984, Moore 1971, 1972) are phoretic on this beetle. Within this population, the closest associates of *D. frontalis* are three species of mites, *Tarsonemus ips* Lindquist, *T. krantzi* Smiley & Moser, and *T. fusarii* Cooreman (Acarina: Tarsonemidae) and three fungal species, *O. ranaculosus* Perry & Bridges, *Entomocorticium* sp. *A* and *O. minus* (Hedgcock) H. & P. Sydow. The fungi are carried in the mycangia (glandular invaginations) of the beetles (Barras & Perry 1972, Barras & Taylor 1973) or on the exoskeleton of the beetles (Barras & Perry 1975) while the mites are transported on the external surfaces of the beetles and do not undergo any feeding or ontogenesis during this period of transport (Lindquist 1969, Smiley & Moser 1974).

The mites feed upon *O. minus* and *O. ranaculosus* in the beetle galeries and also carry these two fungal species within their sporothecae (Bridges & Moser 1983, Dowding 1969, Lombardero *et al*. 2000). The mites benefit from the beetles in obtaining transport to new suitable host material (Klepzig *et al*. 2001). The mites normally have little or no direct effect on the bark beetles that transport them (Kinn & Witcosky 1978, Moser 1976, Stephen *et al*. 1993). However, the mites have the potential to interrupt the interactions between the bark beetles and their mutualistic fungi since they transport and inoculate an antagonistic fungus [*Ophiostoma minus* (Hedgc.) H. & P. Sydow], into beetle galleries, which could outcompete other fungi that are potential food to the beetle larvae (Barras 1970, Bridges 1985, Franklin 1970).

The role of the associated fungi in the beetle life cycle may be to serve as nourishment to the beetle larvae (Barras 1970, Bridges & Perry 1985) and assist the beetle in killing the hosts, or at least overcome tree resistance (Klepzig *et al*. 2001). The benefit of this interaction to the fungus is clearer. This component of the symbiosis obtains transport and introduction to a specific target tree species (Dowding 1969, Webber & Gibbs 1989).

5.0 *CERATOCYSTIS* SPP. ON HARDWOOD PLANTATION FORESTRY TREES

Six *Ceratocystis* spp. have been reported to affect hardwood trees in plantations (Table 1). Three of these, *C. eucalypti* Z.Q.Yuan & Kile, *C. moniliformis* (Hedgcock) Moreau and *C. moniliformopsis* Yuan & Mohammed appear not to be pathogenic. Two species, *C. fimbriata*, and *C. albifundus* are well-known pathogens of *Eucalyptus* spp. (Barnes *et al*. 2003b, Roux *et al*. 1999, 2001a, 2004) and *A. mearnsii* (Morris *et al*. 1993, Roux & Wingfield 1997, Roux *et al*. 2001b), respectively. *C. pirilliformis* has been described very recently (Barnes *et al*. 2003a) and very little is known regarding its role as a pathogen under natural conditions. However, it has been shown to be capable of causing lesions during artificial inoculation studies in the greenhouse and field (Roux *et al.* 2004). The following section treats these fungi individually.

5.1 *Ceratocystis albifundus*

Ceratocystis albifundus causes the disease known as Ceratocystis wilt (Wattle wilt) on non-native *A. mearnsii* trees in South Africa (Morris *et al.* 1993, Wingfield *et al.* 1996). Disease symptoms include wilt and die-back of trees, cankers on the stems and branches, blister lesions, and discoloration of the wood (Morris *et al.* 1993, Roux & Wingfield 1997). This fungus is considered to be the most important pathogen of *A. mearnsii* trees in South Africa (Roux & Wingfield 1997).
Ceratocystis albifundus has been reported only from the African continent, and until recently was known only from South Africa. The first records of the fungus (as *C. fimbriata*) were from *Protea*

L. in the area previously known as the Transvaal (Gorter 1977). In the late 1980's, *C. albifundus* was identified as the cause of wilt and die-back of *A. mearnsii* trees in the Kwazulu-Natal province, again as *C. fimbriata* (Morris *et al.* 1993). The fungus was later recognized as a new taxon, clearly distinct from *C. fimbriata*, based on morphological and molecular studies (Wingfield *et al.* 1996). Recently, *C. albifundus* has also been discovered on *A. mearnsii* in Uganda (Roux *et al.* 2001a), Kenya (Roux, personal communication) and Tanzania (Roux *et al*. 2005). Recent studies by Roux *et al*. (2007) also report the fungus from a number of native hardwood trees in South Africa including *Acacia caffra* (Thunb.) Wild., *Burkea africana* Hook, *Combretum molle* R.Br. ex G.Don, *C. zeyheri* Sond., *Faurea saligna* Harv., *Ochna pulchra* Hook, *Ozoroa paniculosa* (Sond) R.&A.Fern, *Terminalia sericea* Burch. Ex DC. and *Protea gaguedii* J.F.Gmelin.

Roux *et al*. (2001b), investigated the possible diversity and origin of *C. albifundus*, by determining the nuclear and mitochondrial gene diversity of a South African population of *C. albifundus* from *A. mearnsii.* A high level of gene diversity was found for both nuclear and mitochondrial DNA. They compared their results to those for populations of native *Ceratocystis* spp., namely *C. eucalypti* Yuan & Kile, *C. virescens* (Davidson) Moreau and *Chalara australis* J. Walker & Kile as published by Harrington *et al*. (1998). Values for the mitochondrial diversity for *C. albifundus* were found to be higher than those of the other species and that of the nuclear DNA similar to that of the native, outcrossing *C. virescens* (Roux *et al.* 2001b). These data, together with the apparent occurrence of *C. albifundus* on native *Protea* spp. supported the hypothesis that *C. albifundus* is endemic to South Africa (Roux *et al.* 2001b).

Recently, the view that *C. albifundus* is native to South Africa has been confirmed using polymorphic simple sequence repeat (SSR) markers (Barnes *et al.* 2005, Nakabonge 2002). In these studies, populations of *C. albifundus* from *A. mearnsii* in South Africa were compared to a population on *A. mearnsii* collected in Uganda. The study revealed a high differentiation between the Ugandan and South African populations, very low gene flow between the two populations and similar genetic diversities. This lead the authors to conclude that *C. albifundus* has been in Africa for a long time and is probably native to Africa and not only South Africa (Barnes *et al.* 2005, Nakabonge 2002). To fully explain the origin of this fungus, it will be necessary to obtain populations from other African countries, as well as from native hosts of *C. albifundus*.

Typical of *Ceratocystis* spp., *C. albifundus* has a close association with wounds on trees. In South Africa, it is especially problematic after hail damage to trees (Roux & Wingfield 1997). In Uganda, *C. albifundus* was commonly isolated from branch stubs and stumps on trees from which branches and stems had been harvested for fuelwood (Nakabonge 2002, Roux *et al*. 2001a). The first report of the pathogen in South Africa was also from trees that had been mechanically damaged through the removal of branches (Morris *et al*. 1993). It is suspected that similar to other *Ceratocystis* spp., insects play some role in the dispersal of *C. albifundus*. At this stage, however, very little is known concerning specific insects associated with the development of disease on *A. mearnsii* in South Africa, Uganda Tanzania and Kenya.

5.2 *Ceratocystis fimbriata*

Ceratocystis fimbriata sensu lato is a well-known plant pathogen in many parts of the world and has a comparatively wide host range including both woody and herbaceous plants (Kile 1993, Upadhyay 1981). There have, however, been few reports of this fungus from Africa. It has been reported as a saprobe on *Hevea* spp. in Uganda (Snowden 1926), and two reports have suggested it as a pathogen on *Hevea* spp. in the Democratic Republic of Congo (Anonymous 1948, Ringoet 1923). In plantation forestry, *C. fimbriata sensu lato* has been reported to cause rapid wilt and death of non-native *Eucalyptus* spp. in the Republic of Congo (Roux *et al.* 1999) and in Uganda (Roux *et al*. 2001a). A single isolate of the fungus was also found on the stump of an *A. mearnsii* tree in South Africa (Roux 1998) and later another from a hail wound on *A. mearnsii* from the same area (Roux, personnal communication). Recently the fungus has also been isolated from artificially inflicted wounds on *E. grandis* in South Africa (Roux *et al.* 2004).

The disease symptoms produced by *C. fimbriata sensu lato* vary according to the type of host it infects. Symptoms include stem cankers, root and stem rots, wilt and vascular stains (Kile 1993). On trees, *C. fimbriata* is primarily a xylem pathogen (Baker & Harrington 2001). Infection typically occurs through fresh wounds (Giraldo 1957, Viegas 1960, Moller *et al.* 1969), either on the stems of trees or through roots (Laia *et al.* 2000, Ribeiro *et al.* 1986, Rossetto & Ribeiro 1990, Roux *et al*. 2004). Mycelium and spores enter wounds and move through the xylem in water-conducting cells and into ray parenchyma cells. Infection results in dark reddish-brown to purple, to deep-brown or black staining in the xylem (Seifert 1993, Walter *et al*. 1940, Walter 1946). In plantation forestry wilting may also occur in the absence of canker development as was the case in disease of ***Eucalyptus*** spp. in the Republic of Congo (Roux ***et al.*** 1999).

Species in the *Ceratocystis fimbriata sensu lato* complex produce a strong fruity odour, which is assumed to be an adaptation for dispersal by insects (Hunt 1956, Moller & Devay 1968). The fungus may be dispersed as fragments of mycelium, conidia, aleurioconidia or ascospores. Aleurioconidia are probably the most common survival units, because they are thick-walled and durable, and they probably facilitate survival in soil (Accordi 1989) and in insect frass (Iton 1960).

It is now well recognized that *C. fimbriata* represents a species complex (Harrington & Baker 2002, Johnson *et al*. 2005, Marin *et al*. 2003, Webster & Butler 1967). There are several apparently host-specialized strains that are sometimes called “types”, “races” or “forms” (Baker *et al*. 2003, Harrington & Baker 2002, Wellman 1972), and many of these may prove to be distinct species. Thorpe *et al*. (2005), for example have shown that *C. fimbriata* isolates from the family Araceae, the only known monocotyledonous family host for *C. fimbriata*, represent three groups of cryptic species in the *C. fimbriata* complex, based on ITS sequences analysis (Thorpe *et al.* 2005). In recent years several new species, previously known as *C. fimbriata*, have been described. They are *C. albifundus* (Wingfield *et al*. 1996), *C. pirilliformis* (Barnes *et al*. 2003a), *C. polychroma* (Van Wyk *et al*. 2004a), *C. cacaofunesta* Engelbrecht & Harrington, *C. platani* (Baker Engelbrecht & Harrington 2005), *C. variospora* (Davids.) C. Moreau, *C. populicola* J.A. Johnson & Harrington, *C. caryae* J.A. Johnson & Harrington, and *C. smalleyi* J.A. Johnson & Harrington (Johnson *et al*. 2005). Most likely, many more species in this genus will be described and this will mainly emerge from phylogenetic inference. Some are likely to be host specific species while others will no doubt be generalists.

Barnes *et al*. (2001), investigated the diversity of *C. fimbriata sensu lato* by developing PCR based microsatellite markers for *C. fimbriata* collected from a wide geographical and host range. Results based on microsatellites were also compared with those from sequence data of the internal transcribed spacer region (ITS). The phylogenetic trees generated from the microsatellite and ITS data clearly resolved isolates of *C. fimbriata* into distinct groups based on hosts and geographical origin (Barnes *et al*. 2001, Barnes 2002). These results were in agreement with previous studies emerging from hybridization experiments (Webster & Butler 1967), host specificity (Kojima *et al*.

1982), and pathogenicity assays (Leather 1966, Pontis 1951, Walter *et al*. 1952). Recent studies by Roux *et al*. (2004), also showed that isolates of *C. fimbriata* from South Africa are different from isolates from North America but share the same ancestor with isolates from the rest of the world. In addition, isolates from South Africa grouped in a clade made up of isolates from the same host, *Eucalyptus* spp. (Roux *et al*. 2004).

The origin of *C. fimbriata* in South Africa has recently been investigated using DNA based microsatellite markers previously developed specifically for the fungus (Barnes *et al*. 2001, Barnes 2002, Steimel *et al*. 2004, Van Wyk *et al*. 2006b). Gene diversity for *C. fimbriata* on *Eucalyptus* in South Africa was low and the population was found to be predominantly clonal. These results suggested that, *C. fimbriata* has been introduced accidentally into South Africa (Van Wyk *et al*. 2006b). No definitive data regarding origin are yet available for *C. fimbriata* from other countries or hosts. Barnes (2002), in a preliminary study showed that *C. fimbriata* isolates from *Eucalyptus* in Congo and Uruguay were phylogenetically most closely related to each other, but more distantly related from the Colombian population of *C. fimbriata* (Barnes 2002).

5.3 *Ceratocystis pirilliformis*

Ceratocystis pirilliformis was first isolated from Australia, where it was discovered on artificial wounds on *Eucalyptus nitens* Dean & Maiden trees (Barnes *et al.* 2003a). Infection of wounds in Australia is characterized by vascular staining that may decrease timber value (Barnes *et al.* 2003a). Recently, *C. pirilliformis* has also been recorded from South Africa where it was collected from artificial wounding trials on *E. grandis* (Roux *et al.* 2004). The only known reports of *C. pirilliformis* are thus from wounds on *Eucalyptus* trees. The first isolate of this fungus collected in South Africa was from the stump of a freshly harvested *E. grandis* tree (Roux *et al*. 2004). Although *C. pirilliformis* is relatively new to science, studies by Roux *et al*. (2004), have shown that it has the potential to cause disease of *E. grandis* and *E. grandis* hybrids. In both greenhouse and field experiments extensive bark and cambial discolouration was observed, similar to that of the known pathogen *C. fimbriata* (Roux *et al*. 2004). This potential pathogen clearly deserves more intensive study.

5.4 *Ceratocystis eucalypti*

Ceratocystis eucalypti was first isolated in Australia from artificially inflicted wounds on *Eucalyptus* spp. (Kile *et al.* 1996). The fungus was described as a non-pathogenic wound colonist, but may change the wood color as infection of wounds in Australia are characterized by discolouration of the sapwood (Kile *et al.* 1996). The fungus also causes vascular stain of living *Eucalyptus* trees in Victoria and Tasmania (Kile *et al.* 1996). It has to date not been reported from any other hosts or geographic areas.

5.5 *Ceratocystis moniliformis*

Ceratocystis moniliformis was first described from hardwood trees in Texas and Arkansas in the United States of America (Hedgcock 1906). The fungus occurs on a wide range of plants including angiosperms, fruits, vegetables and other crops (Grylls & Seifert 1993). In plantation forestry, the first report of *C. moniliformis* was from artificially inflicted wounds on *Eucalypts* spp. (Roux *et al.* 2004). No more information is available about its possible role on *Eucalyptus* spp. *C. moniliformis* is not known as a pathogen, but may cause a discoloration of the sapwood and was said to cause superficial grey or brown stain of angiosperms in Europe, North America, Africa and India (Davidson 1935, Farr *et al.* 1989, Seifert 1993).

In recent years, many fungi resembling *C. moniliformis* have been described. They include *C. bhutanensis* (Van Wyk *et al*. 2004b), *C. moniliformopsis* (Yuan & Mohammed 2002), *C. omanensis* Al-Subhi, M. J. Wingfield, Van Wyk & Deadman (Al-Subhi *et al*. 2006), and *C. tribiliformis* Van Wyk & Wingfield (Van Wyk *et al*. 2006a). These fungi were tentatively identified as *C. moniliformis* based on cultural characteristics. They formed part of the *C. moniliformis sensu lato* complex, which is characterized based on species possessing hat-shaped ascospores, a disk-shape at the base of the ascomatal neck and ascomatal bases ornamented with short conical spines (Al-Subhi *et al*. 2006, Van Wyk *et al*. 2004b, Van Wyk *et al*. 2006a, Yuan & Mohammed 2002).

5.6 *Ceratocystis moniliformopsis*

Ceratocystis moniliformopsis occurs in Australia where it was first isolated from the cut ends of *E. obliqua* L'Her. logs, and described as a non pathogenic wound colonist (Yuan & Mohammed 2002). The only report of *C. moniliformopsis* is from *Eucalyptus* wounds from Australia. Infection of wounds in Australia is characterized by discolouration of the sapwood (Yuan & Mohammed

2002). *C. moniliformopsis* is morphologically similar to *C. moniliformis* with the ascomatal bases of both species covered with pigmented spines and both producing hat shaped ascospores (Yuan & Mohammed 2002). However, in *C. moniliformopsis*, these spines are different in size and shape, and are referred to as setae (Yuan & Mohammed 2002). It is also phylogenetically distinct from other species in the *C. moniliformis* sensu lato species complex (Van Wyk *et al*. 2006a).

6.0 *OPHIOSTOMA* SPP. ON NON-NATIVE PLANTATION HARDWOOD TREES

Few *Ophiostoma* spp. have been reported from non-native hardwood trees in plantations. Most publications treating *Ophiostoma* spp. consider species from the Northern hemisphere on conifers such as pine and spruce. Reports from the Southern hemisphere are very few and most are from South Africa. Although not associated with diseases in the southern hemisphere, some *Ophiostoma* spp. recorded in South Africa form part of an economically important group of fungi associated with sapstain of timber, reducing its commercial value (De Beer *et al*. 2003a).

In the Southern Hemisphere, *O. quercus* is the only *Ophiostoma* sp. that has been consistently reported from hardwoods. The fungus occurs on both hardwoods and softwoods and on both native and non-native trees (De Beer *et al*. 2003b). In South Africa for example, *O. quercus* has been reported from *Olinia* sp., *E. grandis* and *Quercus robur* L. (De Beer *et al*. 1995). The only report of *O. quercus* in Uruguay is from *Eucalyptus* spp. (Harrington *et al*. 2001) while in Ecuador, the fungus occurs on diseased *Schizolobium parahybum* (Vell.) Blake (Geldenhuis *et al*. 2004).

Very little is known regarding the biodiversity and pathogenicity of *Ophiostoma* spp. in plantation forestry on the African continent, outside South Africa. Given the increased establishment of plantations in many African countries and the sporadic reports of potential disease symptoms observed in plantations in many African countries, we believe that the extent of epidemic in Africa could be the same as the scenario observed in many countries of the northern hemisphere. There is great need of surveys in African countries in order to gain a full understanding of the species, distribution and impact of *Ophiostoma* spp. on the continent.

7.0 CONCLUSIONS

- Forestry diseases require urgent attention if plantation forestry is to be practiced successfully on the African continent. These diseases can be caused by both native and introduced pathogens.
- In Africa, very little information is known regarding Ophiostomatoid fungi native to the continent and many undescribed and possibly pathogenic species most likely await discovery. The increased reliance on non-native tree species and increased trade could also have resulted in the introduction of several non-native Ophiostomatoid fungi.
- To develop sustainable forestry on the African continent and to protect our native biodiversity, considerably more research needs to be conducted with the collaboration of other countries. One of the groups of pathogens and potential pathogens that deserve more attention are the Ophiostomatoid fungi, including species of *Ceratocystis* sensu lato and *Ophiostoma* sensu lato that infect plantation-grown trees.
- The Ophiostomatoid fungi include many economically important pathogens. In many cases, losses caused by these fungi are greatly underestimated, since they include only losses due to tree mortality. However, losses caused by growth reduction and the impact on fibre quality and yield, are probably much greater but are difficult to measure. Other important losses not normally considered include loss in timber value, watershed, wilderness, recreation and habitat values.
- Wounds, either natural or from human activities are important infection courts for some members of the Ophiostomatoid fungi.
- There is relatively limited knowledge pertaining to the occurrence, biodiversity and pathogenicity of the Ophiostomatoid fungi on hardwoods in Africa. Obtaining knowledge on these aspects will contribute to the way the forestry industry develops improved methods of managing diseases caused by Ophiostomatoid fungi.
- This thesis focuses on the study of *Ceratocystis* and *Ophiostoma* species that infect wounds particularly on native trees in Southern Africa. Some work is also included on these fungi infecting wounds on *Eucalyptus* and other hosts in other parts of the world. Knowledge of the species on native African trees as well as on related hosts will be valuable in the development of strategies to manage these fungi.

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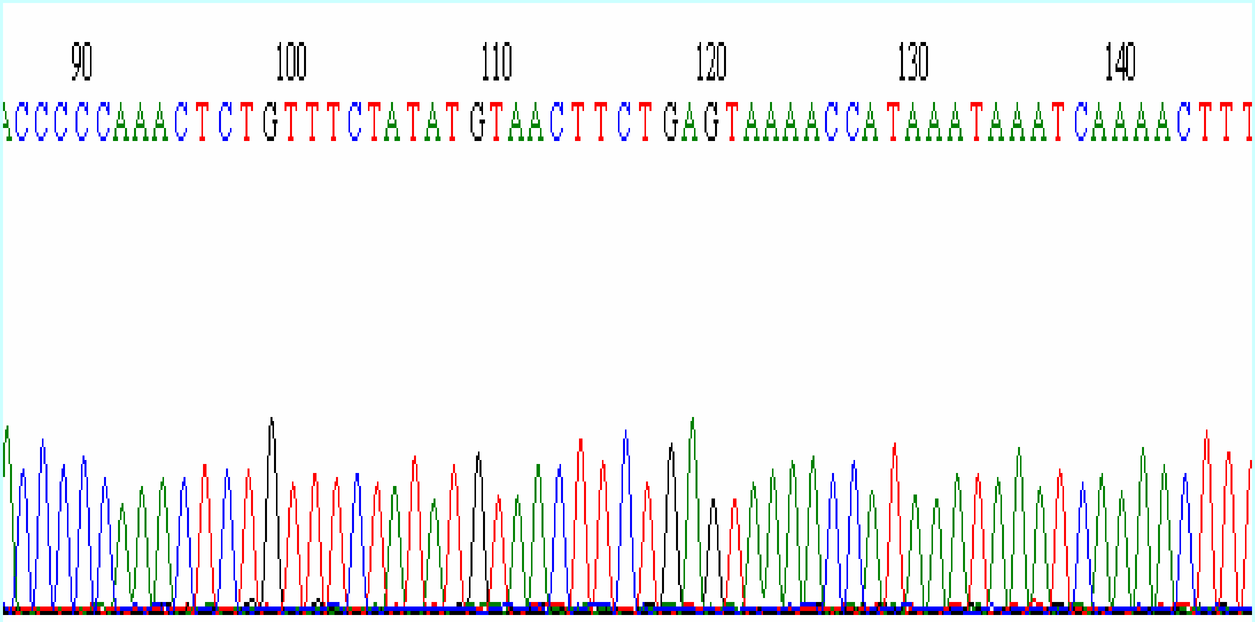
Table 1: Current taxonomic status of Ophiostomatoid fungi and the characteristics that distinguish them.

	Ceratocystis sensu stricto	*Ophiostoma sensu lato*			
Characters	***Ceratocystis***	***Ophiostoma***	***Ceratocystiopsis***	***Grosmannia***	***Gondwanamyces***
Kingdom	Fungi	Fungi	Fungi	Fungi	Fungi
Division	Eumycota	Eumycota	Eumycota	Eumycota	Eumycota
Phylum	Dikaryomycota (Ascomycota)	Dikaryomycota (Ascomycota)	Dikaryomycota (Ascomycota)	Dikaryomycota (Ascomycota)	Dikaryomycota (Ascomycota)
Class	Ascomycetes	Ascomycetes	Ascomycetes	Ascomycetes	Ascomycetes
Order	Microascales	Ophiostomatales	Ophiostomatales	Ophiostomatales	Microascales
Family	Ceratocystidaceae	Ophiostomataceae	Ophiostomataceae	Ophiostomataceae	Ceratocystidaceae
Genus	*Ceratocystis* sp.	*Ophiostoma* sp.	*Ceratocystiopsis* sp.	*Grosmannia* sp.	*Gondwanamyces* sp.
Type species	*Ceratocystis fimbriata*	*Ophiostoma piliferum*	*Ceratocystiopsis minuta*	*Grosmannia penicillata*	*Gondwanamyces protea*
Anamorph	*Thielaviopsis*	*Pesotum, Sporothrix*	*Hyalorhinocladiella*	*Leptographium*	*Knoxdaviesia*
Conidiogenesis	Enteroblastic	Holoblastic	Holoblastic	Holoblastic	Holoblastic
Cell Wall Composition	Absence of Cellulose	Presence of Cellulose	Presence of Cellulose	Presence of Cellulose	?
	Absence of Rhamnose	Presence of Rhamnose	Presence of Rhamnose	Presence of Rhamnose	Absence of Rhamnose
Biochemistry	Cycloheximide sensitive	Cycloheximide insensitive	Cycloheximide insensitive	Cycloheximide insensitive	Cycloheximide sensitive
Centrum Development	Asci line inner ascomata	Asci line periphery of ascomata	Asci line periphery of ascomata	Asci line periphery of ascomata	?

CHAPTER 2

Distribution and population biology of *Ceratocystis pirilliformis* in South Africa



1.0 ABSTRACT

Ceratocystis pirilliformis is a fungus, first isolated from wounds on *Eucalyptus nitens* in Australia, and subsequently found in a similar niche on *E. grandis* in South Africa. Artificial inoculation studies under field conditions in South Africa resulted in bark lesions and sap-stain suggesting that the fungus is a pathogen of potential importance to forestry in this country. Because *Eucalyptus* spp. are native to Australia and *C. pirilliformis* was first found in the absence of disease, it has been assumed that the fungus is native to Australia. The aim of this study was to expand on our knowledge of the distribution and population biology of *C. pirilliformis* in South Africa. Wounds were made on the stems of *Eucalyptus* spp. growing in six of the most important forestry areas in South Africa. PCR-based microsatellite markers, developed for the closely related tree pathogen, *C. fimbriata sensu lato* were used to assess the population structure and diversity of the isolates collected. *Ceratocystis pirilliformis* was found in four areas planted to *Eucalyptus,* substantially expanding its known distribution. Of the twenty-seven available microsatellite markers, eighteen amplified the desired loci of *C. pirilliformis*, however only seven were polymorphic. The gene and genotypic diversity of the *C. pirilliformis* isolates was very low and the populations tended towards a high degree of clonality. Although only a small number of isolates of the fungus were available from Australia, they displayed a higher level of diversity than those from South Africa. *Ceratocystis pirilliformis* clearly has a wide distribution in South Africa and results support the view that it is not native in this country.

2.0 INTRODUCTION

The genus *Ceratocystis* includes several economically important species that are pathogens of crop plants, especially trees. Important tree pathogens include *C. albifundus* De Beer, Wingfield & Morris, the cause of wilt and canker on non-native *Acacia mearnsii* de Wild in South Africa (Morris *et al.* 1993, Wingfield *et al.* 1996), *C. fimbriata* Ellis & Halsted *sensu lato*, the cause of wilt and canker stain disease of *Eucalyptus* spp. in Africa and South America (Roux *et al*. 1999, 2001a, 2004, Barnes *et al*. 2003a) and *C. fagacearum* (Bretz) Hunt, the causal agent of Oak wilt in the United States of America (Dietz & Young 1948, Griswold 1958, Henry *et al*. 1944).

Two main phylogenetic groups have been identified for species of *Ceratocystis*. One of these, referred to as the *Ceratocystis coerulescens sensu lato* species complex (Harrington *et al*. 1996, Witthuhn *et al*. 1998), includes species such as *C. moniliformis* (Hedgcock) Moreau, *C. fagacearum* (Bretz) J. Hunt and *C. virescens* (Davidson) Moreau. The other major group includes species such as *C. fimbriata sensu stricto*, *C. albifundus* (Witthuhn *et al*. 1999) and *C. pirilliformis* I. Barnes & M. J. Wingfield (Barnes *et al*. 2003b). Until ten years ago, *C. fimbriata* was the only species name used in this second group. However, it has been recognised that this important pathogen represents a species complex (Barnes *et al*. 2001, Marin *et al*. 2003, Baker *et al*. 2003). Thus, a number of new species, initially thought to represent *C. fimbriata* have been described. These include among others, *C. albifundus* (Morris *et al.* 1993, Wingfield *et al.* 1996), *C. platani* (Walter) Baker Engelbrecht & Harrington (Baker Engelbrecht & Harrington 2005), *C. polychroma* M. van Wyk & M. J. Wingfield (Van Wyk *et al*. 2004) and *C. pirilliformis* (Barnes *et al*. 2003b). Currently only *C. fimbriata* isolates from sweet potato (*Ipomoea batatas* Lam) are recognised as representing *C. fimbriata sensu stricto* and others are most appropriately treated in the broad sense as *C. fimbriata sensu lato*. Of these *Ceratocystis* spp., *C. albifundus* has been shown to most likely be native to the African continent (Roux *et al.* 2001b, Barnes *et al.* 2005).

Ceratocystis pirilliformis is a recently described species residing in the larger *C. fimbriata sensu lato* clade (Barnes *et al*. 2003b). It produces dark, pear-shaped ascomatal bases with dark necks from which hat shaped ascospores exude. *Ceratocystis pirilliformis* can be distinguished from other *Ceratocystis* spp. with hat-shaped ascospores by the distinct pear-shaped ascomatal bases and slight size differences in important morphological features (Barnes *et al*. 2003b). It was first described from Australia where it was found infecting artificially induced wounds on native *Eucalyptus nitens* Dean & Maiden trees (Barnes *et al*. 2003b). It was subsequently found in South Africa on wounds made on the stems of *E. grandis* (Hill) Maiden (Roux *et al*. 2004). Studies by Roux *et al*. (2004), have shown that *C. pirilliformis* is capable of causing disease on one-year-old *E. grandis* trees under field conditions. However, it has not yet been associated with naturally dying trees in either South Africa or Australia and its status as a pathogen remains unclear.

Introduced pathogens including species of *Ceratocystis* have caused substantial losses to forest ecosystems around the world. The accidental introduction of *C. platani* (Walter) Baker Engelbrecht & Harrington into Europe is one example where large-scale mortality of plane trees (*Platanus* spp.) has occurred (Anonymous 1986, Baker Engelbrecht & Harrington 2005). However, for most of these fungi, little is known regarding their areas of origin. In the case of *C. albifundus* and *C. platani*, analysis of gene diversity using microsatellite markers has contributed to elucidating their possible origins in Africa (Barnes *et al*. 2005) and the United States (Baker *et al*. 2003, Baker Engelbrecht *et al*. 2004) respectively.

Very little is known regarding the distribution, impact or origin of *C. pirilliformis* in South Africa. It has been collected from only a few locations and it has been hypothesised that the fungus is native to Australia, where it would have co-evolved with its only known host, *Eucalyptus* (Barnes *et al*. 2003b, Roux *et al*. 2004). This view emerges from the fact that the fungus has been isolated from native *E. nitens* in the absence of disease (Barnes *et al*. 2003b). The aim of this study was thus to increase our knowledge regarding the host range and geographic distribution of *C. pirilliformis* in South Africa. An additional aim was to consider the possible origin of the pathogen by evaluating its population diversity in South Africa.

3.0 MATERIALS AND METHODS

3.1 Distribution in South Africa

Surveys for *C. pirilliformis* were conducted in six areas of South Africa where *Eucalyptus* spp. are commercially propagated. These areas included Bushbuckridge, Kwambonambi, Paulpietersburg, Pietermaritzburg, Sabie and Tzaneen. In all areas other than in Pietermaritzburg, wounds were made on the stems of *Eucalyptus* trees, as described by Barnes *et al.* (2003b). Wounds were left for a minimum of one week and in some cases up to two months before pieces of bark and wood were collected from them. The length of time between wounding and sampling depended on the presence of fungal structures on the surfaces of the wounds, which was influenced by climatic conditions. Samples from Pietermaritzburg were collected from the stumps (one-month-old) of recently felled trees. For the Bushbuckridge, Kwambonambi, Paulpietersburg, Sabie and Tzaneen areas both stumps and chisel wounds were sampled.

Pieces of bark and wood bearing fungal structures were collected from wounds and stored in brown paper bags. All the samples were transported to the laboratory in plastic bags that served as moist chambers. Isolations were made directly from samples on which freshly produced fungal fruiting bodies were found. Dry samples were sprayed with sterile distilled water, sealed in plastic bags and incubated at room temperature (~25ºC) to induce sporulation of the fungi. Samples were inspected daily for the presence of fruiting bodies.

Single drops of spores produced at the apices of ascomata were transferred to Petri dishes containing 2% malt extract agar (MEA: 20g malt extract, 15g agar, Biolab, Midrand, South Africa and 1Lt. deionised water) with 0.05g/l streptomycin (SIGMA-ALDRICH, Steinheim, Germany). Plates were incubated at 24ºC for seven days to obtain pure cultures. Duplicates of all isolates have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. A number of *C. pirilliformis* isolates collected during previous studies in Australia (Barnes *et al*. 2003b, Roux, unpublished) were also included in this study, where genetic diversity of the fungus was considered (Table 1).

3.2 DNA Extraction

Cultures were grown on 2% MEA for 7-10 days. Mycelium was collected by scraping the surface of the agar plates using a sterile scalpel and then transferred to 1.5ml Eppendorf tubes. DNA was extracted using the protocol described by Möller *et al*. (1992), except that 10µl of RnaseA was added at the final step and the samples were incubated overnight at room temperature to digest the RNA. The presence of DNA was verified by separating an aliquot (5µl) of the extraction mixture on 1% agarose gels stained with ethidium bromide and visualizing under ultraviolet (UV) light.

3.3 Microsatellite amplification and allele scoring

Twenty-seven sets of microsatellite primers previously shown to be polymorphic for *C. fimbriata* (Barnes *et al*. 2001, Steimel *et al*. 2004) and *C. albifundus* (Barnes *et al*. 2005) were tested on three randomly selected isolates (CMW12680, CMW16521, CMW16471) of *C. pirilliformis*. The PCR reaction mixes and thermal cycling conditions were the same as those described previously (Barnes *et al*. 2001) for all the primers tested, except that the annealing temperature range was modified for certain reactions. Primers that successfully amplified the desired size fragments (Table 2) were used to amplify DNA for the remaining isolates of *C. pirilliformis* collected from South Africa and Australia. Primers that did not amplify, that produced double bands or were inconsistent with amplification, were discarded. To verify the approximate sizes of the amplicons, 5µl aliquots of the PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized under UV light.

To determine the allele sizes of the amplicons, various PCR products were mixed together based on the expected size of amplicons and the type of fluorescent label attached to the primer (Table 2). Each sample mix included 2µl of the combined DNA (Table 3) and 0.14µl internal standard Genescan-500 Liz and 10µl formamide (Applied Biosystems, Foster City, USA). Sample mixes were separated on a 36cm capillary using POP^{TM4} polymer on an ABI Prism 3100 sequencer. Allele sizes for DNA fragments were determined using Genomapper version 3.0 (Applied Biosystems, Foster City, USA).

Alleles that had only one base pair difference in their lengths within a locus were sequenced to confirm the authenticity of the allele scoring. PCR products were purified using Sephadex G-50 Gel (SIGMA-ALDRICH, Steinheim, Germany), as recommended by the manufacturer. An accurate concentration of the purified PCR product was determined using the 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), according to the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Between 60-100ng PCR product was used to prepare a 10µl sequencing PCR that also contained 2µl of ready reaction mixture (Big dye), 2µl of 5X reaction buffer, 1µl of either the reverse or forward, non-fluorescent primer (10mM) and enough Sabax water to complete the volume of 10µl. The same primers were used as those described for the PCR amplifications. Both DNA strands were sequenced. Sequence data were aligned and compared manually using Sequence Navigator version 1.01 (ABI PRISM, Perkin Elmer). Alleles that showed one base pair difference in the genescan analyses were compared with each other to determine the validity of the extra base pair observed in the length of the allele. This was especially due to the fact that most of the microsatellite repeat units within these loci are either di-, tri- or tetra- nucleotide repeats.

3.4 Statistical Analysis - Genetic diversity

All the isolates screened in this study were scored based on the presence or absence of an allele at each of eighteen loci. The frequency of each allele was calculated by taking the number of times the allele was present in the population and dividing it by the population sample size. Allele frequencies were used to calculate the gene diversity using the formula described by Nei (1973): $H=1-\sum_k x_k^2$, where x_k is the frequency of the K^{th} allele.

Multilocus genotypes for all isolates were determined based on the combination of alleles at each polymorphic locus. The genotypic diversity was thus calculated using the formula: $G = 1/\sum [f_{(x)}(x/n)^2]$, where G is the effective number of frequent genotypes, n is the sample size and $f_{(x)}$ is the number of multilocus genotypes occurring x-times in the population (Stoddart & Taylor 1988). To confirm that sufficient numbers of isolates and markers have been used in the population analyses to make sound conclusions, the software program Multilocus v1.3b (Agapow & Burt 2001) was used to plot genotypic diversity against the number of loci, with 1000 resampling repetitions.

The Index of association (I_A) (Taylor *et al.* 1999) was used to test the mode of reproduction of the fungus. Two tests were conducted for I_A; the standard test including all the isolates for all the genotypes recorded and secondly, the test for the clone corrected population containing only one representative isolate for each genotype. An input file containing the multilocus genotypes for the isolates was constructed and used to compute the I_A (1000 randomly recombining data sets) using the program Multilocus (Agapow & Burt 2001). Data obtained from this analysis were used to construct a distribution range for 1000 randomly recombining data sets. The observed value of I_A obtained was compared with this distribution range. Where the observed value of I_A fell within the distribution range of the combined data sets, then the null-hypothesis that the population was undergoing recombination was accepted. Where the observed value of I_A fell outside the distribution range with a significant P value (P<0.05), then the population was considered to be clonal (Taylor *et al*. 1999).

4.0 RESULTS

4.1 Distribution in South Africa

A total of thirty-nine isolates of *C. pirilliformis* were collected from different *Eucalyptus* trees in four geographical areas of South Africa (Figure 1, Table 1). Of these, nineteen were from Sabie (Bergvliet Plantation) (S25º03.242' E030º51.680'), eleven were from Paulpietersburg (Eersteling Plantation) (S27º31.843' E030º48.123'), eight were from the Bushbuckridge (Waterhoutboom) (S24º56.829' E030º55.213') area and one was from Kwambonambi (Figure 1, Table 1). Isolates from the Sabie area were collected from *E. grandis* x *camaldulensis* hybrid clones. Isolates from Paulpietersburg, Kwambonambi and Bushbuckridge, were from artificially induced wounds inflicted on *E. grandis* and *E. grandis* x *camaldulensis* respectively. No *C. pirilliformis* isolates were obtained from the Pietermaritzburg or Tzaneen areas. Isolates obtained from wounds were in some cases associated with blue to brown streaks in the cambium, spreading upward from the wounds. However, no other disease symptoms were observed.

All isolates were identified as *C. pirilliformis* based on the morphology of pure cultures as described by Barnes *et al*. (2003b). A total of sixteen isolates from Australia were obtained from the culture collection (Table 1). These included thirteen isolates from *E. nitens* growing near Canberra and three isolates from *E. nitens* near Brisbane.

4.2 Microsatellite amplification and allele scoring

Eighteen of the twenty-seven sets of microsatellite primers (Table 2) amplified products within the expected size ranges (Table 4). At four of the loci, some alleles were scored (based on length) that were only one base pair different in length. In locus AG1/2 alleles 274 and 275 were scored, in locus AG7/8 alleles 276 and 277 were scored, in locus CF17/18, alleles 273 and 274 were scored and in locus CF21/22, alleles 248 and 249 were scored. These alleles were sequenced but no differences were observed in sequence within the microsatellite or flanking regions. They were thus treated as single alleles with sizes 249, 274, 275 and 277 at loci CF21/22, CF17/18, AG1/2 and AG7/8 respectively.

After correction and confirmation of alleles, a total of thirty and thirty-three alleles were obtained from the eighteen loci amplified for the South African and Australian isolates of *C. pirilliformis* respectively (Table 4). Twenty-two alleles were shared by isolates from the two areas. Eleven alleles were unique to the Australian collection while eight alleles were unique to the South African isolates. Only seven of the twenty-seven loci were polymorphic and allelic frequencies obtained for all loci were recorded (Table 4). Locus CF15/16 was the most polymorphic containing a total of eight alleles. A gene diversity value (H) of H=0.099 and H=0.213 was obtained for the South African and Australian isolates respectively, using the allele frequencies of the loci (Table 4).

From a collection of fifty-five isolates, twenty-four different genotypes were identified including sixteen from South Africa and eight from Australia (Table 5). No genotypes occurred in both Australian and South African isolates. The genotypic diversity was $G_{st} = 1.79$ and $G_{st} = 1.46$ for the South African and Australian isolates of *C. pirilliformis* respectively. The genotypic diversity versus the number of loci gave rise to a curve that is approaching a plateau (Figure 2).

Thirteen (81.3%) of the South African genotypes differed at only one allele at each of the seven polymorphic loci. Ten (62.2%) genotypes were present in isolates from the Sabie area and of these, five were exclusive to this collection. Eight (43.8%) genotypes were present in Paulpietersburg, three of which were unique to this area. Five (37.5%) genotypes were present in the Bushbuckridge area and one genotype was unique. The only isolate from Kwambonambi represented a unique genotype different from those in any other area.

Both tests for the index of association gave the same result, whether all the genotypes were used in the analyses or only the clone corrected genotypes. In both cases the observed value, with significant support, fell outside the distribution range of a randomly recombining population. Thus, there is strong evidence suggesting a clonal mechanism of propagation of this fungus for both the Australian and South African populations (Figure 3, Figure 4).

5.0 DISCUSSION

Results of this study have shown, for the first time, that *C. pirilliformis* occurs in several *Eucalyptus*-growing areas of South Africa. In addition, analyses of genetic diversity suggest that the fungus tends towards clonality in the country. This, and the low diversity in the population, is consistent with the view that *C. pirilliformis* was probably introduced into South Africa.

Ceratocystis pirilliformis was collected widely in this study but it was never associated with disease symptoms. The areas in which *C. pirilliformis* was collected included those with temperate (Paulpietersburg; temperatures below 0ºC in winter and regular frosts) and sub-tropical (Bushbuckridge, Sabie Bergvliet, Kwambonambi) climates, suggesting that, *C. pirilliformis* can exist effectively under a wide range of environmental conditions. Likewise, Australian isolates of the fungus originate from Canberra which has a cold winter (temperatures even below 0ºC and regular frosts) and Brisbane which has a sub-tropical climate. It thus, appears that *C. pirilliformis* could become established in most *Eucalyptus* growing areas of South Africa. The absence of the fungus from Pietermaritzburg and Tzaneen is best explained by the fact that it may not yet have spread to those areas.

Surveys for *C. pirilliformis* in this study gave rise to large numbers of isolates of *C. fimbriata sensu lato*. These isolates were used in a previous population diversity study (Van Wyk *et al*. 2006). Although *C. fimbriata* isolates from these collections were found to have a relatively low population diversity (H=0.36, G_{st}=0.99), this was higher than that found for *C. pirilliformis* in the present study. Furthermore, *C. fimbriata* was found in all areas surveyed, and it was also much more common. It was also commonly found in the Tzaneen area and in the absence of *C. pirilliformis*. In the Kwambonambi area, where only one isolate of *C. pirilliformis* was collected, thirty-one isolates of *C. fimbriata* were obtained. These results suggest that *C. fimbriata sensu lato* has been present in South Africa for a longer period of time than *C. pirilliformis* and that it is much better established in the country.

The fact that microsatellite markers produced for *C. fimbriata sensu lato* (Barnes *et al*. 2001, Steimel *et al*. 2004) were effective on *C. pirilliformis* is not surprising. This fungus resides in the *C. fimbriata sensu lato* clade and the two fungi are clearly closely related. Various of these markers have also been effectively applied to studies on *C. albifundus* (Barnes *et al*. 2005), showing that they have a broad potential application with the *C. fimbriata sensu lato* clade of *Ceratocystis*.

Population diversity of *C. pirilliformis* isolates showed very low levels of gene diversity (H=0.099) for the fungus in South Africa. The genotypic diversity was low (G_{st} = 1.79) and the population appears to have predominantly a clonal mechanism of propagation. In comparison, the small collection of Australian isolates originating from fewer than ten trees in only two different areas, displayed much higher levels of gene diversity (H=0.213). This is more than twice the diversity in a population of half the size. The Australian isolates also had more alleles in the collection. The Australian collection contained 33 alleles compared to the 30 in the South African collection, which was more than twice as large. The gene diversity of the Australian collection could have been underestimated in this study due to the lower sample size and could thus be much higher than suggested here. The data available at present suggest that *C. pirilliformis* is native to Australia. The fact that Australian and South African isolates shared more than 60% of their alleles also adds to the view that Australia represents the source of the South African population.

Recently introduced populations are generally recognized by their low gene diversity in contrast to native populations that typically have very high gene diversity in their natural environment (Gordon *et al*. 1996, McDonald 1997). However, multiple introductions of an organism, such as a fungus, into a new ecosystem would also result in high gene diversity (Burdon & Roelfs 1985, Burgess *et al*. 2001). The gene diversity exhibited by the South African population of *C. pirilliformis* in this study was very low when compared with that of recent studies of this aspect using the same markers; H=0.41 and 0.38 for the Ugandan and South African population of *C. albifundus* respectively (Barnes *et al*. 2005) and H=0.36 for the South African population of *C. fimbriata* (Van Wyk *et al*. 2006). For these reasons, we assume that *C. pirilliformis* has been introduced into South Africa only recently and also that there have been few introductions. This is also supported by the relatively limited geographic distribution of the fungus in South Africa.

Results of this study, based on the Index of Association, showed that the South African population of *C. pirilliformis* has a predominantly clonal reproduction. Clonality is generally recognised by low genotypic diversity, widespread occurrence of identical genotypes, absence of recombinant genotypes and correlations between independent sets of genetic markers (Anderson & Kohn 1995, Kohn 1995, Milgroom 1996). The genotypic diversity of *C. pirilliformis* was also very low. Only sixteen genotypes were identified and many of these were shared between areas where the fungus was collected. This is significant as some of these areas are more than 300km apart from each other.

Ceratocystis pirilliformis is a relatively newly discovered fungus in South Africa and very little is known regarding its biology or origin. Species of *Ceratocystis* are known to have close associations, mainly with casual insects (Jewell 1956, Juzwik and French 1983, Juzwik *et al.* 1998). *Ceratocystis fimbriata sensu lato*, has for example been shown to be vectored by drosophylid flies (*Chymomyza procnemoides* Wheeler) and nitidulid beetles (*Carpophilus freemani* Dobson) (Moller & Devay 1968). The spread of *C. pirilliformis* within South Africa could thus be facilitated by insects, or the movement of infected timber. Fresh mycelium and fruiting bodies of *C. pirilliformis* were found abundantly under bark flaps during the survey period. Under favourable conditions such as high humidity, the fungus could thus easily be spread from one geographic area to another, under bark that has not been completely removed from cut timber.

Ceratocystis pirilliformis was not found to be associated with tree mortality or canker formation in this study. The fungus has, however, been shown to be capable of causing significant bark and cambium lesions on one-year-old *E. grandis* trees under field conditions (Roux *et al*. 2004). Whether *C. pirilliformis* is able to kill trees naturally remains unclear, as in the current study only wood staining was observed. The same is also true for *C. fimbriata sensu lato* on *Eucalyptus* in South Africa, but this fungus has resulted in serious disease of *Eucalyptus* trees in Uruguay and Congo (Barnes *et al*. 2003a, Roux *et al*. 1999). Clearly, further studies are requested to clarify the potential impact of *C. pirilliformis*, as well as *C. fimbriata* in South Africa.

2.5 REFERENCES

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Table 1: Isolates of *C. pirilliformis* from South Africa and Australia used in this study.

Origin	Sample size	Isolate number	Hosts	Collectors
South African population				
Sabie	19	CMW16511-16512, 16514-16526, 16746, 16747, 16749, 16750	*E. grandis* X *camaldulensis*	G. Kamgan & J. Roux
Paulpietersberg	10	CMW16463, 16466, 16467, 16469-16471, 16527-16529, 12281	*E. grandis*	G. Kamgan & R. Heath
	1	CMW12671	*E. grandis*	J. Roux & H. Hatting
Bushbuckridge	8	CMMW12673, 12675-12677, 12699, 12680, 11699, 17914	*E. grandis* X *camaldulensis*	J. Roux
Kwambonambi	1	11722	*E. grandis* X *camaldulensis*	J. Roux
Australian collection				
Canberra	13	CMW6566, 6574, 6575, 6579, 6583, 6670, 6569, 6556, 6586, 6576, 6577, 6571, 6563	*E. nitens*	MJ. Wingfield
Brisbane	3	19341, 19344, 19334	*E. nitens*	J. Roux & G. Pegg

Table 2: PCR-based microsatellite markers used in this study.

Primers	Primer Names	Fluorescent label & expected size range (base pairs)	Authors
AG1/2	I1	PET (255-266)	Barnes *et al*. 2001
AG7/8	I2	VIC (284-304)	
CF11/12	I6	FAM (216-230)	
CF13/14	I7	PET (402-415)	
CF15/16	I8	VIC (218-267)	
CF17/18	I9	PET (266-292)	
CF21/22	I10	NED (250-259)	
CF23/24	I11	PET (154-168)	
AAG8	T1	NED (187)	Steimel *et al*. 2004
AAG9	T2	VIC (413)	
CCAA9	T3	FAM (253)	
CCAA15	T5	NED (342)	
CAA38	T6	NED (240)	
DBVCAT	T8	VIC (272)	
CAGDL2-5	T12	PET (342)	
CCAG15	T13	FAM (269)	
CAG900	T14	PET 196	
GACA6K	T16	VIC (212)	

Table 3: Multiplex and organisation of PCR products for genescan analysis and alleles scoring. Two lanes on a 36cm capillary were used for each isolate whose loci were amplified with eighteen microsatellite markers.

Lane 1				**Lane 2**			
Mix A1		**Mix B1**		**Mix A2**		**Mix B2**	
Primers	**Vol. (µl)**	**Primers**	**Vol. (µl)**	**Primers**	**Vol. (µl)**	**Primers**	**Vol. (µl)**
T5	1	T12	1	T1	1	T2	1
T6	1	T13	1	T3	1	T8	1.5
I7	1	T16	1	I11	1	H_2O	247.5
I9	2	I8	1	I10	1	**Total**	250
H_2O	15	T14	1.5	H_2O	6		
Total	20	I2	1.5	**Total**	10		
		I6	1.5				
		H_2O	191.5				
		Total	200				
Composition of wells in lane 1				**Composition of wells in lane 2**			
1µl of MixA1 +1µl of Mix B1 + 0.14µl Liz + 10µl formamide				1µl of MixA2 + 1µl of MixB2 + 1µl of I1 (Primer I1) + 0.14µl Liz + 10µl formamide			

Table 4: Allele frequency and gene diversity (H) values for South African populations of *C. pirilliformis*. Unique alleles are in bold.

Locus	Allele length	Allele configuration	Allele frequencies	
			Australia	South Africa
AG7/8	277	A	1	1
			H=0	H=0
CF23/24	155	A	1	1
			H=0	H=0
CF17/18	274	A	1	1
			H=0	H=0
CF13/14	382	A	1	1
			H=0	H=0
CF21/22	349	A	1	1
			H=0	H=0
AAG9-F	385	A	1	1
			H=0	H=0
CCAA9-F	138	A	1	1
			H=0	H=0
CAA38-F	92	A	1	1
			H=0	H=0
DBVCAT-1F	217	A	1	1
			H=0	H=0
CAG900-1F	192	A	1	1
			H=0	H=0
GACA6K-1F	175	A	1	1
			H=0	H=0
AG1/2	265	A	0.313	0.974
	275	B	0.687	0.026
			H=0.429	H=0.05
CF11/12	205	A	**0.063**	
	206	B	0.75	0.282
	207	C	0.188	0.077
	208	D		**0.487**
	209	E		**0.154**
			H=0.398	H=0.654
CF15/16	460	A	**0.063**	
	462	B	0.188	0.59
	464	C	**0.375**	
	466	D	0.25	0.051
	488	E		**0.026**
	490	F		**0.308**
	496	G	**0.125**	
	498	H		**0.026**
			H=0.742	H=0.554
AAG8-1F	159	A	**0.313**	
	165	B	**0.063**	
	173	C	0.375	0.974

	176	D		**0.026**
	182	E	**0.25**	
			H=0.695	H=0.05
CCAA15-F	269	A	**0.063**	
	284	B	0.75	0.256
	293	C	**0.188**	
	299	D		**0.744**
			H=0.398	H=0.381
CAGDL2-5-1F	306	A	0.5	0.974
	315	B	0.5	0.026
			H=0.5	H=0.05
CCAG15-1F	262	A	**0.313**	
	276	B		**0.026**
	281	C	**0.313**	
	284	D	0.375	0.974
			H=0.664	H=0.05
MEAN			**H=0.213**	**H=0.099**
Sample size			16	39
Number of allele			33	30
Unique alleles			11	8
Polymorphic loci			7	7

Table 5: Multilocus genotypes across the seven polymorphic loci of *C. pirilliformis* from South Africa and Australia.

Isolates	**Multilocus genotypes**						
				South African population			
CMW11722	B	B	B	D	B	B	B
CMW12677	A	C	F	C	D	A	D
CMW16522	A	C	F	C	D	A	D
CMW16521	A	E	B	C	B	A	D
CMW16514	A	E	B	C	D	A	D
CMW12676	A	E	B	C	D	A	D
CMW12680	A	E	B	C	D	A	D
CMW16469	A	E	B	C	D	A	D
CMW16515	A	E	F	C	D	A	D
CMW11699	A	B	B	C	B	A	D
CMW12281	A	B	B	C	B	A	D
CMW16523	A	B	D	C	B	A	D
CMW16527	A	B	D	C	D	A	D
CMW16463	A	B	F	C	D	A	D
CMW16520	A	B	F	C	D	A	D
CMW16529	A	B	F	C	D	A	D
CMW16517	A	B	F	C	D	A	D
CMW16525	A	B	F	C	D	A	D
CMW16526	A	B	F	C	D	A	D
CMW16466	A	C	B	C	D	A	D
CMW16516	A	D	H	C	D	A	D
CMW12673	A	D	F	C	D	A	D
CMW12675	A	D	F	C	D	A	D
CMW12671	A	D	E	C	B	A	D
CMW16749	A	D	B	C	B	A	D
CMW16528	A	D	B	C	B	A	D
CMW16470	A	D	B	C	B	A	D
CMW16747	A	D	F	C	B	A	D
CMW12679	A	D	B	C	D	A	D
CMW 17914	A	D	B	C	D	A	D
CMW16467	A	D	B	C	D	A	D
CMW16471	A	D	B	C	D	A	D
CMW16511	A	D	B	C	D	A	D
CMW16512	A	D	B	C	D	A	D
CMW16518	A	D	B	C	D	A	D
CMW16519	A	D	B	C	D	A	D
CMW16524	A	D	B	C	D	A	D
CMW16746	A	D	B	C	D	A	D
CMW16750	A	D	B	C	D	A	D
				Australian collection			

CMW6670	A	A	B	E	B	A	C
CMW6566	B	C	B	E	B	A	C
CMW6571	B	C	B	E	B	A	C
CMW6579	B	B	C	A	B	B	A
CMW6574	B	B	C	A	B	B	A
CMW6575	B	B	C	A	B	B	A
CMW6556	B	B	D	C	B	B	D
CMW6569	B	B	D	E	B	A	D
CMW6583	B	B	D	C	B	B	D
CMW6586	B	B	D	C	B	B	D
CMW6576	B	B	C	A	B	B	A
CMW6577	B	B	C	A	B	B	A
CMW6563	A	C	C	B	A	A	D
CMW19344	A	B	G	C	C	A	C
CMW19334	A	B	G	C	C	A	C
CMW19341	A	B	A	C	C	A	D

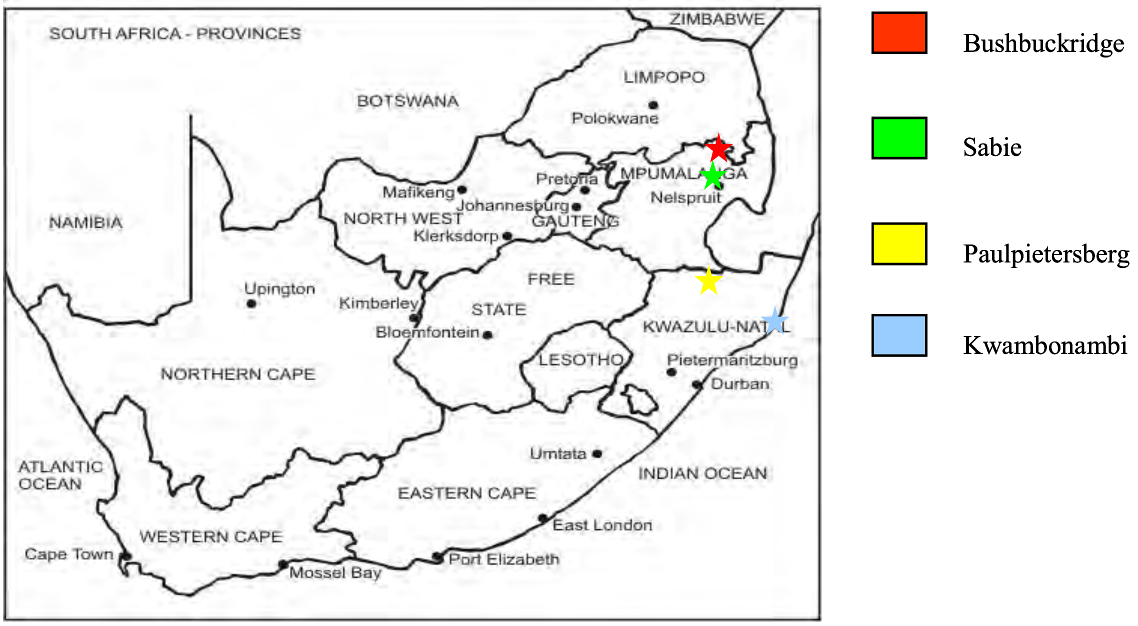


Figure 1: Map of South Africa highlighting the four areas from which *C. pirilliformis* samples were obtained (in coloured stars).

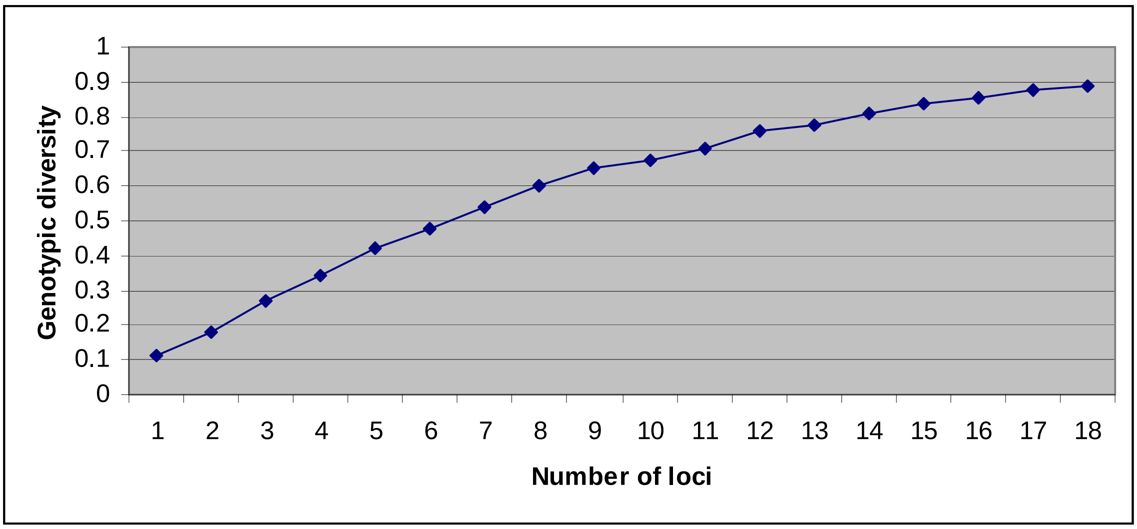


Figure 2: Genotypic diversity against the number of loci. The results indicate that the collection of isolates of *C. pirilliformis* from South Africa were not large enough to represent the true population of the fungus since the plateau was not reached.

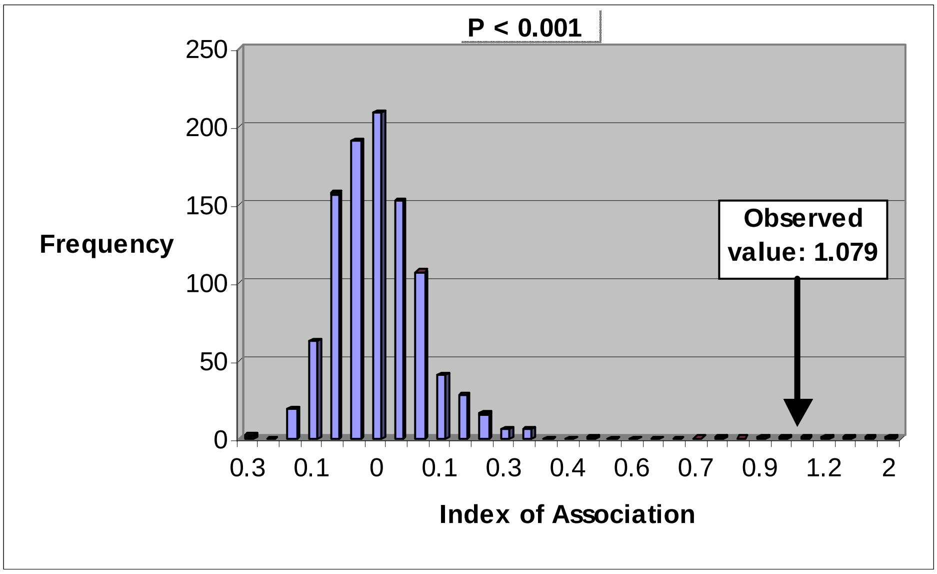


Figure 3: Histogram of the frequency distribution representing multilocus disequilibium estimate I_A for 1000 randomised datasets for the South African population of *C. pirilliformis*.

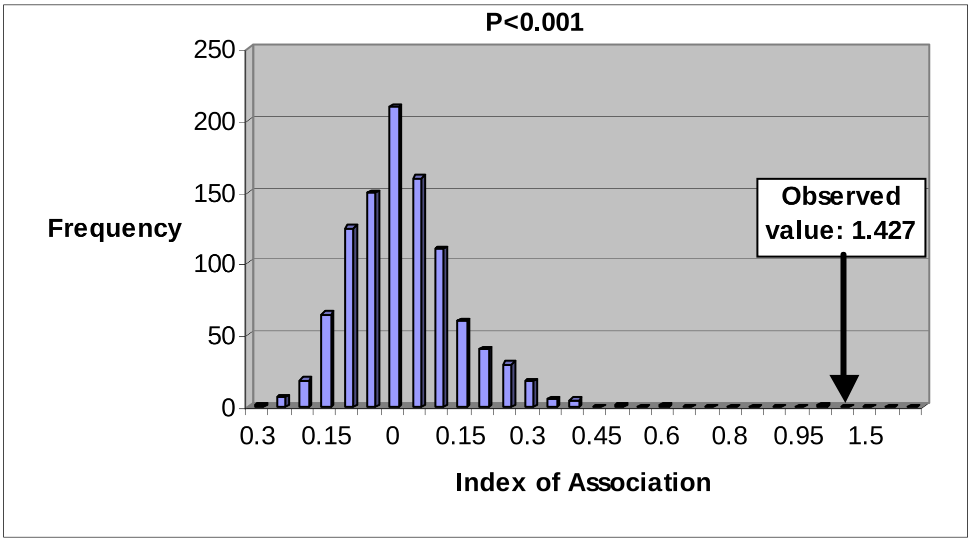
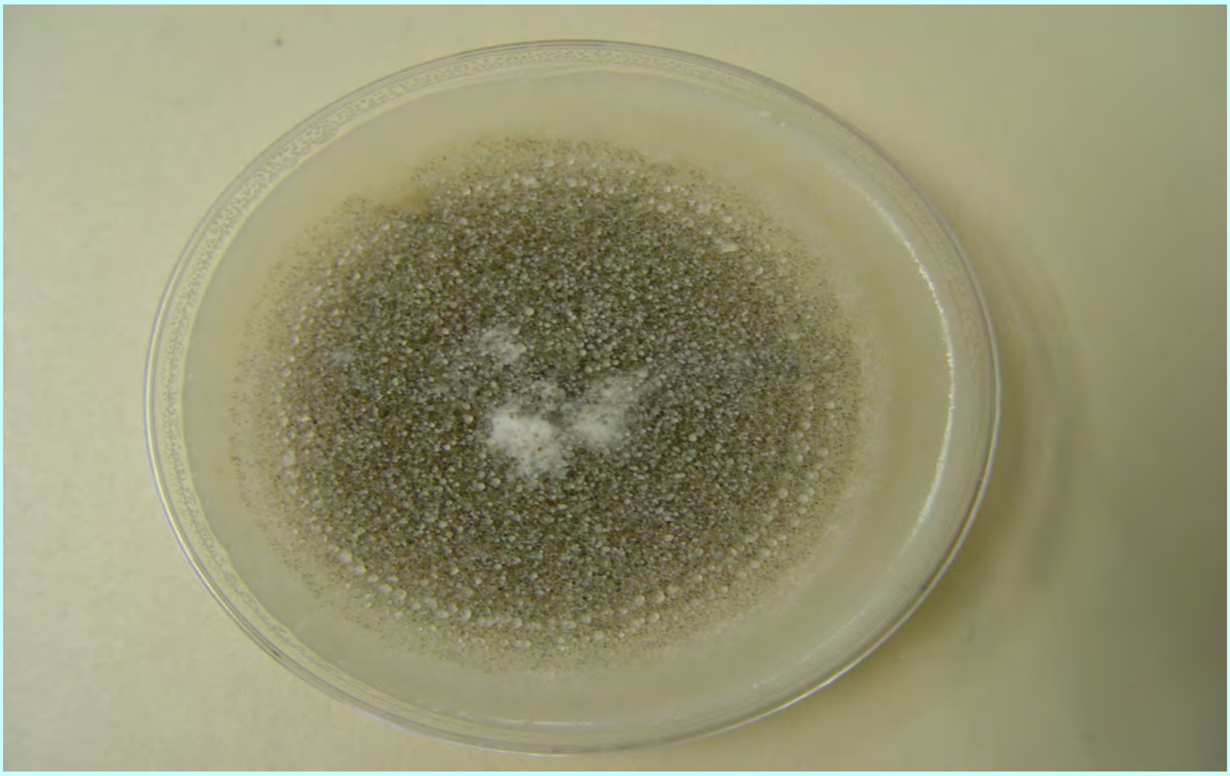


Figure 4: Histogram of the frequency distribution representing multilocus disequilibium estimate I_A for 1000 randomised datasets for the Australian population of *C. pirilliformis*.

CHAPTER 3

Pesotum species including *Pesotum australi prov. nom.* associated with *Acacia mearnsii* trees in Uganda and Australia



1.0 ABSTRACT

Pesotum accommodates synnematal anamorphs of ***Ophiostoma*** spp. with sympodially proliferating conidiogenous cells. ***Pesotum ulmi***, the anamorph of the Dutch Elm disease pathogen, represents the type species and is one of a number of economically important species in the genus. These fungi are usually closely associated with wounds on trees and the insects that visit them. During tree disease surveys in Uganda, as well as studies of fungi infecting wounds on ***Acacia mearnsii*** trees in Uganda and Australia, many isolates resembling species of ***Pesotum*** were collected. The aim of this study was to identify these fungi using both morphological and DNA sequence comparisons. ***Pesotum quercus***, anamorph of ***Ophiostoma quercus***, a fungus closely related to the Dutch Elm disease pathogens, was the only species collected from multiple collections in Uganda. Collections from Australia represent a new species of ***Pesotum*** described here as ***P. australi prov. nom.***

2.0 INTRODUCTION

The genus *Ophiostoma* accommodates virulent pathogens such as *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which result in tree death (Brasier 1990, 2000, Wingfield *et al*. 1993). It also includes many species that result in sapstain of lumber, which can lead to great losses in revenue (Münch 1907, Lagerberg *et al.* 1927, Seifert 1993, Uzunovic & Webber 1998). *Ophiostoma* spp. require wounds for infection and most species are closely associated with insects such as bark beetles (Curculionidae: Scolytinae) that act as wounding agents (Grossmann 1931, 1932, Harrington 2005, Kirisits 2004, Six 2003). These beetles often carry a wide variety of fungi, including *Ophiostoma* spp., in mycangia, on the surfaces of their bodies, or in their guts (Grossmann 1931, 1932, Harrington 2005, Kirisits 2004, Six 2003). In some cases, the relationship between these fungi is relatively specific and in others they are carried by casual insects such as flies and nitidulid beetles that are attracted to the sap associated with freshly made wounds on trees (Jewell 1956, Juzwik and French 1983, Juzwik *et al.* 1998).

Ophiostoma sensu lato is a polyphyletic genus, including at least three genera. These include *Ophiostoma* H. & P. Sydow sensu stricto with *Pesotum* Crane and *Sporothrix* Hektoen & Perkins anamorphs, *Ceratocystiopsis* Upadhyay & Kendrick with *Hyalorhinocladiella* Upadhyay & Kendrick anamorphs and *Grosmannia* Goidanich with *Leptographium* Lagerberg & Melin anamorphs (Upadhyay 1981, Zipfel *et al.* 2006). Sexual forms of these fungi commonly produce ascomata with long erect necks giving rise to sticky spore drops that facilitate dispersal by insects (Malloch & Blackwell 1993). Asexual structures are typically erect conidiophores with sticky spores at their apices (*Hyalorhinocladiella* sp., *Pesotum* sp. and *Leptographium* sp.) or dry spores (*Sporothrix* sp.) that can be wind-dispersed (Crane & Schoknecht 1973, Ingold 1971, Malloch & Blackwell 1993).

The anamorph genus *Pesotum* was established to accommodate species that produce both synnematous and mononematous conidiophores, with sympodially proliferating conidiogenous cells (Crane & Schoknecht 1973). However, its taxonomic placement in *Ophiostoma* has been the source of considerable debate. Okada *et al*. (1998) treated *Pesotum* to include all synnematal anamorphs with affinities to *Ophiostoma*. In a more recent treatment based on DNA sequence comparison, Harrington *et al*. (2001) recommended that *Pesotum* should be restricted only to anamorphs of the *O. piceae* (Munch) H. & P. Sydow complex.

Acacia mearnsii de Wild is a woody legume of the family Mimosaceae (Orchard & Wilson 2001). It is endemic to Australia and has been introduced into many countries for tannins that can be extracted from its bark and for high value, short fiber wood used in pulp and fuel production (Acland 1971, Gibson 1975, Sherry 1971). As with other leguminous plants, *A. mearnsii* fixes atmospheric nitrogen in symbioses with soil bacteria, which makes it suitable for planting in poor quality soil. For this reason the tree is often planted in rotation with agronomic crop plants (Sherry 1971, Acland 1971). In many developing countries, such as Uganda, *A. mearnsii* trees are utilized extensively for fuel wood, often growing in dense clumps of naturally regenerating trees.

Very little is known regarding the occurrence of *Ophiostoma* spp. in parts of the world other than Europe and North America. In the Southern Hemisphere, reports on *Ophiostoma* spp. are restricted to a few countries and from a limited number of studies. Other than those from South Africa, there are no reports of *Ophiostoma* spp. from Africa. Reports of *Ophiostoma* spp. from Australia are also relatively limited with a few species known to be associated with introduced pine-infesting bark beetles (Stone & Simpson 1987, 1991, Vaartaja 1967) and *O. quercus* has been recorded from *Pinus radiata* D. Don (Harrington *et al*. 2001). The aim of this study was to identify *Pesotum* spp. collected from artificially induced wounds on *A. mearnsii* trees in Australia, where this tree is native, and those collected from non-native *A. mearnsii* in Uganda. For this purpose both morphological and DNA sequence comparisons were used.

3.0 MATERIALS AND METHODS

3.1 Cultures

Cultures from *A. mearnsii* in Uganda were collected from stumps of *A. mearnsii* trees shortly after harvesting, in the Kabale area of south western Uganda and from stem cankers on these trees in the same area. Isolates from Australia were obtained from artificially induced wounds made on the stems of *A. mearnsii* trees near Cann River in the state of Victoria, collected as part of a previous study (Barnes *et al*. 2003). All cultures used in this study have been preserved 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 with the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands (CBS).

3.2 Morphology

Isolates for morphological characterisation were grown on 2% Malt extract agar (MEA, 20gl^{-1} malt extract and 15gl^{-1} agar) (Biolab, Midrand, South Africa) containing the antibiotic streptomycin sulphate (0.05g/l) (SIGMA-ALDRICH, Steinheim, Germany) at 24ºC for seven days. Single drops of conidia or segments of mycelium were transferred from pure cultures to Oatmeal agar medium (OMA, 30g Oats 20g Biolab agar and 1Lt. deionised water) to promote sporulation and for comparisons with previously published descriptions. Cultures were incubated at 24ºC until sporulation and then grouped into morphotypes based on differences in colony colour (Rayner 1970), arrangement of fruiting bodies and morphology. Fruiting structures (synnemata and conidia) were mounted in 80% lactic acid on microscope slides and measured using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements were made for each structure from each isolate chosen as the type of new species and ten measurements were made for additional isolates and the means were computed for relevant morphological structures. Measurements were noted as (minimum-) lower – upper (-maximum). To induce the production of sexual fruiting structures, cultures were grown on 1.5% water agar (15g Biolab agar and 1Lt. deionised water) supplemented with sterile pieces of *A. mearnsii* wood. Plates were incubated at room temperature and inspected weekly for the appearance of ascomata and ascospore production.

3.3 DNA extraction and PCR amplification

A selection of isolates, representing each of the different groups identified based on culture and morphological characteristics were selected for DNA sequence comparisons. Single spore drops from synnemata in pure cultures were grown on 2% MEA for 7-10 days. Mycelium was then transferred to 1.5ml Eppendorf tubes using a sterile scalpel. DNA was extracted using the protocol described by Möller *et al.* (1992), except that 10µl of RnaseA were added at the final step and incubated overnight at room temperature to digest RNA. The presence of DNA was verified by separating an aliquot of 5µl on 1% agarose gels containing ethidium bromide and visualized under Ultraviolet light. The Internal Transcribed Spacer regions (ITS1 and ITS2) and 5.8S gene of the ribosomal RNA operon were amplified using an Eppendorf Mastercycler (Merck, Hamburg, Germany) and primers ITS1 and ITS4 (White *et al.* 1990). Parts of two other gene regions comprising the nuclear large sub-unit rDNA and the beta tubulin gene were also amplified, using primers LROR (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') (http://www.biology.duke.edu/fungi/mycolab/primers.htm) for the large sub-unit, and primers T10

(5'-ACGATAGGTTCACCTCCAGAGAC-3') (O'Donnell & Cegelnik 1997) and Bt2b (5'-GGTAACCAAATCGGTGCTGCTTTC-3') (Glass & Donaldson 1995) for the β-tubulin regions. DNA template (60ng) was used to prepare a 25µl Polymerase Chain Reaction (PCR), that also contained 2.5µl of 10X reaction buffer with $MgCl_2$ (25mM) (Roche Diagnostics, Mannheim, Germany), 2.5µl $MgCl_2$ (25mM) (Roche Diagnostics, Mannheim, Germany), 1U of Taq polymerase (Roche Diagnostics, Mannheim, Germany), 2.5µl of deoxynucleotide triphosphate mix (dNTP) (10mM) and 0.5µl of each primer (10mM). The conditions used for the thermal cycling were as follows: an initial denaturation of the DNA at 96ºC for 2min, followed by 35 cycles consisting of denaturation at 94ºC for 30s, annealing at 55ºC for 30s, primer extension at 72ºC for 1min and a final extension at 72ºC for 10min. An aliquot of 5µl of the PCR products were separated on a 1% agarose gel and visualized under UV light after staining with ethidium bromide. For a few isolates, multiple bands were obtained. In each of these cases, the annealing temperatures were adjusted until a single band was obtained.

3.4 DNA sequencing

PCR products were purified using Sephadex G-50 Gel (SIGMA-ALDRICH, Steinheim, Germany), as recommended by the manufacturer. Purified products (1µl) were separated by electrophoresis in a 1% agarose gel to estimate the concentration of DNA. Subsequently an accurate concentration of the purified PCR product was 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), according to the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Between 60-100ng PCR product was used to prepare 10µl sequencing reactions that also contained 2µl of ready reaction mixture (Big Dye), 2µl of 5X reaction buffer, 1µl of primer (10mM) and enough water to complete the volume of 10µl. The same primers were used as those used for the PCR amplifications. Both DNA strands were sequenced.

3.5 Phylogenetic analyses

A preliminary identity for isolates from Uganda and Australia was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov) using ITS sequence data. Thereafter, sequences from both strands for each isolate were checked visually and combined using the programme Sequence Navigator version 1.01 (ABI PRISM, Perkin Elmer), by comparing the nucleotides and their corresponding peaks. Additional sequences of related ***Pesotum*** spp. were obtained from the GenBank database (Table 3.1). Sequences were then aligned with those from GenBank using Mafft ver.5.851 (Katoh *et al*. 2002). Phylogenetic analyses were performed using PAUP*4.0b10 (Swofford 1998). For isolates from Australia, the nuclear large sub-unit (LSU) and the beta tubulin genes were also included in the analyses. Heuristic searches using maximum parsimony with 10 random addition sequence replicates, branch swapping and Tree Bisection Reconstruction (TBR) were performed. Trees were rooted using *O. piliferum* H. Syd. & P. Syd. as an outgroup taxon. Confidence levels of the branching points in the phylogenetic trees were estimated with the bootstrap method (1000 replications) (Felsenstein 1985).

3.6 Mating studies

To produce a tester strain that could be used for isolate identification, 15 single ascospore cultures were prepared from ascomata produced by an isolate of *P. quercus* (CMW5826) from Uganda on sterilized *A. mearnsii* wood. The single ascospore cultures were crossed in every possible combination on MEA supplemented with *A. mearnsii* wood pieces. To induce the production of ascomata, these cultures were first incubated at 24^{o}C for two weeks, and then at 20^{o}C for three weeks and checked weekly using a dissection microscope. Some crosses gave rise to ascomata, and it was then possible to select tester strains of opposite mating type. The tester strains were used in crosses with three single conidium cultures prepared from each of fourteen other isolates from Uganda, and which did not produce ascomata on the wood. They had also not been subjected to DNA sequence comparisons and the aim was to determine their identity based on mating compatibility. A few isolates from Uganda that had been compared based on DNA sequences were also included in the mating tests to serve as controls. Isolates from Australia were not used in the mating tests as there were only four isolates and DNA sequence comparisons could be made for all of them. The tester strains [CMW17256, CMW17257 (+) and CMW17258, CMW14307 for (-)] are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI).

3.7 Growth in culture

Disks of agar (9mm diam.) bearing mycelium of selected isolates (CMW6606, CMW6589), of an unknown species were transferred from the actively growing margins of seven-day-old cultures and placed upside down at the center of 90mm Petri dishes containing 2% MEA. Plates were incubated in the dark for 10 days at temperatures ranging from 5 to 35ºC at five degree intervals. Five replicates of each isolate were used at each temperature. Growth of cultures after ten days was measured using two diameter measurements perpendicular to each other for each plate at each temperature tested. The averages of the ten measurements were then computed.

4.0 RESULTS

4.1 Morphology

A total of 21 isolates (17 from Uganda and 4 isolates from Australia) resembling *Pesotum* spp. were collected from *A. mearnsii* and examined. These isolates could be assigned to one of four different morphotypes, one from Australia and three from Uganda, based on colony colour and the production of fruiting structures on OMA. Morphotype A included isolates with brown colonies and synnemata scattered over the plates. Morphotype B was comprised of isolates with white or lightly coloured mycelium and synnemata organized in a circular pattern. Morphotype C included isolates with light-brown colonies, with synnemata scattered at the edges of the plates, but forming circular rings towards the middle of the plates and Morphotype D included only isolates from Australia, which could be distinguished from Ugandan isolates on OMA by their cream colored colonies and synnemata with slimy heads, arranged in concentric rings. On WA supplemented with wood chips, only isolate CMW5826 from Uganda produced sexual fruiting structures. These were characteristic of an *Ophiostoma* sp. and this isolate was used to produce tester strains for the mating studies.

4.2 Analyses of ITS DNA sequences

All isolates selected for DNA sequencing produced PCR products of approximately 650 bp, using the primers ITS1 and ITS4. Blast searches suggested that the *A. mearnsii* isolates from Uganda and Australia represent *O. quercus*. Comparison of Ugandan and Australian isolates with those from GenBank and analysis in PAUP resulted in a total of 605 characters including gaps, with 94 constant characters, 37 parsimony-uninformative and 474 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option, resulted in 100 trees with branch length of 710. The consistency index (CI) value and the retention index (RI) value were 0.927 and 0.961 respectively. All eight isolates from Uganda clustered with *O. quercus*, supported by a bootstrap value of 58% (Figure 3.1). Isolates from Australia did not group with any of the representative *Ophiostoma* reference strains (Figure 1), suggesting that they represent a previously undescribed species, most closely related to *O. quercus*. Sequence comparisons using Australian isolates and the LSU (Figure 2) and β-tubulin (Figure 3) gene regions produced trees of similar topology to those of the ITS, confirming that it represents an undescribed taxon.

4.3 Mating studies

Nine isolates from Uganda that did not produce sexual fruiting structures, and which were not sequenced were crossed with two tester strains of opposite mating type (Table 2). These had been identified as *O. quercus* based on DNA sequence comparison. Five other isolates from Uganda that had been identified based on DNA sequences were also subjected to mating compatibility tests. Ten isolates gave positive results with the (-) tester strain (CMW14307) while four isolates gave positive results with the (+) tester strain (CMW14257), confirming that all 14 isolates from Uganda represent *O. quercus*.

4.4 Taxonomy

Phylogenetic studies based on a number of gene regions including part of the ITS genes and the 5.8S gene region (Figure 1), the large subunit (Figure 2), as well as the beta tubulin gene regions (Figure 2) showed that isolates from Australia represent an undescribed taxon. This was further supported by morphological comparisons and a new species is, therefore, described as follows:

Pesotum australi Kamgan-Nkuekam, Jacobs & Wingfield, ***prov. nom***. (Figure 4, Figure 5)
Etymology: Refers to the country where the fungus was first collected.

Coloniae umbrinae, capitula cremea mucosa in annulis concentricis disposita formantes. Conidiophorae synnematae, erectae, basin atrobrunneae, apicem versus pallescentes, (202-) 224.5 - 275.5 (-324.5) µm altae, basin (16.5-) 20 - 37.5 (-60) µm latae. Rhizoidea adsunt. Capitulum conidiogenum maxime (47-) 63 - 96 (-122) µm diametro, laete brunneum apicem versus hyalinescens. Cellulae conidiogenae (17.5-) 25.3 - 65.4(-133.7) µm longae, (1.6-)1.8 – 2.6(-3.5) µm latae, apicem versus angustatae. Conidia aseptata, hyalina, oblonga vel cylindrica, 1.5-2 (-2.5) x 0.5 -1 µm.

Colonies umber (13m) on OMA with conidiophores forming cream-colored slimy heads arranged in concentric rings, reverse dark mouse grey (13’’’’’k) to almost black. On MEA colonies avellaneous (17’’’b) with conidiophores forming cream-colored slimy heads arranged in concentric rings, reverse colonies tawny olive (17’’i). Colony diameter reaching 14 mm in 10 days on MEA at 25 ºC. Optimal growth temperature 20 ºC, no growth at 5 ºC or above 30 ºC. *Conidiophores* synnematal, erect, dark brown at the bases, becoming lighter towards the apex, (202-) 224.5 - 275.5 (-324.5) µm long, (19-) 17 - 42 (-31) µm wide in the middle, (16.5-) 20 - 37.5 (-60) µm wide at the base. *Rhizoids* present. Conidiogenous heads (47-) 63 - 96 (-122) µm across the widest part, light brown becoming hyaline towards the apex. Conidogenous cells, hyaline (17.5-) 25.3 - 65.4(-133.7) µm long, (1.6-) 1.8 – 2.6(-3.5) µm wide tapering towards the apex. *Conidia* produced through holoblastic, annellidic development. Conidia aseptate, hyaline, oblong to cylindrical, accumulating in slimy heads on the apices of the synnemata, 1.5-2 (-2.5) µm x 0.5 -1 µm.

Specimens examined: Australia, isolated from wounds on *Acacia mearnsii.* Cann River, NSW, November 2000, MJ. Wingfield, **holotype** PREM 59426, living culture CMW6606/CBS****

Additional specimens: Australia, isolated from wounds on *Acacia mearnsii.* Cann River, NSW, November 2000, MJ. Wingfield, **paratype**, Living cultures CMW6589, CMW6588, CMW6590.

5.0 DISCUSSION

In this study we expand the host and geographic range of *P. quercus* and the new species, *Pesotum australi* is described. These two fungi were isolated from *A. mearnsii* trees either in Australia or Uganda, both countries where few studies on *Ophiostoma* spp. have been conducted in the past. Both *Pesotum* spp. reported in this study group within the larger *O. piceae* complex. This is a group of morphologically similar species that are well-known to be difficult to identify and that have been the subject of considerable taxonomic confusion (Harrington *et al*. 2001, Okada *et al*. 1998, Przybyl & De Hoog 1989).

Pesotum australi prov. nom. is phylogenetically most closely related to *O. quercus*. However, DNA sequence data for several gene regions, including the ITS1 & ITS 4, 5.8S, beta tubulin and the large sub-unit gene have shown that this fungus is distinct from other *Pesotum* spp. In these analyses, it forms a well-resolved clade, supported by a bootstrap value of 92% on the parsimony tree and 88% on the neighbor-joining tree for ITS data. It is most closely related to members of the *O. piceae* complex that had previously been recognized to include nine species (Harrington *et al*. 2001). Species in the complex are morphologically similar to each other, leading to considerable taxonomic confusion (Przybyl & de Hoog 1989, Harrington *et al*. 2001). Indeed, recognition of *O. quercus* as distinct from *O. piceae* only became clear twelve years ago (Brasier 1993, Pipe *et al*. 1995, Halmschlager *et al*. 1994). Thus, many species identified as either *O. piceae* or *O. quercus* before the advent of DNA sequence comparisons may represent other species in the complex.

Pesotum australi prov. nom. can be distinguished from other members in the *O. piceae* complex and from *O. quercus*, its closest phylogenetic relative, by the fact that it produces only a *Pesotum* anamorph in culture. All other members of the *O. piceae* complex form *Sporothrix* synanamorphs in addition to the *Pesotum* state and this separates the complex from other species of *Ophiostoma* (Harrington *et al*. 2001). Additionally, *O. quercus* grows at 32°C (Brasier & Stephens 1993, Harrington *et al*. 2001) while *P. australi prov. nom.* does not grow at 30°C or above. The optimum growth temperature for *P. australi prov. nom.* is 20°C on MEA, while the maximum growth temperature is 25°C. Most isolates of *O. quercus* form concentric rings of aerial mycelium on MEA (Halmschlager *et al*. 1994, Harrington *et al*. 2001) with synnemata bearing viscous drops of ellipsoid to ovoid conidia. *P. australi prov. nom.* has a similar culture morphology to *O. quercus* on OMA, however, its synnemata terminate in creamy masses of oblong to cylindrical conidia that are also shorter than those of *O. quercus*.

In this study, we were able to develop positive and negative mating tester strains from an isolate identified as *P. quercus* based on DNA sequence comparisons. Crosses between the tester strains and fourteen other isolates from Uganda produced ascomata confirming that these all represent *P. quercus*.

O. quercus was a common inhabitant of wounds on *A. mearnsii* in Uganda. This is interesting, given the fact that this fungus was not isolated from *A. mearnsii* in Australia. Neither was *O. australi* found on this tree in Uganda. *O. quercus* has, however, been recorded from *P. radiata* in Australia (Harrington *et al*. 2001) and the results of this study might imply that *P. australi prov. nom.* preferentially colonises wounds on *A. mearnsii*. However, collections in this study were from very limited areas and sampling of *A. mearnsii* from different countries and from a wider range of areas in Australia where the tree is native, are needed to better understand the host specificity of *P. australi prov. nom.*

This study represents the first record of *O. quercus* from Uganda. Its occurrence in this country is not surprising as the fungus occurs worldwide, predominantly on hardwoods, but also on conifers in the Northern Hemisphere (Brasier & Kirk 1993, Halmschlager *et al*. 1994, Harrington *et al*. 2001, Kim *et al*. 1999, Morelet 1992, Pipe *et al*. 1995). *O. quercus*, along with others factors has been associated with the decline of oak trees in central and Eastern Europe (Anonymous 1990). It has also been reported in many countries of the Southern Hemisphere, from both native and non-native trees (De Beer *et al*. 2003). The only previous reports of the fungus from Africa are from South Africa, where it has been found on native *Olinia* sp. (De Beer *et al*. 1995), non-native *Eucalyptus grandis* (Hill) Maiden and *Quercus robur* L. (De Beer *et al*. 1995) and from three bark beetle species infesting *Pinus* spp. in the country (Zhou *et al*. 2001).

The origin of *O. quercus* in the Southern Hemisphere has been a matter of controversy. It has been suggested that the fungus was introduced from the Northern Hemisphere, where it is probably native (Brasier & Kirk 1993, Harrington *et al*. 2001). However, the fact that *O. quercus* is common on various native trees in the Southern Hemisphere might also suggest that it is also native to this part of the world (De Beer *et al*. 2003). Furthermore, *O. quercus* grows at high temperature ranges up to 32^{o}C (Brasier & Stephens 1993), which suggests that it is well adapted to warmer climates (De Beer *et al*. 2003). Recent reports of the fungus on native *S. parahybum* in Ecuador (Geldenhuis *et al*. 2004) also suggest that it has a wide natural distribution, beyond the boreal region.

The taxonomy of *O. quercus* appears not to be fully resolved. The differences in branch lengths for isolates treated as *P. quercus* in the phylogenetic component of this study and the low bootstrap values for these branches lead us to hypothesize that *O. quercus* represents a complex of species with a wide geographic distribution. There are likely different strains or sub-species among those currently treated as *O. quercus* occurring on different hosts and under different geographical and climatic conditions. This hypothesis deserves further study, particularly at the population level where gene diversity among *O. quercus* strains collected from different parts of the world and from different substrates can be considered.

This study has extended the host and geographic range of *P. quercus* and it has identified a new species closely related to it. It is clear that native hardwood species in Australia represent a substrate where new *Ophiostoma* spp. are likely to be found. Thus, surveys of fungi occurring, particularly on wounds on native Australian tree species will probably result in the description of many new Ophiostomatoid fungi.

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Table 1: *Ophiostoma* spp. included in DNA sequence comparison studies.

Species	Isolate numbers	Genbank accession number			Hosts	Collectors	Origin
		ITS	β-Tubulin	LSU			
O. catonianum	C1084, CBS263.35	AF198243	NA	NA	*Pyrus communis*	G. Goidanich	Italy
O. floccosum	C1086, CBS799.73	AF198231	NA	NA	NA	A. Käärik	Sweden
	CMW7661	AF493253	NA	NA	*Pinus elliottii*	ZW. de Beer	South Africa
	KAS708	NA	AY305691	NA	NA	NA	NA
	NZFS637	NA	AY789141	NA	NA	NA	New Zealand
	CMW1713	NA	NA	DQ294367	NA	NA	USA
O. himal-ulmi	C1183, CBS374.67; ATCC36176; ATCC36204	AF198233	NA	NA	*Ulmus* sp.	HM. Heybroek	India
	C1306, HP27	AF198234	NA	NA	*Ulmus* sp.	CM. Brasier	India
O. kryptum	DAOM229702 (IFFFBW/1)	AY304434	NA	NA	*Larix decidua*	T. Kirisits & MJ. Wingfield	Austria
	IFFFHasd/1	AY304437	NA	NA	*Larix deciduas*	T. Kirisits & MJ. Wingfield	"
	DAOM229702	NA	AY305686	NA	*L. decidua*	MJ. Wingfield & T. Kirisits	"
	DAOM229701	NA	AY305685	NA	*L. decidua*	T. kirisits	"
O. multiannulatum	CBS124.39	AY934512	NA	NA	NA	NA	NA
O. novo-ulmi	C510	AF198236	NA	NA	*Ulmus* sp.	NA	Iowa, USA
	C1185, CBS298.87; WCS637	AF198235	NA	NA	*Ulmus* sp.	HM. Heybroek	Russia
	CMW10573	NA	DQ296095	NA	NA	NA	Austria
	CMW10373	"	NA	DQ294375	"	"	"
O. perfectum	C1104, CBS636.66	DQ062970	NA	NA	NA	NA	NA
O. piceae	C1087; CBS108.21	AF198226	NA	NA	NA	E. Munch	Germany
	CMW7648, C967; H2181	AF493249	NA	NA	*Picea sitchensis*	DB. Redfern & JF. Webber	United Kingdom
	CMW7648	NA	AY789152	NA	NA	NA	"
	NZFS332.01	NA	AY789151	NA	NA	NA	New Zealand

	CMW8093	NA	DQ296091	DQ294371	NA	NA	Canada
O. piliferum	CBS129.32	AF221070	NA	NA	*Pinus sylvestris*	H. Diddens	United Kingdom
	NA	AF221071	NA	NA	NA	NA	NA
	CMW7877	NA	DQ296098	DQ294378	"	"	"
	CMW7879	NA	DQ296097	NA	"	"	"
	CBS12932			DQ294377	"	"	"
O. pluriannulatum	MUCL18372	AY934517	NA	NA	NA	NA	USA
	C1033, NZ-150	DQ062971	NA	NA	*P. radiata*	Farrell	New Zealand
	C1567, UAMH9559; WIN(M)869	DQ062972	NA	NA	*Podocarpus* sp.	Reid	New Zealand
O. quercus	C970, CBS102353, H1039	AF198239	NA	NA	*Quercus* sp.	PT. Scard & JF. Webber	United Kingdom
	CMW7656	AF493250	NA	NA	*Q. robur*	MJ. Wingfield	South Africa
	CMW2463, 0.96	AF493239	NA	NA	*Fagus sylvatica*	M. Morelet	France
	CMW7650, C969; CBS102352; H1042	AF198238	NA	NA	*Quercus* sp.	PT. Scard & JF. Webber	United Kingdom
	CMW7645, W3; HA367	AF493246	NA	NA	*Q. robur*	T. Kirisits & E. Halmschlager	Austria
	C970	NA	AY789157	"	NA	NA	UK
	KUC2210	"	AY789155	"	"	"	NZ
	NZFS3182	"	AY789156	"	"	"	"
	CMW3110	"	DQ296096	"	"	"	USA
	CBS118713	"	NA	DQ294376	"	"	"
	*CMW5826	NA	NA	NA	*A. mearnsii*	J. Roux	Uganda
	*CMW5928	EF408598	"	"	"	"	"
	*CMW5932	NA	"	"	"	"	"
	*CMW5952	"	"	"	"	"	"
	*CMW5948	EF408600	"	"	"	"	"
	*CMW5679	NA	"	"	"	"	"
	*CMW5955	"	"	"	"	"	"
	*CMW5943	EF408599	"	"	"	"	"
O. setosum	AU16053	AF128927	NA	NA	NA	NA	Canada
	AU16038	AF128929	NA	NA	NA	NA	"
	NZFS3652	NA	AY789159	NA	NA	NA	NA
	AU160-53	NA	AY305703	NA	NA	NA	Canada
O.	CBS188.86	AY934522	NA	NA	NA	NA	NA

subannulatum							
O. tetropii	CBS428.94	AY194507	NA	NA	*Picea abies*	T. Kirisits	Austria
	DAOM229566 (C01-015)	AY194493	NA	NA	*P. glauca*	G. Alexander	McNabs Island, Canada
	CBS428.94	NA	AY305702	NA	NA	NA	Austria
	C00-003	NA	AY305701	NA	NA	NA	Canada
O. ulmi	C1182, CBS102.63; IMI101223; JCM9303	AF198232	NA	NA	*Ulmus* sp.	WF. Holmes & HM. Heybroek	Netherlands
	CMW1462	NA	DQ296094	DQ294373	NA	NA	USA
P. australi	*CMW6590	EF408601	NA	NA	*A. mearnsii*	MJ. Wingfield	Australia
	*CMW6588	EF408604	"	"	"	"	"
	*CMW6606	EF408603	EF408606	EF408608	"	"	"
	*CMW6589	EF408602	EF408605	EF408607	"	"	"

* Isolates sequenced in this study

Table 2: Results of mating compatibility tests using tester strains and isolates from *Acacia mearnsii* in Uganda.

Tester Strains	Isolates from Uganda crossed													
	CMW5679	CMW5910	CMW5948	CMW5900	CMW5825	CMW5933	CMW5917	CMW5902	CMW5651	CMW5955	CMW5930	CMW5952	CMW5922	CMW5943
CMW14307*														
1	+	+	-	-	+	+	+	+	+	+	+	-	+	-
2	+	+	-	-	+	+	+	+	+	+	+	-	+	-
3	+	+	-	-	+	+	+	+	+	+	+	-	+	-
CMW17257*														
1	-	-	+	+	-	-	-	-	-	-	-	+	-	+
2	-	-	+	+	-	-	-	-	-	-	-	+	-	+
3	-	-	+	+	-	-	-	-	-	-	-	+	-	+

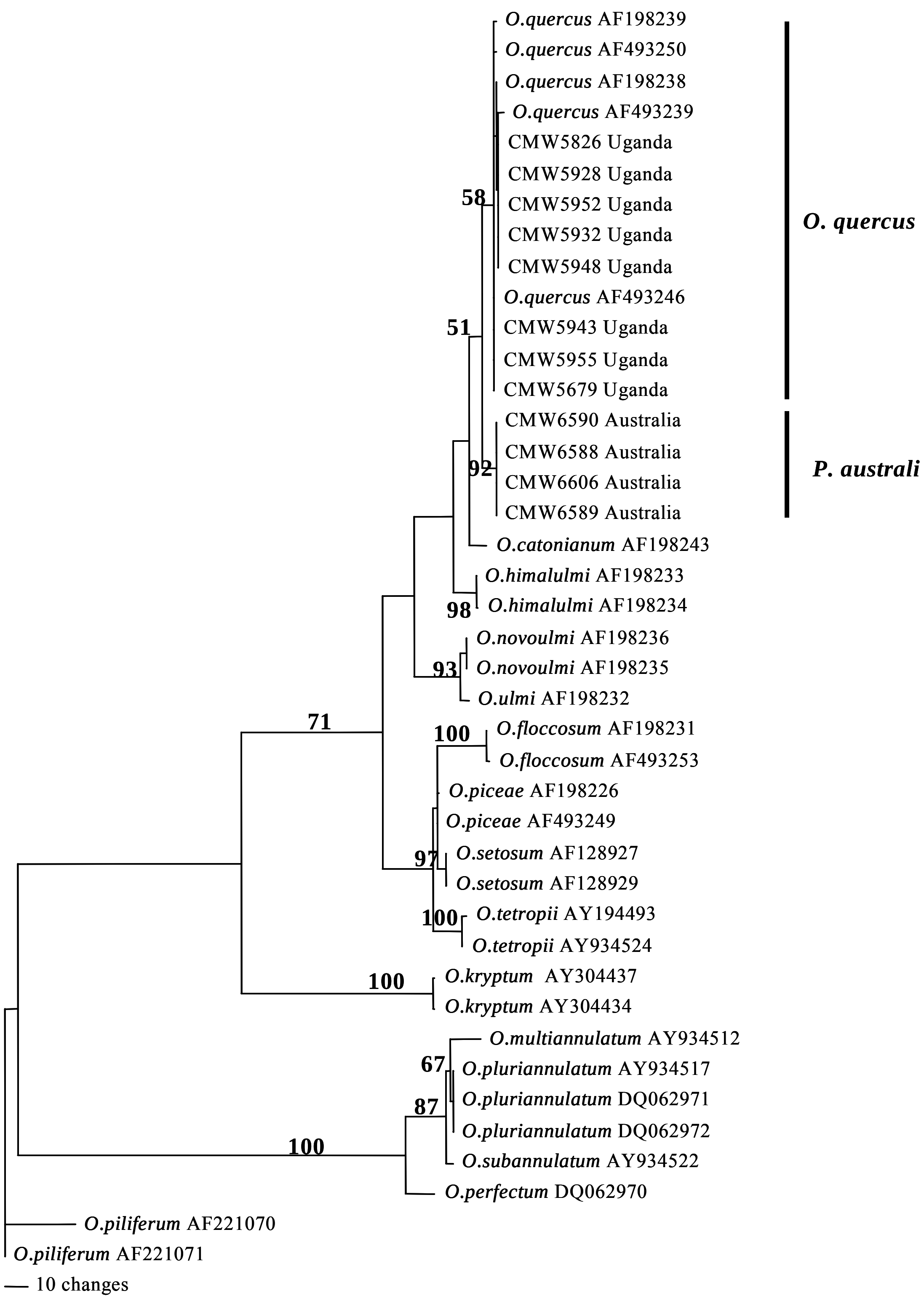

Figure 1: Phylogenetic tree produced by a heuristic search of the ITS sequence data. *Ophiostoma piliferum* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated above the branches of the tree.

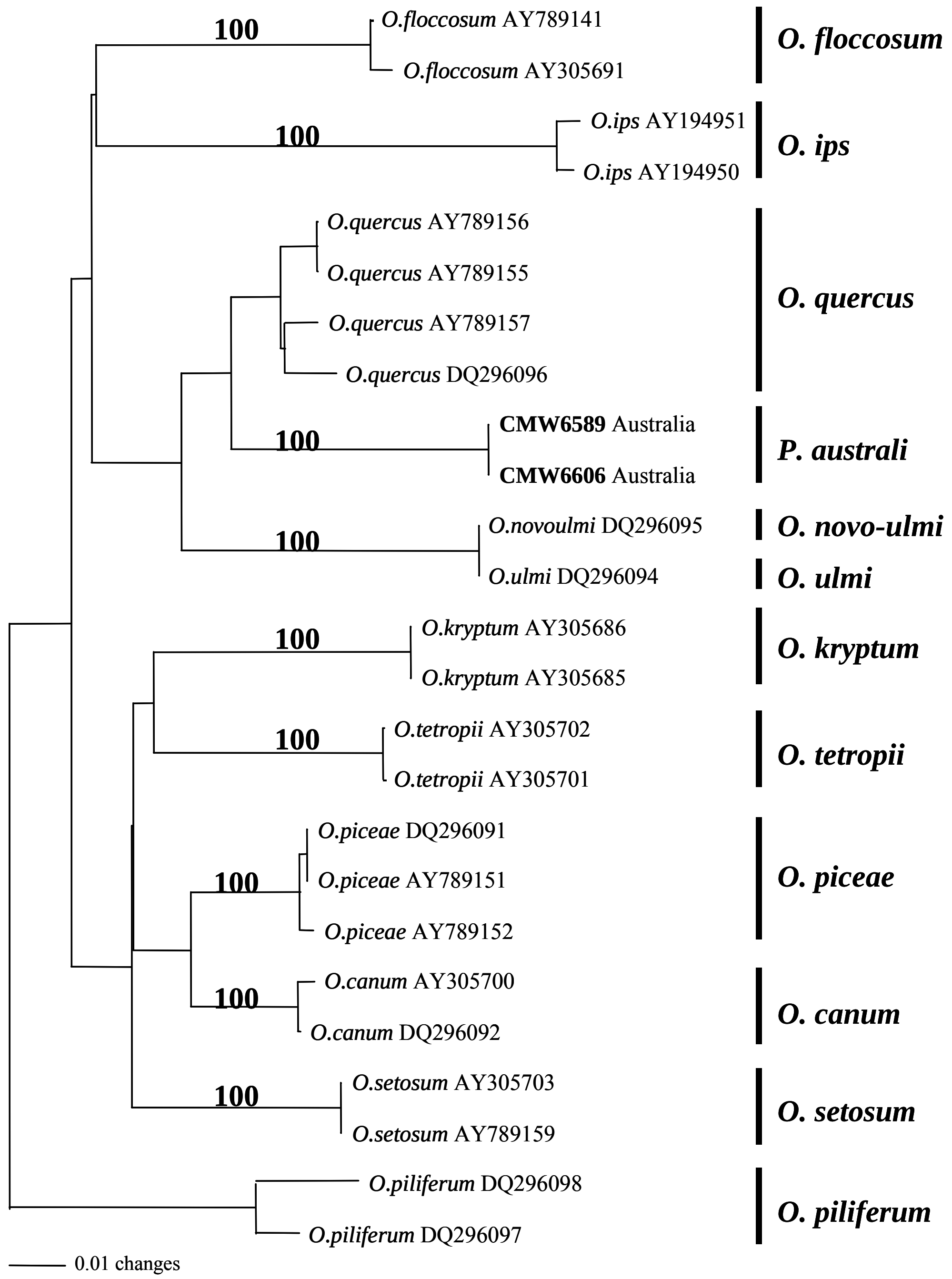

Figure 2: Neighbor-joining tree produced by a heuristic search of the β-tubulin sequence data. *Ophiostoma piliferum* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated above the branches of the tree. Isolates from Australia are in bold.

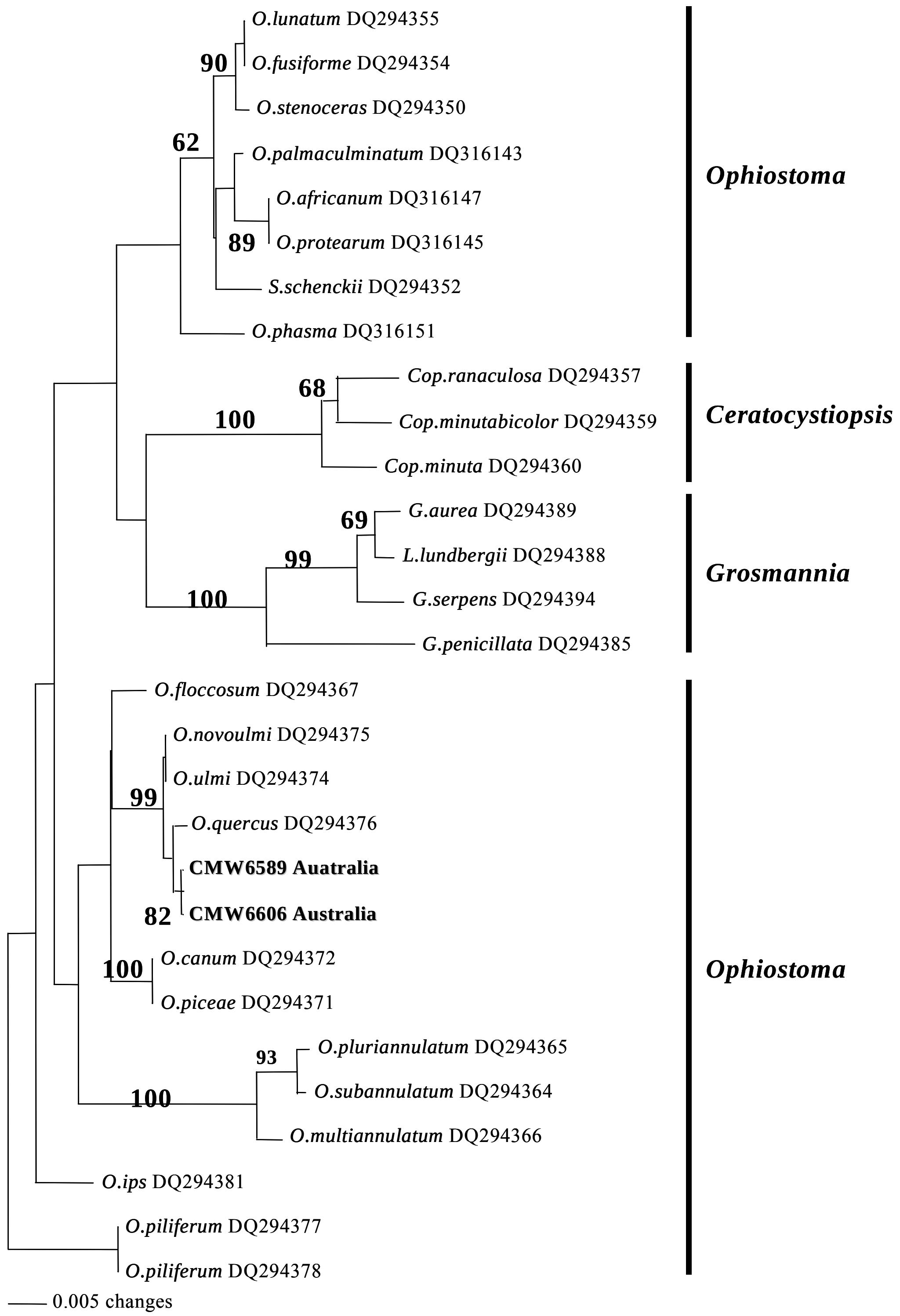

Figure 3: Neighbor-joining tree produced by a heuristic search of the large sub-unit sequence data. *Ophiostoma piliferum* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated above the branches of the tree. Isolates from Australia are in bold.

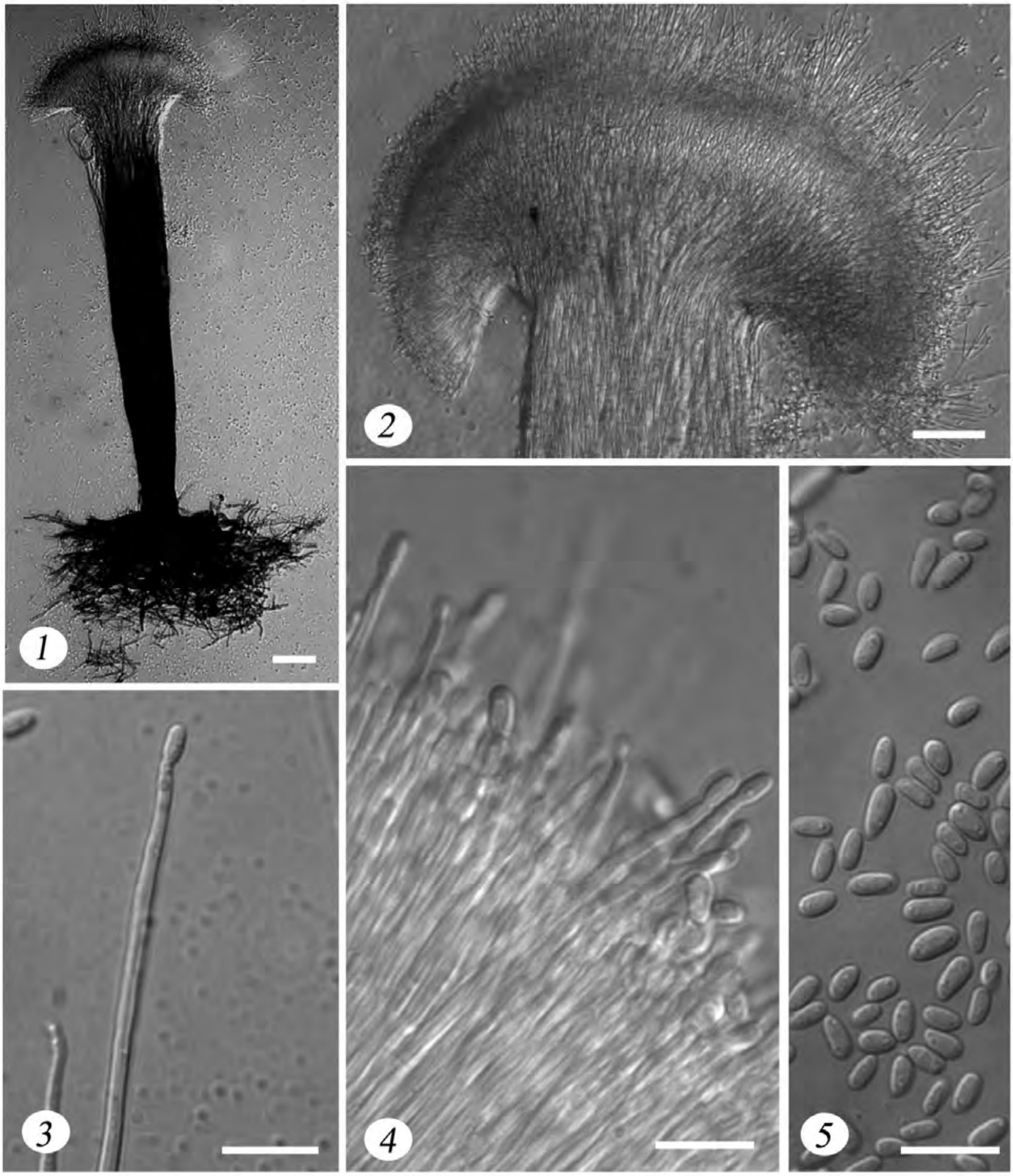

Figure 4: Morphological characteristics of *Pesotum australi prov. nom.* (CMW6606). 1) Synnemata (scale bar = 10µm) showing rhizoids at base, 2) Head of synnema (scale bar = 10µm), 3-4) Conidiogenous cells with conidia at the tips of percurrently proliferating conidiogenous cells (scale bar = 5µm), 5) Conidia (scale bar = 5µm).

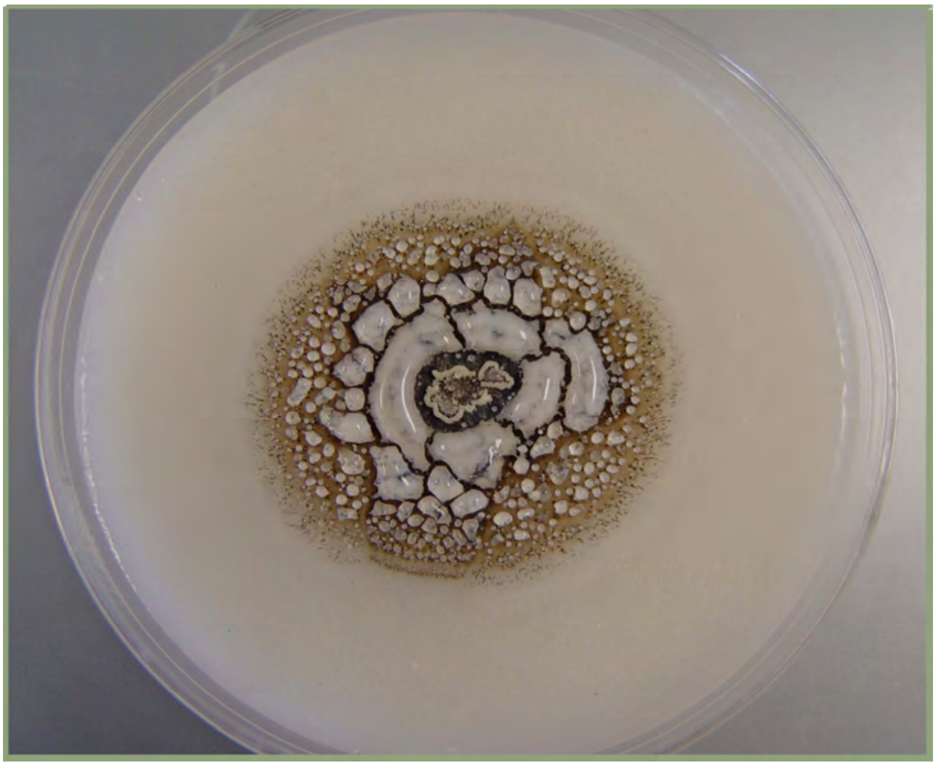

Figure 5. Cultural morphology of *Pesotum australi prov. nom*. on OMA. *Colonies* umber (13m) with conidiophores forming cream-colored slimy heads arranged in concentric rings.

CHAPTER 4

Ceratocystis and *Ophiostoma* species including three new taxa associated with wounds on native South African trees

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1.0 ABSTRACT

Ceratocystis and *Ophiostoma* species are fungi, some of which are important pathogens, associated with insects that typically infect wounds visited or made by these vectors. There are few reports of these fungi from the African continent and little is known of their relative importance in the area. In this study, *Ceratocystis* and *Ophiostoma* species were collected from wounds on native tree species in selected areas of South Africa and they were identified using morphology and DNA sequence comparisons. The pathogenicity of selected species was also tested in artificial inoculation studies under glasshouse conditions. *Ceratocystis* and *Ophiostoma* species were common on wounds of most trees investigated and sometimes they were associated with wood discolouration. Isolates were collected from eight different native trees including seven different families. *Pesotum quercus*, *P. fragrans, P. tropicale*, *C. albifundus* as well as an undescribed *Ophiostoma* sp. and two undescribed *Ceratocystis* spp. were collected. The new *Ceratocystis* spp. are described here as *Ceratocystis savannae prov. nom.* and *Ceratocystis tsitsikammensis prov. nom.* and the *Ophiostoma* sp. as *Ophiostoma longiconidiatum prov. nom.* In the pathogenicity tests, *C. tsitsikammensis prov. nom*. resulted in significant lesions on *Rapanea melanophloeos* trees, while *C. savannae prov. nom.* produced very small lesions on *Acacia nigrescens* and *Sclerocarya birrea* trees. This study greatly expands reports of *Ceratocystis* and *Ophiostoma* species from South Africa and emphasizes the view that the diversity of these fungi, and their role in disease development, is incompletely documented in the country.

2.0 INTRODUCTION

Species of *Ceratocystis* Ellis & Halsted and *Ophiostoma* H. & P. Sydow and their anamorph genera are collectively referred to as the Ophiostomatoid fungi due to their morphological similarities and particularly convergent evolution of structures adapted to insect dispersal (Wingfield *et al*. 1993). Fungi in these genera are characterized by mostly dark, globose ascomata with elongated necks giving rise to sticky spores at their apices. Asci generally disappear early in the development and are seldom seen (Upadhyay 1981). It is now widely accepted that *Ceratocystis* and *Ophiostoma* are distinct genera, in separate orders of the fungi. *Ceratocystis* spp. have *Thielaviopsis* anamorphs with enteroblastic conidiogenesis (Paulin-Mahady *et al.* 2002) and reside in the order Microascales Luttrell: Benny & Kimbr. (Hausner *et al*. 1993, Spatafora & Blackwell 1994, Paulin-Mahady *et al*. 2002). *Ophiostoma sensu lato* is recognized as a generic aggregate in the order Ophiostomatales Benny & Kimbr. (Hausner *et al*. 1993, Spatafora & Blackwell 1994). *Ophiostoma* s. l. includes *Ophiostoma* H. & P. Sydow *sensu stricto* with *Pesotum* Crane and *Sporothrix* Hektoen & Perkins anamorphs, *Ceratocystiopsis* Upadhyay & Kendrick with *Hyalorhinocladiella* Upadhyay & Kendrick anamorphs and *Grosmannia* Goidanich with *Leptographium* Lagerberg & Melin anamorphs (Upadhyay 1981, Zipfel *et al.* 2006). These genera, although phylogenetically distinct, have clearly evolved similar morphologies in response to the similar niches and the survival strategies that they have adapted (Hausner *et al*. 1993, Spatafora & Blackwell 1994), leading to the long-standing confusion in their taxonomy.

Many *Ceratocystis* and *Ophiostoma* species are responsible for significant economic losses to both agricultural and forest crops worldwide. Well-documented examples of tree pathogens are *O. ulmi* (Buismann) Nannf. and *O. novo-ulmi* Brasier, responsible for the Dutch Elm disease pandemics in Europe and the United States of America (USA), *C. fagacearum* (Bretz) Hunt, a damaging pathogen of *Quercus* spp. in the USA (Sinclair & Lyon 2005) and species in the *C. fimbriata sensu lato* complex (Kile 1993). On agricultural crops, *C. fimbriata* Ellis & Halsted *sensu lato*, especially, is a notorious pathogen, causing diseases of sweet potato, diseases of *Colocasia esculenta* (L.) commonly known as taro rot, rot diseases of *Xanthosoma* spp. and many other plants (Kile 1993).

Reports of *Ceratocystis* and *Ophiostoma* species from Africa are very limited. Where they do exist, they are typically unconfirmed and no voucher specimens or cultures have been retained for them.

Some of these fungi from Africa include *C. albifundus* De Beer, Wingfield & Morris from native South African *Protea* spp. (Gorter 1977, Roux *et al*. 2004a, Wingfield *et al*. 1996) and from non-native *Acacia mearnsii* de Wild trees (Morris *et al.* 1993, Wingfield *et al.* 1996) and *C. fimbriata sensu lato* from many different plants including *Crotalaria* sp. (Davet 1962), *Ipomoea* sp. (Kihurani *et al*. 2000), *Hevea* sp. (Snowden 1926, Ringoet 1923), *Eucalyptus* spp. (Roux *et al*. 2000, 2001a) and *A. mearnsii* (Roux *et al*. 2004a). Two other species, *C. pirilliformis* Barnes & Wingfield and *C. moniliformis* (Hedgcock) Moreau have also recently been reported from *Eucalyptus* spp. in South Africa (Roux *et al*. 2004b). Other reports of *Ceratocystis* spp. in Africa are mostly from agronomic crops in South Africa (Crous *et al*. 2000) and there are a few single reports from West Africa (Spaulding 1962, Nag Raj & Kendrick 1975, Upadhyay 1981).

Various ophiostomatoid fungi occur in the flower heads of native South African *Protea* spp. Seven *Ophiostoma* spp. have been reported from this unusual niche and very little is known regarding their ecology (Marais & Wingfield 1994, Marais & Wingfield 1997, Marais & Wingfield 2001, Roets *et al.* 2006a, Wingfield *et al*. 1988, Wingfield & Van Wyk 1993). It has, however, recently been shown that they are associated with insects that visit the infructescences (Roets *et al*. 2006b). Other reports of *Ophiostoma* spp. are those for species that infest *Pinus* spp. and that have been introduced with non-native bark beetles in South Africa (Zhou *et al*. 2001).

The vegetation of South Africa is essentially a woodland savannah with little indigenous forest, covering only 0.56% of the total surface of the country (Lowes *et al*. 2004). These forests are dispersed around the country in an archipelago-like fashion, especially along the southern and eastern seaboard (Lowes *et al*. 2004). Very little information is available regarding diseases of native trees in South Africa and until recently, no *Ceratocystis* and *Ophiostoma* species were known from native trees in the country. In recent studies *C. albifundus*, the cause of wattle wilt of non-native *A. mearnsii* trees in South Africa was reported from seven native tree genera (Roux *et al*. 2004a, Roux *et al*. 2007. They also provided preliminary evidence to show that this fungus could potentially cause disease on selected *Acacia caffra* (Thumb.) Wild. and *Combretum molle* R.Br, G.Don trees. In another study the same authors reported *C. albifundus* as well as an undescribed *Ceratocystis* sp. from several native tree species in Malawi, Zambia and South Africa (Roux *et al*. 2004a, Roux *et al*. 2005).

The aim of this study was to expand our knowledge regarding the biodiversity of *Ceratocystis* and *Ophiostoma* species on native trees in South Africa, consistent with the requirements of the Centre of Excellence in Tree Health Biotechnology (www.fabinet.up.ac.za/cthb/index). Fungi were identified based on their morphology and by means of DNA sequence comparisons for various gene regions. We also tested the ability of the isolated fungi to cause disease of their hosts under greenhouse conditions.

3.0 MATERIALS AND METHODS

3.1 Collection of Isolates

Surveys of naturally occurring and artificially made wounds were conducted during 2004 and 2005 in the Kruger National Park (Mpumalanga Province), Leeuwfontein Collaborative Nature Reserve (Gauteng Province) and Groenkloof Forest (Tsitsikamma Forests, Western Cape Province). Wounds from which samples were collected included damage caused by elephants, kudu, eland, wind as well as wounds made artificially (Barnes *et al*. 2003, Roux *et al*. 2004b) using axes or masonry chisels. Pieces of bark and wood were examined using a 10 X magnification hand lens and those showing signs of fungal growth and discoloration were collected and stored, separately for each tree, in brown paper bags. All the samples were then transported to the laboratory in a plastic bag to retain moisture. Dry samples were sprayed with water, sealed in plastic bags and incubated to induce sporulation of the fungi.

Samples were observed regularly for the presence of *Ceratocystis* and *Ophiostoma* species. These fungi were isolated as they appeared and plated onto 2% malt extract agar (MEA: $20gl^{-1}$ malt extract and $15gl^{-1}$ agar, Biolab, Midrand, South Africa and 1000ml deionised water) containing 0.05g/l of the antibiotic streptomycin (SIGMA-ALDRICH, Steinheim, Germany) at 24ºC, to obtain pure cultures. Isolates have been preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative specimens have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates have also been deposited in the National Collection of fungi (PREM), Pretoria, South Africa.

3.2 Morphological characterization

Fruiting structures (ascomata and ascospores; synnemata and conidia) were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope. *Ceratocystis* and *Ophiostoma* species were initially identified based on morphology. 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-) lower – upper (-maximum). The means were then calculated for relevant morphological structures.

3.3 Growth in culture

A disk of agar (9mm diam.) bearing mycelium of isolates selected to be tested for their growth in culture, were transferred from the actively growing margins of seven-day-old cultures and placed upside down at the centres of 90mm Petri dishes containing 2% MEA. The plates were incubated in the dark for 10 days at temperatures ranging from 5ºC to 35ºC at 5 degrees intervals. Five replicates of each isolate were used per temperature in each assessment. Two measurements, perpendicular to each other, were taken daily for each colony and the results averaged for each temperature.

3.4 DNA sequence comparisons

Representative isolates of each *Ceratocystis* and *Ophiostoma* species collected in this study were selected for DNA sequence comparisons (Table 1 & 2). Single spore drops collected from the apices of ascomata or conidiophores in pure cultures were grown on 2% MEA for 7-10 days. Mycelium was then transferred to 1.5ml Eppendorf tubes using a sterile scalpel. Mycelium was freeze-dried and ground into a fine powder with a sterile toothpick after addition of liquid nitrogen. DNA was extracted using the protocol described by Möller *et al*. (1992) except that 10µl of RnaseA were added at the final step and incubated overnight at room temperature to digest RNA. The presence of DNA was verified by separating an aliquot of 5µl on 1% agarose gels stained with ethidium bromide and visualized under Ultraviolet light (UV).

The internally transcribed spacer regions (ITS1, ITS4) 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 and the transcription elongation factor-1α gene 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 (25µl) mixtures were prepared using 60ng of the DNA template, 2.5µl of 10X reaction buffer with $MgCl_2$ (25mM) (Roche), 2.5µl $MgCl_2$ (25mM) (Roche), 1U of Taq polymerase (Roche), 2.5µl of deoxynucleotide triphosphate mix (DNTP) (10mM) and 0.5µl of each primer (10mM). The conditions used for the thermal cycling were as follows: an initial denaturation of the DNA at 96ºC for 2min, followed by 35 cycles consisting of denaturation at 94ºC for 30s, annealing at 55ºC for 30s for the ITS and β-tubulin genes and 60ºC for the elongation factor-1α gene, primer extension at 72ºC for 1min and a final extension at 72ºC for 10min. An aliquot of 5µl of the PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualized under UV light.

PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), according to the manufacturer's instructions. Subsequently, the concentration of the purified PCR product was determined using the 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). Between 60-100ng of PCR product was used to prepare 10µl sequencing PCR that also contained 2µl of ready reaction mixture (Big dye), 2µl of 5X reaction buffer, 1µl of primer (10mM) and sufficient sterile water to bring the volume to 10µl. The same primers were used for sequencing as those described for the PCR amplifications. 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 from 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* and *Ophiostoma* species were obtained from the GenBank database. PAUP 4.0b10 was used to construct phylogenetic trees from the distance matrices by pair-wise alignment of the sequences, using the neighbour-joining method (Saitou & Nei 1987). Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein 1985).

3.5 Pathogenicity tests

Pathogenicity tests were conducted in a greenhouse using three different tree species native to South Africa and which were found to be natural hosts for the various test strains. Twenty trees, approximately two-years-old, were inoculated with each test strain and ten other trees of the same age were inoculated with a sterile agar disc to serve as controls. Test strains collected in Kruger National Park (CMW17300) and Leeuwfontein Collaborative Nature Reserve (CMW17575) were used to inoculate *Acacia nigrescens* Oliver and *Sclerocarya birrea* (A. Rich.) Hochst., while test strains collected in Groenkloof (CMW14276, CMW14278) were used to inoculate *Rapanea melanophloeos* (L.) Mez. Inoculations were done by growing test strains on MEA for ten days and inoculating 6mm diam. discs overgrown with the fungi into wounds of equal size made by removing the bark to expose the cambial layer, using a sterile metal cork borer. The wounds filled with agar discs were sealed with Parafilm (Pechiney, Chicago, USA) to protect them against desiccation. Sixty days after inoculation lengths of both bark and cambial lesions were measured. Re-isolations were made from the lesions to meet the requirements of Koch's postulates. Lesion lengths were then analysed using SAS/STAT in SAS (SAS Institute Inc. 1999).

4.0 RESULTS

4.1 Collection of Isolates

A wide diversity of *Ceratocystis* and *Ophiostoma* species were isolated from tree wounds during the course of this study. Isolates were obtained from bark, cambial and wood samples collected from wounds on native tree species spanning eight different genera and six families in three geographical areas of South Africa, including the Kruger National Park, Leeuwfontein Collaborative Nature Reserve and Groenkloof Forest (Table 1 & 2). Tree species from which *Ceratocystis* and *Ophiostoma* isolates were obtained included: *Acacia nigrescens* (Mimosaceae), *Combretum zeyheri* Sond. (Combretaceae), *Sclerocarya birrea* (Anacardiaceae), *Burkea africana* Hook. (Leguminosae), *Faurea saligna* Harvey (Proteaceae), *Ocotea bullata* (Burch.) Baill. (Lauraceae), *Rapanea melanophloeos* (Myrsinaceae) and *Terminalia sericea* Burch. ex Dc. (Combretaceae) (Table 3). In most cases, the fungi were associated with and isolated from wood showing signs of xylem discolouration.

4.2 Morphological characterization

Ceratocystis spp. collected during the course of this study could be assigned to three morphotypes based on colony colour and the production of ascomata on MEA. The first group, representing isolates from Groenkloof produced grey to green-coloured colonies with black ascomata, similar to those of *C. fimbriata sensu lato*. However, these isolates differed from known species in the *C. fimbriata* complex in various characteristics. Isolates of the second morphotype was represented by isolates collected in the Kruger National Park. These fungi produced light-coloured, slow-growing colonies and ascomata with light coloured bases and black necks. Based on these characteristics, the isolates were identified as those of *C. albifundus*. Isolates of the third morphotype were collected in both the Kruger National Park and Leeuwfontein Collaborative Nature Reserve and they produced fluffy colonies with white mycelium when young, turning dark brown as they became older. Ascomata were produced abundantly in these cultures which also had a strong fruity odour. These characteristics are similar to those of *C. moniliformis* and led us to assign this third group of isolates to the *C. moniliformis sensu lato* complex.

Ophiostoma spp. that were collected could be assigned to two morphotypes based on colony colour and the production of sexual fruiting structures. The first group originating from Leeuwfontein, produced pale to grey and fluffy colonies. Ascomata had small, light coloured bases and very long black necks, in most cases bearing single annuli and they were also produced abundantly in culture. The second morphotype produced only a *Pesotum* anamorph.

4.3 DNA sequence comparisons

Selected isolates of the *Ceratocystis* sp. resembling *C. moniliformis*, collected in the KNP and Leeuwfontein (CMW17297, CMW17298, CMW17300, CMW17575) and that represented the third morphotype generated amplicons of about 600, 550, 850 bps for part of the ITS, β-tubulin and translation elongation factor-1α genes (EF-1 α) respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.714, suggesting that the data from the three gene regions can be combined. Comparison of these isolates with those from GenBank and automatic alignment using Mafft ver.5.851 (Katoh *et al*. 2002), followed by analysis in PAUP resulted in a total of 1896 characters including gaps, with 1546 constant characters, 188 variable characters (parsimony-uninformative) and 162 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option resulted in 455 best trees with a consistency index (CI) and retention index (RI) value of 0.886 and 0.902 respectively. Isolates formed a well-resolved clade, supported by a bootstrap value of 100%, separate from any known *Ceratocystis* sp., suggesting that they represent an undescribed species (Figure 1). The closest phylogenetic neighbors of these isolates were *C. bhutanensis* Van Wyk, Wingfield & Kirisits and *C. omanensis* Al-Subhi, M. J. Wingfield, Van Wyk & Deadman.

Selected isolates of the *Ceratocystis* sp. collected from Groenkloof (CMW14276, CMW14278, CMW14280), and representing morphotype one, generated amplicons of about 600, 550 and 900 bp for part of the ITS, β-tubulin and EF-1α gene regions respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.699, suggesting that the data from the three regions could be combined. Comparison of these isolates with those from GenBank and automatic alignment using Mafft ver.5.851, followed by analysis in PAUP resulted in a total of 1955 characters including gaps, with 1550 constant characters, 176 variable characters (parsimony-uninformative) and 229 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option resulted in 511 best trees of which one was retained for representation (Figure 2). The consistency index (CI) and retention index (RI) values were 0.908 and 0.902, respectively. The isolates from Groenkloof formed a well-resolved clade, separate from any known *Ceratocystis* sp. and supported by a bootstrap value of 100%, suggesting that they represent a previously undescribed species. The closest phylogenetic neighbors of these isolates were *C. polychroma* M. Van Wyk, MJ. Wingfield & ECY. Liew and *C. pirilliformis*.

The *Pesotum* and *Ophiostoma* isolates from Groenkloof and Leeuwfontein (CMW14279, CMW20452, CMW20445, CMW20446, CMW20447, CMW14265, CMW17574, CMW17684, CMW17688) for which DNA sequence comparisons were made, produced fragments of approximately 650 bp, using the primers ITS1 and ITS4. Preliminary blast searches suggested that the isolates represent three distinct taxa. Comparison of these isolates with those from GenBank in PAUP resulted in a total of 681 characters including gaps, 302 characters were constant, 20 characters were parsimony-uninformative and 359 characters were parsimony informative.

Phylogenetic analysis using parsimony and the heuristic search option resulted in 767 best trees. Forty-eight of these were retained, of which one was selected for representation (Figure 3). The consistency index (CI) and the retention index (RI) values were 0.782 and 0.957, respectively. Isolates from native trees in South Africa could be separated into four distinct taxa. The first group clustered with strains of *O. quercus*, supported by a bootstrap value of 82%. The second group clustered with *O. tropicale*, supported by a bootstrap value of 99%, while the third group formed a clade close to *P. fragrans*, but clustering with a *P. fragrans*-like isolate with a bootstrap value of 89%. A number of isolates from Leeuwfontein formed a separate fourth clade, clearly separated from other known strains, suggesting that they represent an undescribed species of *Ophiostoma*.

4.4 Pathogenicity tests

Two months after inoculation trees were assessed for disease development based on the length of bark and cambial lesions produced. *C. savannae prov. nom*. and *C tsitsikammensis prov. nom*. produced distinct lesions on the stems of inoculated *A. nigrescens*, *S. birrea* and *R. melanophloeos*, respectively. Some *R. melanophloeos* trees inoculated with *C. tsitsikammensis prov. nom*. produced epicormic shoots below the inoculation points and lesions reached up to 20cm and longer in six weeks. *C. tsitsikammensis prov. nom*. was re-isolated from a large number of trees while re-isolation of *C. savannae prov. nom*. from lesions was not successful. Significant differences ($P<0.0001$) in lesion lengths were found for the two strains of *C. tsitsikammensis prov. nom* as well as *C. savannae prov. nom* when compared to the control inoculations (Figures 4-7).

4.5 TAXONOMY

Based on morphological and DNA sequence comparisons, isolates of the *Ceratocystis* spp. from Groenkloof (CMW14276, CMW14278, CMW14280), Leeuwfontein (CMW17575) and the Kruger National Park (CMW17300, CMW17297, CMW17298) represent two undescribed species distinct from all other described species. Likewise, one of the *Ophiostoma* sp. (CMW14265, CMW17574, CMW17684, CMW17688) isolated from Leeuwfontein represents a new taxon. The following descriptions are, therefore provided for them.

Ceratocystis savannae Kamgan-Nkuekam & Jol. Roux***, prov. nom*.** **(**Figure 8 & 11)

Etymology: Name refers to the Savanna vegetation type in which the trees from which the fungus was collected occur.

Coloniae ad 58 mm diametro in 4 diebus in MEA in 30°C crescentes. Crescit optime in 30°C, in 35°C coloniae ad 30 mm in 4 diebus crescit, infra 5°C non crescit. Mycelium superficiale et inclusum, in agaro tegem grassam formans. *Bases* ascomatum atrobrunneae globosae vel obpyriformes (155-) 181 – 227.3 (-248) µm longae (155-) 178 – 217 (-248) µm latae, cum spinis atris conicis (1.42-) 2.9 – 8.1 (-13.4) µm longis et indumento hyphali. *Colla* ascomatum atrobrunnea (359.6-) 455 – 703 (-775) µm longa, *hyphis ostiolaribus* divergentibus (17-) 24.5 – 39.8 (-46) µm longis cum vinculis ad basin disciformibus, basi (37.2-) 48.2 – 59.3 (-62) µm latis. *Asci* evanescentes, *ascosporae* pileiformes hyalinae non septatae, vaginatae (4.5-) 4.6 - 5.3 (-5.8) × (2.2-) 2.6 - 3.2 (-3.7) µm. Forma anamorpha *Thielaviopsis. Conidiophorae* in mycelio singulae phialidicae hyalinae tubulosae basi leviter incrassatae (15.9-) 19.5 – 31.3 (-52.2) × (2.4-) 2.6 – 3.8 (-5) µm; collulis manifestis (0.59-) 0.98 – 1.97 (-2.7) µm. *Conidia* hyalina non septata biformia, oblonga (3.84-) 4.6 – 5.7 (-6.16) × (2.2-) 2.6 – 3.4 (-3.7) µm et bacilliformia basibus rotundatis (5.35-) 6.04 – 8.1 (-10.27) × (1.71-) 2 – 2.9 (-3.94) µm.

Colonies smoke grey (21''''d), fluffy on MEA, reverse smoke grey (21''''d) almost pale. Colony diameter reaching 58mm in 4 days on MEA at 30ºC. Optimal growth at 30ºC, growth at 35ºC with colony diameter reaching 30mm in 4 days. No growth below 5ºC. Mycelium, forming thick mat on agar. Hyphae smooth, not constricted at septa. *Ascomata* scattered over the surface of the colonies or embedded in mycelium. *Ascomatal* bases dark brown, globose to obpyriform (155-) 181 – 227.3 (-248) µm long and (155-) 178 – 217 (-248) µm wide, with dark and conical spines (1.42-) 2.9 – 8.1 (-13.4) µm and hyphal hair. *Ascomatal* necks dark brown (359.6-) 455 – 703 (-775) µm long, middle of necks (23.9-) 29.9 – 37.1 (-39.3) µm wide, tips of necks (13.1-) 16.2 – 20.9 (-23.6) µm wide, producing sticky and hyaline spore drops at the tips of divergent *ostiolar hyphae* (17-) 24.5 – 39.8 (-46) µm long and with disc-like (disciform) bases (37.2-) 48.2 – 59.3 (-62) µm wide at bases. *Asci* rarely seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (4.5-) 4.6 - 5.3 (-5.8) X (2.2-) 2.6 - 3.2 (-3.7) µm, accumulating in round, straw yellow (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular with a slight swelling at bases (15.9-) 19.5 – 31.3 (-52.2) X (2.4-) 2.6 – 3.8 (-5) µm; colarettes visible (0.59-) 0.98 – 1.97 (-2.7) µm. *Conidia* hyaline, aseptate, two types, oblong (3.84-) 4.6 – 5.7 (-6.16)

X (2.2-) 2.6 – 3.4 (-3.7) µm and bacilliform with rounded bases (5.35-) 6.04 – 8.1 (-10.27) X (1.71-) 2 – 2.9 (-3.94) µm. *Chlamydospores* absent.

Specimen examined: **South Africa,** Mpumalanga Province, Kruger National Park, isolated from wound on *Acacia nigrescens*, 09/02/2005, G. Kamgan Nkuekam, **holotype** PREM 59423, living culture CMW17300, CBS****

Additional specimens: **South Africa**, Mpumalanga Province, Kruger National Park, from wound on *Combretum zeyheri*, 09/02/2005, G. Kamgan Nkuekam, **paratype**, living culture CMW17297, CMW17298, CMW17575.

Ceratocystis tsitsikammensis Kamgan Nkuekam & Jol. Roux, ***prov. nom.*** (Figure 9 & 12)
Etymology: The name describes the Tsitsikamma forests of South Africa (Groenkloof) where this fungus was found. The word <<Tsitsikamma>> comes from the Hottentot words of <<tse-tsesa>> (meaning clear) and << gami>> (meaning water). It is possible that the name Tsitsikamma refers to the clear water in the Tsitsikamma River which runs through the forest.

Crescit optime in 25°C; infra 10°C et supra 30°C non crescit. *Bases* ascomatum nigrae, globosae vel obpyriformes (105-) 129–211 (-279) µm longae (124-) 143–175 (-186) µm latae sine spinis ornamentisque. *Colla* ascomatum nigra (217-) 321–425 (-465) µm longa, hyphis ostiolaribus divergentibus (22.5-) 27.7 – 37.5 (-41.7) µm longis. *Ascosporae* piliformes. Forma anamorpha *Thielaviopsis*, conidiophoris singulis, *conidiis* bacillariformibus (15.4-) 18.3 – 22.7 (-28.5) × (2.6-) 3.3 - 4.5 (-5.5) µm, in catenis factis. *Chlamydosporae* ovoideae laeves, singulae factae, terminales, iuventute hyalinae, maturitate atrescentes (9.91-) 11.27 – 14.01 (-15.61) × (7.49-) 8.57 – 10.78 (-11.95) µm.

Colonies greenish olivaceous (23’’’i) on MEA, reverse grey olivaceous (23’’’’i) almost dark coloured. Colony diameter reaching 19 mm in 10 days on MEA at 25ºC. Optimal growth at 25ºC, no growth below 10ºC and above 30ºC. Colonies surfaces scattered with black coloured ascomata. Mycelium immersed and superficial, producing white-grey aerial mycelium. Hyphae smooth, not constricted at septa. *Ascomatal* bases black, globose to obpyriform (105-) 129–211 (-279) µm long and (124-) 143–175 (-186) µm wide. Spines or ornamentations absent. *Ascomatal* necks black (217-

) 321–425 (-465) µm long, bottom of necks smooth (31-) 32.1 – 47.1(-62) µm wide, middle of necks (23.4-) 25.3 – 29.7 (-32.5) µm wide, tips of necks (14.3-) 16.75 – 20.9 (-23.4) µm wide. *Ostiolar hyphae* present, divergent (22.5-) 27.7 – 37.5 (-41.7) µm long. *Asci* evanescent. *Ascospores* hat-shaped, invested in sheath, aseptate (4.4-) 5 - 6.3 (-6.7) µm long and (2.4-) 3.1 – 4 (-4.5) µm wide. Ascospores accumulating in round, hyaline spore drops when fresh, turning pale luteous (19d) when old.

Anamorph state: *Thielaviopsis*. *Conidiophores* occurring singly, phialidic (34.2-) 46.9 – 110.2 (-162.1) X (3.6-) 4.4 – 6.7 (-8.7) µm, tubular with slight thin bases making them almost constricted at septa, hyaline, colarettes absent. *Conidia* bacilliform-shaped (15.4-) 18.3 – 22.7 (-28.5) X (2.6-) 3.3 - 4.5 (-5.5) µm produced in chains. *Chlamydospores* (aleuroconidia) ovoid, smooth, formed singly, terminal, hyaline when young, becoming dark when mature (9.91-) 11.27 – 14.01 (-15.61) X (7.49-) 8.57 – 10.78 (-11.95) µm.

Specimens examined: **South Africa**, Groenkloof. Isolated from wounds on *Rapanea melanophloeos*, 28/01/2005, G. Kamgan Nkuekam, **holotype** PREM 59424, living culture CMW14276/CBS****

Additional specimens: **South Africa**, Groenkloof. Isolated from wounds on *Rapanea melanophloeos*, 28/01/2005, G. Kamgan Nkuekam, **paratype**, living culture CMW14278, CMW14280, CMW14274.

Ophiostoma longiconidiatum Kamgan-Nkuekam, Jacobs & Jol. Roux, ***prov. nom.*** (Figure 10 & 13)
Etymology: The name refers to the long conidia found in the anamorph state.

Coloniae ad 20 mm diametro in 10 diebus in MEA in 25°C crescunt; infra 10°C et supra 30°C non crescunt. *Ascomata* in annulis concentricis in mediis artefactis dispositis, guttas hyalinas mucilagineas sporarum in apicibus collorum ascomatum facientes. *Colla* ascomatum atrobrunnea (279-) 352 – 698 (-868) µm longa saepe cum annulis singulis. Bases ascomatum globosae (267-) 415 – 797 (-992) µm longae (62-) 74 – 115 (-155) µm latae, laete flavescentes, sine ornamentis. Basis colli laevis (31-) 30 - 43 (-50) µm lata. *Hyphae ostiolares* desunt. *Asci* evanescentes, in evolutione praecoque deliquescentes. *Ascosporae* allantoideae non septatae hyalinae (3-) 3.5 – 4 (-4.4) × (1.09-) 1.1 - 1.4 (-1.6) µm. Anamorpha *Sporothrix, conidiophoris* tubulosis hyalinis (3.7-) 5.5–9.3 (-10.8) X (0.8-) 0.9–1.3 (-1.6) µm. Conidia oblonga vel cylindrica basibus rotundatis obtusis hyalinis (6.3-) 7.7–15.9 (-21) × (1.5-) 1.8–2.4 (-2.8) µm.

Colonies pale mouse grey (15'''''d) almost brown, fluffy on MEA. Reverse mouse grey (15'''''I). Colony diameter reaching 20mm in 10 days on MEA at 25°C. Optimal growth at 25°C. No growth below10°C and above 30°C. *Ascomata* arranged in concentric rings on agar surface producing hyaline, slimy spore drops at the necks apices. *Ascomatal* necks dark brown (279-) 352 – 698 (-868) µm long, often with single annuli. Ascomatal bases globose (267-) 415 – 797 (-992) µm long and (62-) 74 – 115 (-155) µm wide, light-yellowish without ornamentations. Neck base smooth, (31-) 30 - 43 (-50) µm wide. *Ostiolar hyphae* absent. *Asci* rarely seen, evanescent, deliquescing early in the development. *Ascospores* allantoid, aseptate, hyaline (3-) 3.5 – 4 (-4.4) x (1.09-) 1.1 - 1.4 (-1.6) µm.

Anamorph: *Sporothrix,* conidiophores, hyaline, cylindrical tapering towards the apex, (3.7-) 5.5–9.3 (-10.8) X (0.8-) 0.9–1.3 (-1.6) µm, prominent denticles present. Conidia, aseptate, hyaline, oblong, occasionally acerose, proximal end distinctly foot-shaped in some cases (6.3-) 7.7–15.9 (-21) X (1.5-) 1.8–2.4 (-2.8) µm.

Specimens examined: **South Africa**, Gauteng Province, Leeuwfontein Collaborative Nature Reserve, isolated from wound on *Terminalia sericea*,03/02/2004, G. Kamgan Nkuekam, **holotype** PREM 59425, living culture CMW17574, CBS****
Additional specimens: **South Africa**, Gauteng Province, Leuwfontein Collaborative Nature Reserve, from wound on *Terminalia sericea*, 03/02/2004, G. Kamgan Nkuekam, **paratype**, living culture CMW14265.

5.0 DISCUSSION

Three new fungal species from native South African trees were discovered in this study. Two of these were species of *Ceratocystis* and one is a new *Ophiostoma* sp., for which the names *C. tsitsikammensis prov. nom.*, *C. savannae prov. nom*. and *O. longiconidiatum prov. nom*. have been provided. In addition to these new species, *O. quercus*, *O. tropicale* and a fungus similar to *P.*

fragrans were found. Of the isolated fungi *C. tsitsikammensis prov. nom*. displayed a high level of pathogenicity in inoculation trials on *R. melanophloeos* and it could be an important pathogen.

The genus *Ceratocystis* as it currently stands, represents an aggregate genus that includes species in very distinct monophyletic lineages (www.fabinet.up.ac.za/ophiostoma/abstracts, BD. Wingfield *et al*. 2006). Species of *Ceratocystis* as they are currently treated, reside in two large phylogenetic groups. One of these accommodates *C. fimbriata* and many related species that have hat-shaped ascospores and of which many are important pathogens (Witthuhn *et al.* 1999, Wingfield *et al.* 2006). The other clade includes *C. coerulescens* and its relatives (Witthuhn *et al.* 1999, Wingfield *et al*. 2006). The latter group might be further sub-divided (BD. Wingfield *et al*. 2006, www.fabinet.up.ac.za/ophiostoma/abstracts) and includes species related to *C. coerulescens* (Withuhn *et al*. 1999) and *C. moniliformis sensu lato* (Van Wyk *et al* 2006). *Ceratocystis tsitsikammensis prov. nom*., one of the new species discovered in this study was most closely related to *C. fimbriata sensu lato. C. savannae prov. nom*., the other new species of *Ceratocystis* , was most closely related to *C. moniliformis sensu lato*.

C. tsitsikammensis prov. nom. resembles species in the *C. fimbriata sensu lato* clade, producing hat shaped ascospores and with a colony morphology very similar to that of *C. pirilliformis* and *C. fimbriata sensu stricto.* This new species can be distinguished from others in the *C. fimbriata sensu lato* clade, based on a number of morphological characteristics. It differs from *C. fimbriata* and *C. pirilliformis* in that it produces divergent ostiolar hyphae. Furthermore, *C. pirilliformis* has distinct pear-shaped ascomatal bases, different to the globose bases of other species in this group (Barnes *et al.* 2003). *Ceratocystis tsitsikammensis prov. nom*. differs from *C. polychroma* in that its ascomata are smaller (217 – 465 µm long) than those of the latter species (837 – 1187 µm long). *Ceratocystis polychroma* also grows much faster (90mm/16d at 25°C) than *C. tsitsikammensis prov. nom*. (19mm/10d at 25°C), and the conidiophores of *C. tsitsikammensis prov. nom*. are tubular to almost obpyriform, while those of *C. polychroma* are cylindrical. Also, *C. pirilliformis* and *C. polychroma* produce two types of conidiophores and two types of conidia (Barnes *et al.* 2003, Van Wyk *et al*. 2004a), in contrast to *C. tsitsikammensis prov. nom*. and *C. fimbriata* (Baker Engelbrecht & Harrington 2005) that produces only one type of conidiophore and one conidia form.

Multiple T rich regions were present in DNA sequence data for the ITS and 5.8S gene regions of *C. tsitsikammensis prov. nom*. This also serves to confirm that this fungus represents a species distinct from any other species in the larger *C. fimbriata* clade. For phylogenetic analyses we used sequences for the ITS, β-tubulin and EF-1α gene regions. Comparisons of sequences for these regions showed that *C. tsitsikammensis prov. nom*. is different from morphologically similar *Ceratocystis* spp. *C. tsitsikammensis prov. nom*. falls within a clade comprising *C. fimbriata*, *C. pirilliformis* and *C. polychroma*. However, within this group, *C. tsitsikammensis prov. nom*. forms a well-resolved clade clearly separated from the other three taxa. The closest phylogenetic neighbor of *C. tsitsikammensis* in a combined tree is *C. polychroma*, while in non-combined trees, its closest phylogenetic neighbor was a strain of *C. fimbriata sensu stricto* from Papua New Guinea.

Ceratocystis savannae prov. nom., described for the first time in this study, is morphologically similar to species in the *C. moniliformis* complex, within the larger *C. coerulescens* clade (Witthuhn *et al*. 1999) of *Ceratocystis*. Species in the *C. moniliformis* complex are morphologically similar to each other with hat-shaped ascospores, a disk shaped attachment point at the bases of the ascomatal necks, short conical spines on the ascomatal bases and the production of both cylindrical and barrel shaped conidia (Al-Subhi *et al*. 2006, Van Wyk *et al*. 2004b, Van Wyk *et al*. 2006, Yuan & Mohammed 2002). Other than *C. moniliformis* Hedgcock (Hedgcock 1906), this complex includes *C. bhutanensis* Van Wyk, Wingfield & Kirisits (Van Wyk *et al*. 2004b), *C. moniliformopsis* (Yuan & Mohammed 2002), *C. omanensis* Al-Subhi, M. J. Wingfield, Van Wyk & Deadman (Al-Subhi *et al*. 2006) and *C. tribiliformis* Van Wyk & Wingfield (Van Wyk *et al*. 2006). Based on phylogenetic comparisons *C. savannae prov. nom*. is different from other species in the *C. moniliformis* complex, residing in a separate clade and most closely related to *C. bhutanensis* and *C. omanensis* (97% bootstrap value).

Ceratocystis savannae prov. nom. is most closely related to *C. bhutanensis* and *C. omanensis* based on DNA sequence comparisons. It can however be distinguished from these species by a few phenotypic traits. *Ceratocystis savannae prov. nom*. produces tubular phialides while *C. omanensis* and *C. tribiliformis* produce phialides with characteristically swollen bases (Al-Subhi *et al*. 2006, Van Wyk *et al*. 2006). Colonies of *C. savannae prov. nom*. are a smokey grey colour while those of *C. omanensis* and *C. tribiliformis* are white to wood-brown (Al-Subhi *et al*. 2006, Van Wyk *et al*. 2006). The mycelia of *C. tribiliformis* is nearly embedded in agar, not as abundant and fluffy as *C.*

savannae prov. nom. *C. savannae prov. nom*. differs from *C. bhutanensis* in that it has globose to obpyriform ascomatal bases, oblong and bacilliform conidia, and smoke grey colonies while *C. bhutanensis* has globose ascomatal bases, cylindrical and barrel-shaped conidia and cream-buff to dark olive colonies. Among species in the *C. moniliformis* complex, these morphological differences are not always reliable characteristics for identification, therefore, comparison of identities based on DNA sequence comparisons are important.

Isolation of *C. albifundus* from wounds on native hardwood trees in this study was not surprising. The fungus has previously been reported from many native tree genera in South Africa (Roux *et al*. 2004b, Roux *et al*. 2007). *C. albifundus* is well-known as a pathogen of non-native *A. mearnsii* trees in South Africa (Morris *et al.* 1993, Roux & Wingfield 1997, Wingfield *et al.* 1996). Its occurrence in the the Kruger National Park, on native trees in isolation from non-native hosts such as *A. mearnsii*, supports the view that the fungus is most likely native to South Africa (Barnes *et al*. 2005, Roux *et al*. 2001b).

Four *Ophiostoma* spp. were collected from native hardwood tree species in this study. These included three known species and the newly described *O. longiconidiatum prov. nom*. This newly described species is closely related to species in the *O. pluriannulatum* complex, most commonly *O. pluriannulatum*, *O. multiannulatum* (Hedgcock & Davidson) N. Fries and *O. subannulatum* Livingston & RW. Davisdon, based on DNA sequence comparisons. Based on morphology, *O. longiconidiatum* can, however, be distinguished from other species in the complex by its light coloured ascomatal bases and the lack of ostiolar hyphae. Also, the ascomatal necks of *O. longiconidiatum prov. nom*. are much shorter (279 - 868μm) than those of other species in the *O.pluriannulatum* complex.

Ophiostoma tropicale, *O. quercus* and a species resembling *P. fragrans* were also found in this study. It was not surprising to collect *O. tropicale*. The fungus was first described in South Africa from discoloured hardwood material collected from three countries including South Africa, Ecuador and Indonesia (De Beer 2001). In South Africa, the fungus was first reported from *Ocotea bullata* (native), *Macaranga capensis* (Baill.) Benth. Ex Sim (native), *Jacaranda mimosifolia* D. Don (non-native), and a non-native *Eucalyptus grandis* Hill Maiden tree (De Beer 2001). However, strains of *O. tropicale* reported in this paper were isolated from two native hardwood tree species including *F.*

saligna and *T. sericea*. This has increased the host and geographic range (Groenkloof, Western Cape) of the fungus in South Africa as previous reports were from the Mpumalanga Province, the Southern Cape Province and Kwazulu-Natal (De Beer 2001).

Pesotum fragrans was first described in Sweden from the galleries of *Ips sexdentatus* Boerner, infesting *Pinus sylvestris* (Mathiesen & Käärik, 1953). The fungus was later also reported from Australia, California, Canada and New Zealand (Harrington *et al*. 2001, Jacobs *et al*. 2003). In South Africa, *P. fragrans* were first reported from *Pinus patula* Schiede Schlecht. & Cham. where it was consistently isolated from an introduced conifer-infesting bark beetle *Hylastes angustatus* Herdst. (Zhou *et al*. 2006). In this study we report strains of a *P. fragrans*-like isolate that were isolated from *R. melanophloeos* and *Rhus chirendensis*. These isolates grouped with a *P. fragrans*-like reference strain in a major clade that also includes the true *P. fragrans*. More work will be required to clarify the species delimitation for these isolates.

It was not surprising to find *O. quercus* on native hardwood trees in this study. The fungus was previously reported from South Africa on native *Olinia* sp. and on non-native *E. grandis* and *Quercus robur* L. (De Beer *et al*. 1995). Its occurrence on wounds on *R. melanophloeos* and *T. sericea* in this study has expanded the host range of the fungus in South Africa. *Ophiostoma quercus* has a cosmopolitan distribution on hardwoods and is found both in the northern and southern Hemisphere (Brasier & Kirk 1993, Halmschlager *et al*. 1994, Kim *et al*. 1999, Morelet 1992, Pipe *et al*. 1995, Harrington *et al*. 2001, De Beer *et al*. 2003). Its occurrence in South Africa, on a number of native tree species further expands the already wide host range of this fungus. Hypotheses regarding its origin include both the northern and southern Hemisphere (Brasier & Kirk 1993, Harrington *et al*. 2001, De Beer *et al*. 2003) but additional research is required to clarify this question. Nonetheless, studies such as the one presented here greatly contributes to unravelling these questions.

Inoculation studies with *C. savannae prov. nom.* showed that this fungus can cause small lesions on young *A. nigrescens* and *S. birrea* trees. This is likely a native fungus with a very low level of pathogenicity. However, pathogenicity tests conducted with strains of *C. tsitsikammensis prov. nom* have showed that it is highly pathogenic. Artificial inoculation of *R. melanophloeos* trees resulted in severe lesions in both the bark and the xylem within eight weeks after inoculation. At the time when the lesion lengths were recorded, many trees had developed epicormic shoots below the inoculation points as a result of stem-girdling. *Ceratocystis tsitsikammensis prov. nom* was also consistently re-isolated from the lesions. Additional studies in the field are required to determine the impact of this fungus under natural conditions.

This study represents the most comprehensive consideration of *Ceratocystis* and *Ophiostoma* species on native hardwood trees in Africa ever to have been undertaken. The number of new taxa encountered, clearly emphasizes the importance of expanding these surveys to include additional tree species and a wider geographic area within South Africa and the rest of the African continent. The high level of pathogenicity found in *C. tsitsikammensis*, and that of the better-known *C. albifundus* also supports the view that the group includes important pathogens that have yet to be discovered.

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Table 1: List of *Ceratocystis* isolates and their accession numbers used for DNA sequence comparisons.

Species	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
C. albifundus	CMW5329	AF388947	ITS	NA	*A. mearnsii*	J. Roux	Uganda
	CMW4068	DQ520638	ITS	NA	"	J. Roux	South Africa
	CMW5364	DQ371650	BT	NA	*A. mearnsii*	J. Roux	South Africa
		AY528977	EF				
	CMW2473	DQ371648	BT	NA	*Acacia dealbata*	M. Morris	South Africa
		AY528976	EF				
C. bhutanensis	CMW8399	AY528959	ITS	CBS115772, BH 8/8	*Picea spinulosa*	T. Kirisit & Dal Bahadur Chhetri	Bhutan
		AY528964	BT				
		AY528954	EF				
	CMW8215	AY528958	ITS	CBS114290, PREM57805	*Picea spinulosa*	T. Kirisit & Dal Bahadur Chhetri	Bhutan
		AY528963	BT				
		AY528953	EF				
C. fimbriata	CMW1547	AF264904	ITS	NA	*Ipomoea batatas*	NA	Papua N. Guinea
		NA	BT	NA			
		NA	EF	NA			
	CMW15049	DQ520629	ITS	CBS141.37	*I. batatas*	CF. Andrus	USA
		NA	BT				
		NA	EF				
C. moniliformis	CMW9590	AY431101	ITS	CBS116452	*E. grandis*	J. Roux	South Africa
		AY528985	BT				
		AY529006	EF				
	CMW8379	AY528995	ITS	NA	*Cassia fistula*	MJ. Wingfield	Bhutan
		AY529005	BT				
		AY529016	EF				
	CMW8240	AY529000	ITS	NA	*C. istula*	MJ. Wingfield, T. Kirisit & Dal Bahadur Chhetri	Bhutan
		AY528989	BT				
		AY529010	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. 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. pirilliformis	CMW6569	AF427104	ITS	PREM57322, DAR75993	*E. nitens*	M.J. Wingfield	Australia
		DQ371652	BT				
		AY528982	EF				
	CMW6579	AF427105	ITS	PREM57323, DAR75996	*E. nitens*	M.J. Wingfield	Australia

		DQ371653	BT				
		AY528983	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. savannae	*CMW17300	EF408551	ITS	PREM59423	*Acacia nigrescens*	G. Kamgan & J. Roux	South Africa
		EF408565	BT				
		EF408572	EF				
	*CMW17297	EF408552	ITS	NA	*Combretum zeyheri*	G. Kamgan & J. Roux	South Africa
		EF408566	BT				
		EF408573	EF				
	*CMW17298	EF408553	ITS	NA	*T. sericea*	G. Kamgan & J. Roux	South Africa
		EF408567	BT				
		EF408574	EF				
	*CMW17575	EF408554	ITS	NA	*T. sericea*	G. Kamgan & J. Roux	South Africa
		EF408568	BT				
		EF408575	EF				
C. tribiliformis	CMW13015	AY529004	ITS	CBS115949	*Pinus mercusii*	MJ. Wingfield	Indonesia
		AY528994	BT				
		AY529015	EF				
	CMW13013	AY529003	ITS	CBS115866	*P. mercusii*	MJ. Wingfield	Indonesia
		AY528993	BT				
		AY529014	EF				
C. tsitsikammensis	*CMW14276	EF408555	ITS	PREM59424	*Rapanea melanophloeos*	G. Kamgan & J. Roux	South Africa
		EF408569	BT				
		EF408576	EF				
	*CMW14278	EF408556	ITS	NA	*R. melanophloeos*	G. Kamgan & J. Roux	South Africa
		EF408570	BT				
		EF408577	EF				
	*CMW14280	EF408557	ITS	NA	*Ocotea bullata*	G. Kamgan & J. Roux	South Africa
		EF408571	BT				
		EF408578	EF				
C. virescens	CMW3276	DQ061281	ITS	NA	*Quercus* sp.	T. Hinds	USA
		AY528990	BT				
		AY529011	EF				

* Isolates sequenced for this study

Table 2: List if *Ophiostoma* isolates and their accession numbers used for DNA sequence comparisons.

Species	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
O. arduennense	NA	AY573242	ITS	NA	NA	NA	NA
	NA	AY573247	ITS	NA	NA	NA	NA
O. floccosum	C1086	AF198231	ITS	CBS799.73	NA	A. Käärik	Sweden
	CMW7661	AF493253	ITS	NA	*Pinus elliottii*	ZW. de Beer	South Africa
O. himal-ulmi	C1183	AF198233	ITS	CBS374.67; ATCC36176; ATCC36204	*Ulmus* sp.	HM. Heybroek	India
	C1306	AF198234	ITS	HP27	*Ulmus* sp.	CM. Brasier	India
O. kryptum	NA	AY304434	ITS	DAOM229702 (IFFFBW/1)	*Larix decidua*	T. Kirisits & MJ. Wingfield	Austria
	NA	AY304437	ITS	IFFFHasd/1	*L. decidua*	T. Kirisits & MJ. Wingfield	"
O. longiconidiatum	CMW17574	EF408558	ITS	NA	*T. sericea*	G. Kamgan & J. Roux	South Africa
	CMW14265	EF408560	ITS	NA	*Faurea saligna*	G. Kamgan & J. Roux	"
	CMW17688	EF408559	ITS	NA	*T. sericea*	G. Kamgan & J. Roux	"
	CMW17684	EF408561	ITS	NA	*F. saligna*	G. Kamgan & J. Roux	"
O. multiannulatum	NA	CBS357.77	ITS	NA	NA	NA	NA
	NA	AY934512	ITS	CBS124.39	NA	NA	NA
O. novo-ulmi	C510	AF198236	ITS	NA	*Ulmus* sp.	NA	Iowa, USA
	C1185	AF198235	ITS	CBS298.87; WCS637	*Ulmus* sp.	H. M. Heybroek	Russia
O. piceae	NA	AF198226	ITS	C1087; CBS108.21	*Abies or Picea*	E. Münch	Germany
	CMW7648	AF493249	ITS	C967; H2181	*Picea sitchensis*	DB. Redfern & JF. Webber	United Kingdom
O. piliferum	NA	AF221070	ITS	CBS129.32	*Pinus sylvestris*	H. Diddens	"
	NA	AF221071	ITS	NA	NA	NA	NA
O. pluriannulatum	NA	AY934517	ITS	MUCL18372	NA	NA	USA
	C1567	DQ062972	ITS	UAMH9559; WIN(M)869	*Podocarpus* sp.	Reid	New Zealand
O. quercus	CMW7656	AF493250	ITS	NA	*Q. robur*	MJ. Wingfield	South Africa
	CMW2520	AF493241	ITS	NA	NA	NA	NA
	CMW7658	AF493251	ITS	NA	NA	NA	NA
	CMW3119	AF493244	ITS	NA	NA	NA	NA
	CMW2534	AF493242	ITS	NA	NA	NA	NA
	CMW7645	AF493246	ITS	W3; HA367	*Q. robur*	T. Kirisits & E. Halmschlager	Austria
	CMW7650	AF198238	ITS	C969; CBS102352; H1042	*Quercus* sp.	PT. Scard & JF. Webber	United Kingdom
	*CMW17573	EF408562	ITS	NA	*T. sericea*	G. Kamgan & J. Roux	South Africa
	*CMW20452	EF408563	ITS	NA	*R. melanophloeos*	J. Roux	"
	*CMW14279	EF408564	ITS	NA	*R. melanophloeos*	G. Kamgan & J. Roux	"
O. subannulatum	NA	AY934522	ITS	CBS188.86	NA	NA	NA
	NA	NA	ITS	CBS118667	NA	NA	NA
O. tropicale	CMW1251	NA	ITS	NA	*E. grandis*	MJ. Wingfield	South Africa
	CMW368	NA	ITS	NA	*O. bullata*	MJ. Wingfield	"
	CMW4026	NA	ITS	NA	Indigenous hardwood	ZW. de Beer	Indonesia
	*CMW20445	NA	NA	NA	*R. melanophloeos*	J. Roux	South Africa
	*CMW20446	NA	NA	NA	*R. melanophloeos*	J. Roux	"
	*CMW20447	NA	NA	NA	*O. bullata*	J. Roux	"

O. ulmi	C1182	AF198232	ITS	CBS102.63; IMI101223; JCM9303	*Ulmus* sp.	WF. Holmes & HM. Heybroek	Netherlands
P. fragrans	NA	AF198248	ITS	CBS279.54	*P. sylvestris*	A. Mathiesen-Käärik	Sweden
	NA	AY194518	ITS	NA	NA	NA	NA
	NA	DQ396790	ITS	NA	NA	NA	NA
P. fragrans-like	NA	DQ062977	ITS	NA	NA	NA	NA
	*CMW20673	NA	NA	NA	*R. melanophloeos*	J. Roux	South Africa
	*CMW20671	NA	NA	NA	*Rhus chirendensis*	J. Roux	"

* Isolates sequenced for this study

Table 3: List of isolates collected in this study

Species	Isolate number	Hosts	Area	Collectors
Ceratocystis albifundus	CMW14287	*Terminalia sericea*	Kruger National Park	G. Kamgan & J. Roux
	CMW14289	*T. sericea*	Kruger National Park	G. Kamgan & J. Roux
	CMW14288	*Combretum zeyheri*	Kruger National Park	G. Kamgan & J. Roux
	CMW14290	*C. zeyheri*	Kruger National Park	G. Kamgan & J. Roux
C. savannae	CMW17300	*Acacia nigrescens*	Kruger National Park	G. Kamgan & J. Roux
	CMW17297	*C. zeyheri*	Kruger National Park	G. Kamgan & J. Roux
	CMW17298	*T. sericea*	Kruger National Park	G. Kamgan & J. Roux
	CMW17301	*Sclerocarya birrea*	Kruger National Park	G. Kamgan & J. Roux
	CMW17306	*S. birrea*	Kruger National Park	G. Kamgan & J. Roux
	CMW17302	*S. birrea*	Kruger National Park	G. Kamgan & J. Roux
	CMW17575	*T. sericea*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW17576	*Burkea africana*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
C. tsitsikammensis	CMW14276	*Rapanea melanophloeos*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
	CMW14278	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
	CMW14280	*Ocotea bullata*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
	CMW14275	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
	CMW14274	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
	CMW13981	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
	CMW13982	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
O. longiconidiatum	CMW17574	*T. sericea*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW14265	*Faurea saligna*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW17688	*T. sericea*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW17684	*F. saligna*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW17685	*F. saligna*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW17689	*F. saligna*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
O. quercus	CMW17573	*T. sericea*	Leeuwfontein Collaborative Nature Reserve	G. Kamgan & J. Roux
	CMW20452	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
	CMW14279	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	G. Kamgan & J. Roux
O. tropicale	CMW20445	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
	CMW20446	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
	CMW20447	*O. bullata*	Groenkloof Forest, Tsitsikamma	J. Roux
P. fragrans-like	CMW20673	*R. melanophloeos*	Groenkloof Forest, Tsitsikamma	J. Roux
	CMW20671	*Rhus chirendensis*	Groenkloof Forest, Tsitsikamma	J. Roux

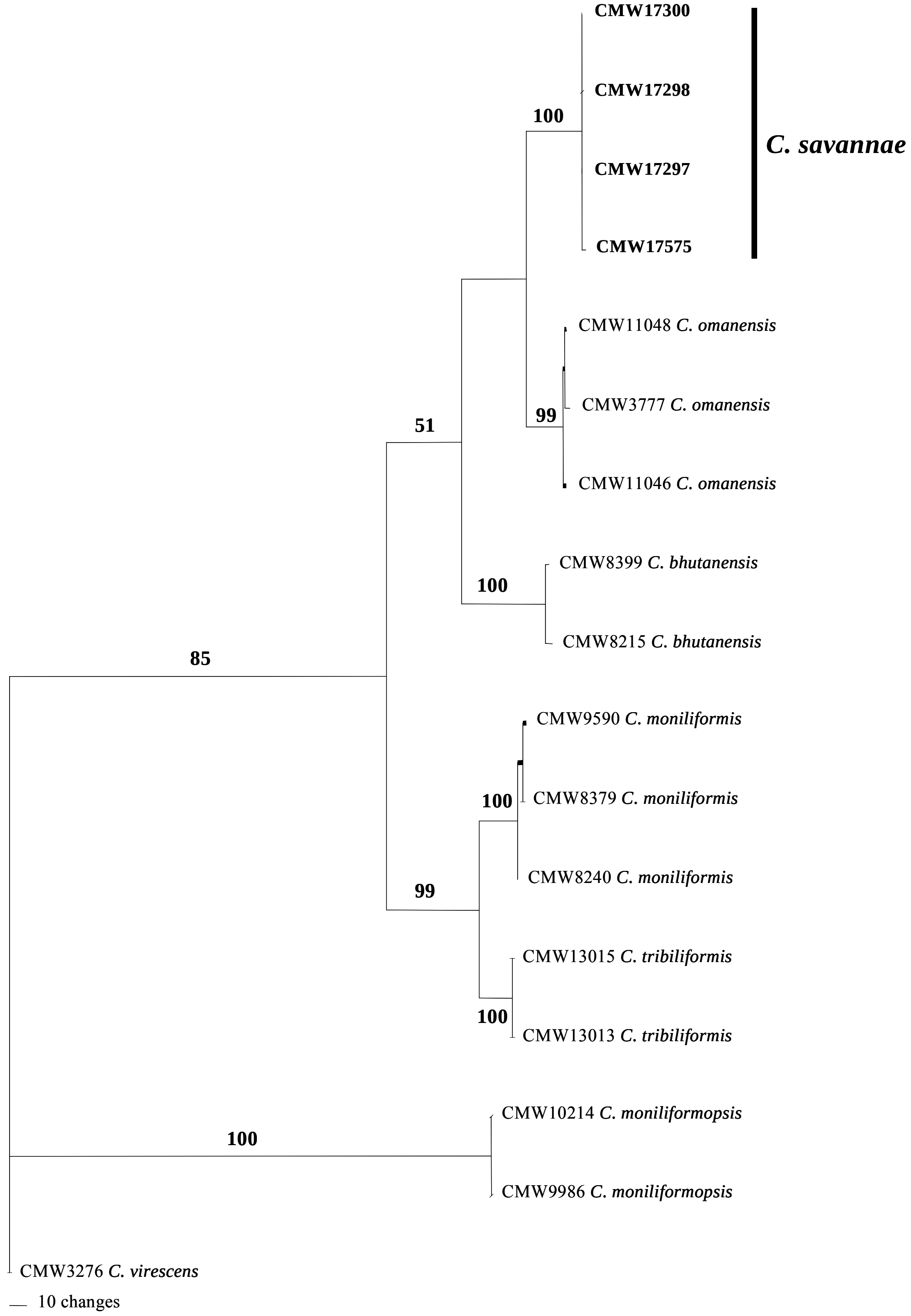

Figure 1: Phylogenetic tree produced from a heuristic search of the combined ITS, β-tubulin and Elongation factor-1α sequence data, showing the relationship between *C. savannae prov. nom* from native tree species in the Kruger National Park and Leeuwfontein Collaborative Nature Reserve and other *Ceratocystis* 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.

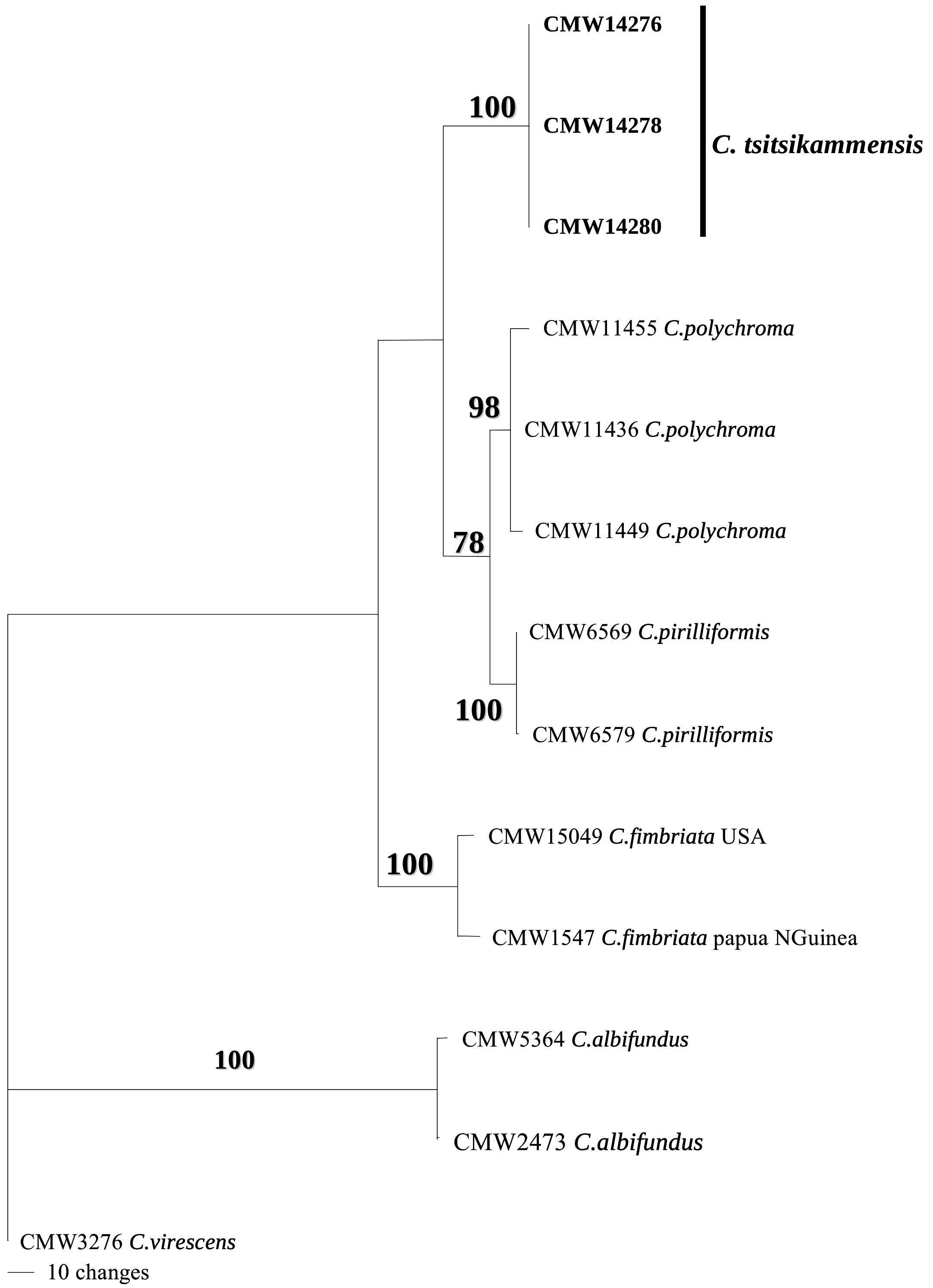

Figure 2: Phylogenetic tree produced from a heuristic search of the combined ITS, β-tubilin and Elongation factor-1α sequence data, showing the relationship between *C. tsitsikammensis prov. nom* from native tree species in Groenkloof and other *Ceratocystis* spp. resembling *C. fimbriata*. *C. virescens* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade.

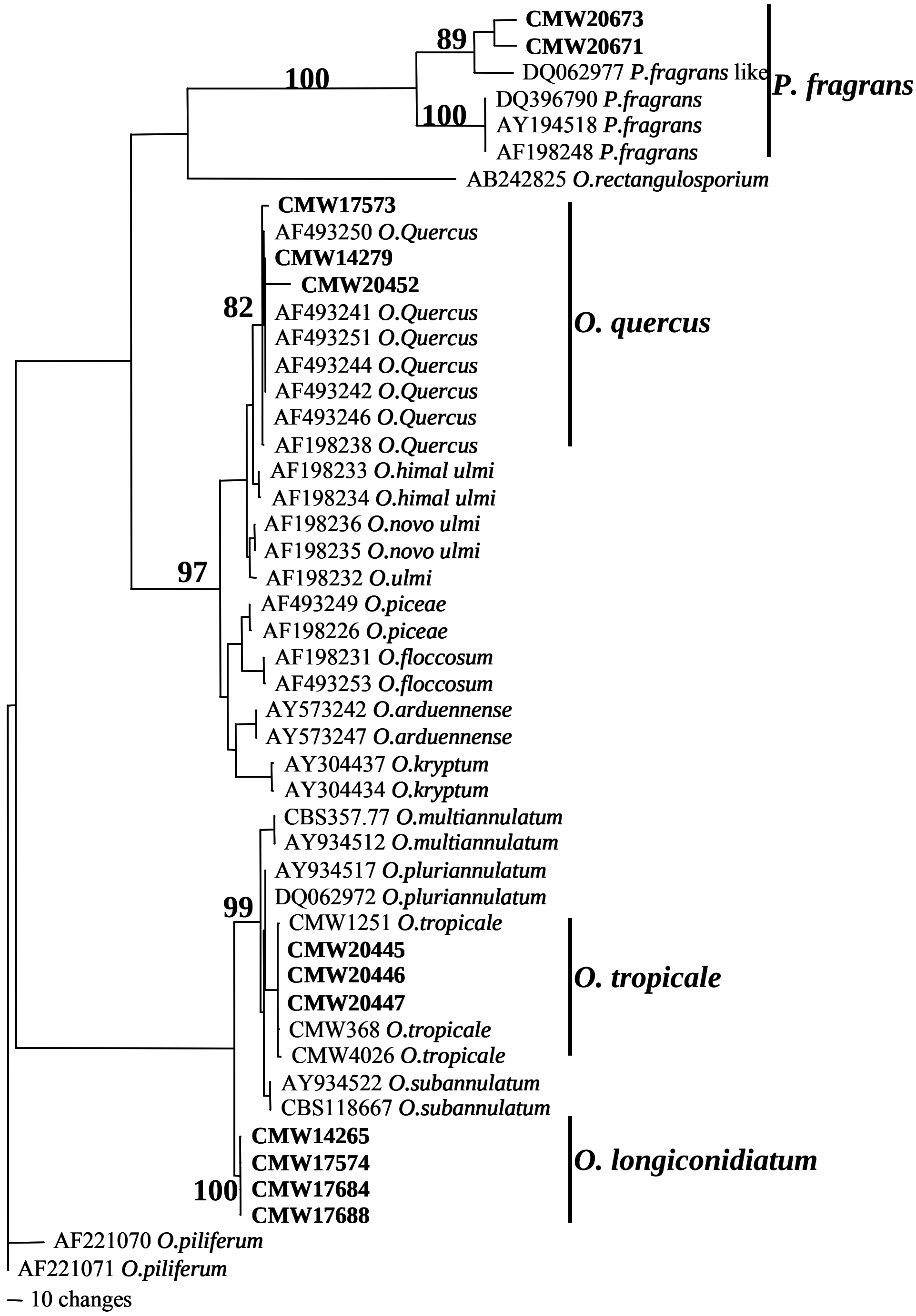

Figure 3: Phylogenetic tree produced from a heuristic search of the ITS sequence data. *Ophiostoma piliferum* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade.

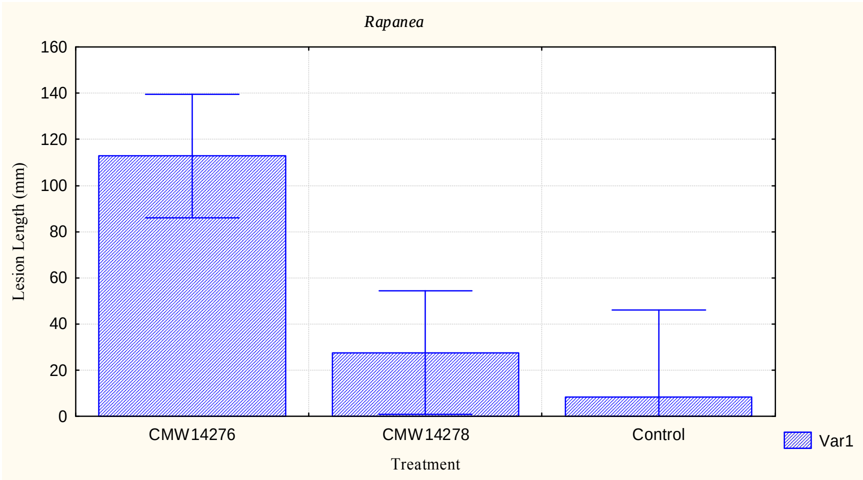

Figure 4: Histogram showing results of 1st inoculation trial (bark lesion) with *C. tsitsikamensis* (CMW14276, 14278) on *R. melanophloeos* trees. Lsmean = 57.82, R = 0.38, CV = 102.58, P<0.0001, Confidence limit = 95%. Average lesion lengths (27.6 – 112.75) mm

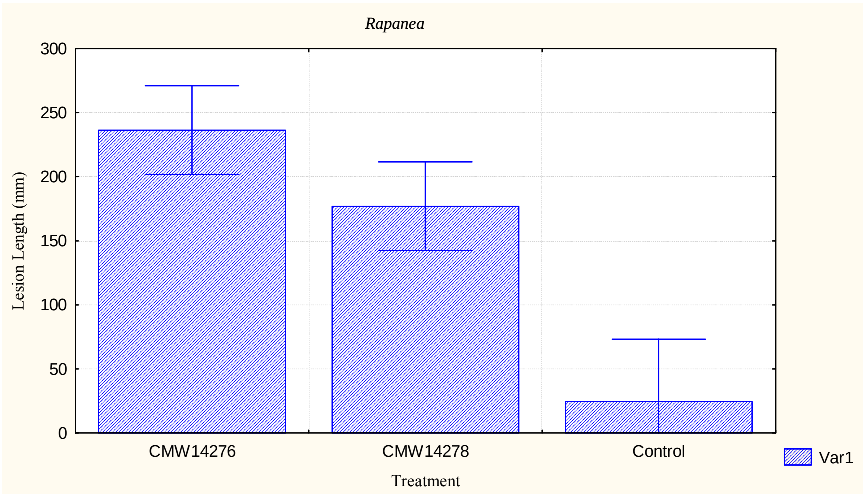

Figure 5: Histogram showing results of 2nd inoculation trial (xylem lesion) with *C. tsitsikamensis* (CMW14276, 14278) on *R. melanophloeos* trees. Lsmean = 170.26, R = 0.52, CV = 45.07, P<0.0001, Confidence limit = 95%. Average lesion lengths (177 – 236.4) mm

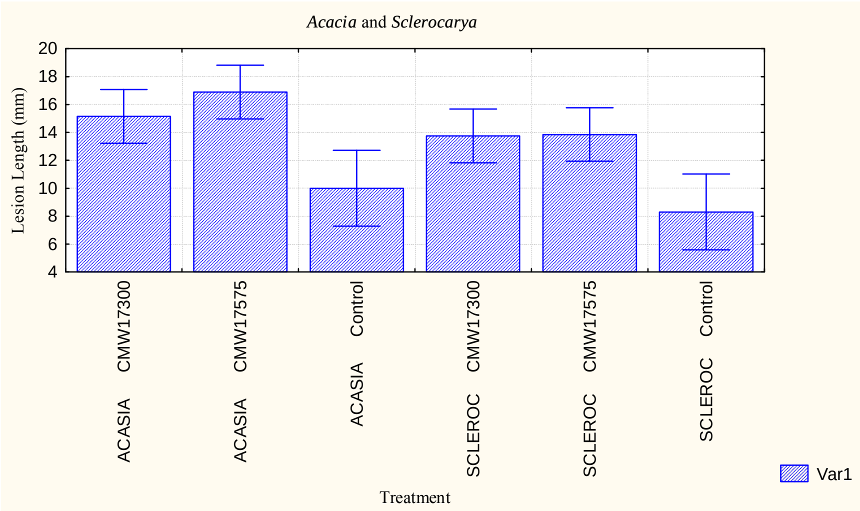

Figure 6: Histogram showing results of 1st inoculation trial (bark lesion) with *C. savannae* (CMW17300, 17575) on *A. nigrescens* and *S. birrea* trees. Lsmean = 13.7, R = 0.27, CV = 31.4, P<0.0001, Confidence limit = 95%. Average lesion lengths (13.75 – 15.35) mm

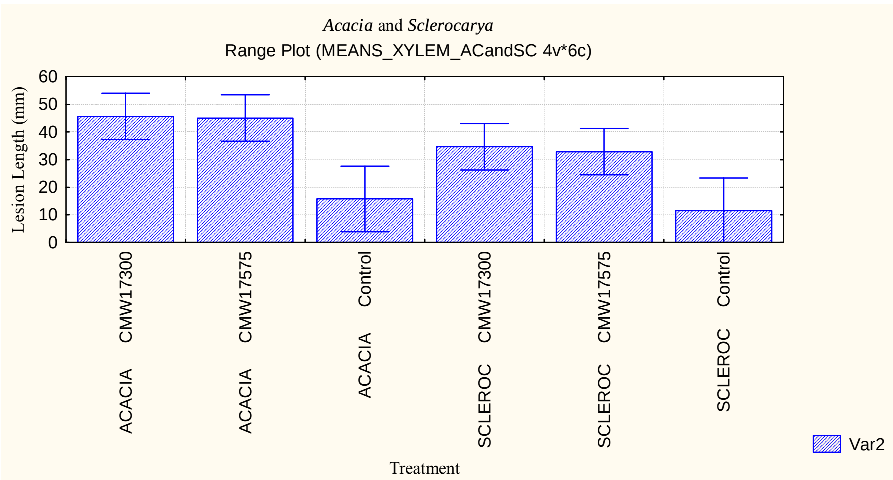

Figure 7: Histogram showing results of 2nd inoculation trial (xylem lesion) with *C. savannae* (CMW17300, 17575) on *A. nigrescens* and *S. birrea* trees. Lsmean = 34.37, R = 0.28, CV = 55.07, P<0.0001, Confidence limit = 95%. Average lesion lengths (34.7 – 45.65) mm

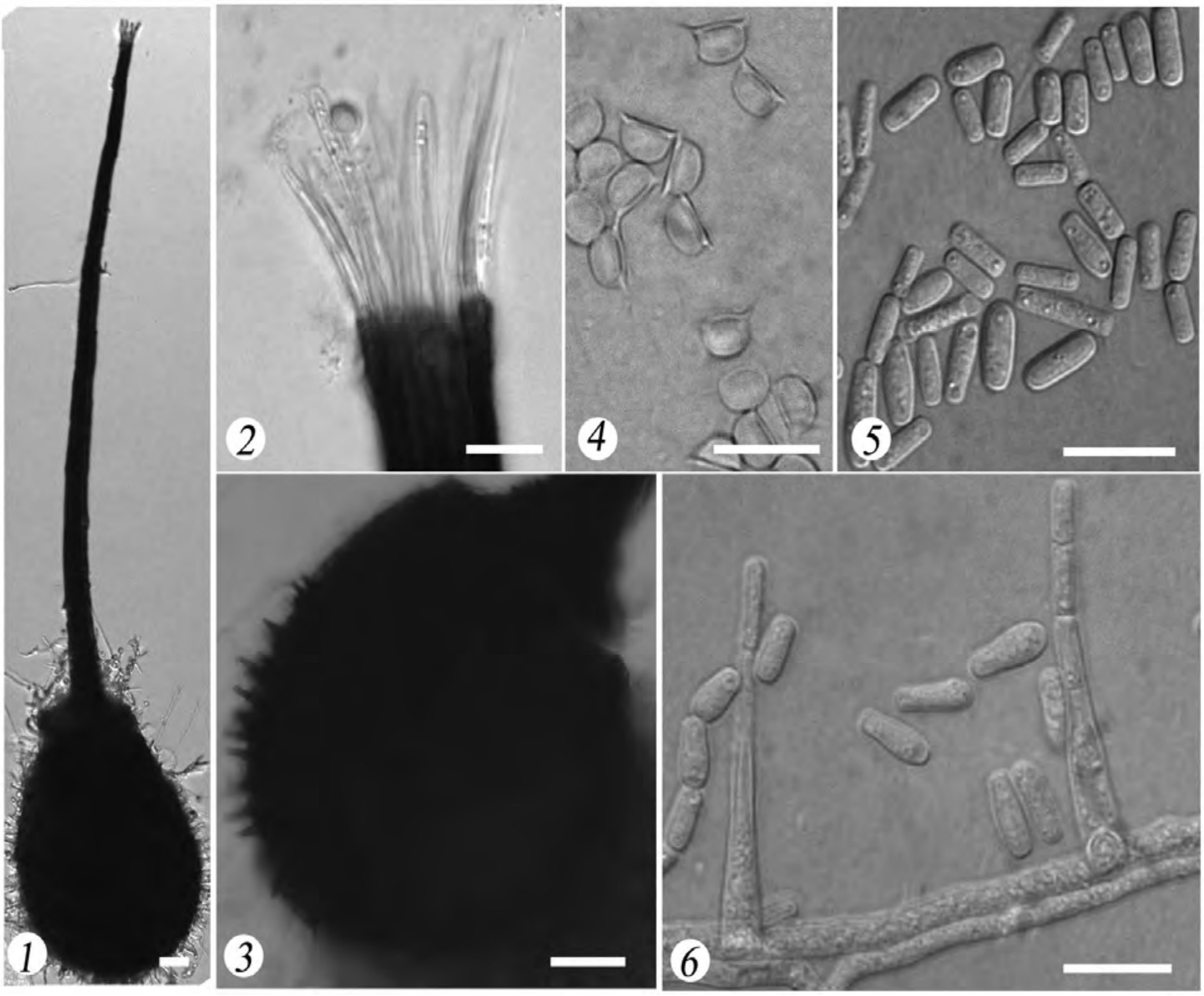

Figure 8: Morphological characteristics of *Ceratocystis savannae prov. nom*. 1) Globose to obpyriform ascomatal base (scale bar = 10µm), 2) Divergent ostiolar hyphae (scale bar = 10µm), 3) Ascomatal base with conical spines (scale bar = 10µm), 4) Hat-shaped ascospores (scale bar = 5µm), 5) Oblong and Bacilliform shaped conidia (scale bar = 5µm), 6) Phialidic conidiogenous cell with emerging bacilliform conidia (scale bar = 5µm).

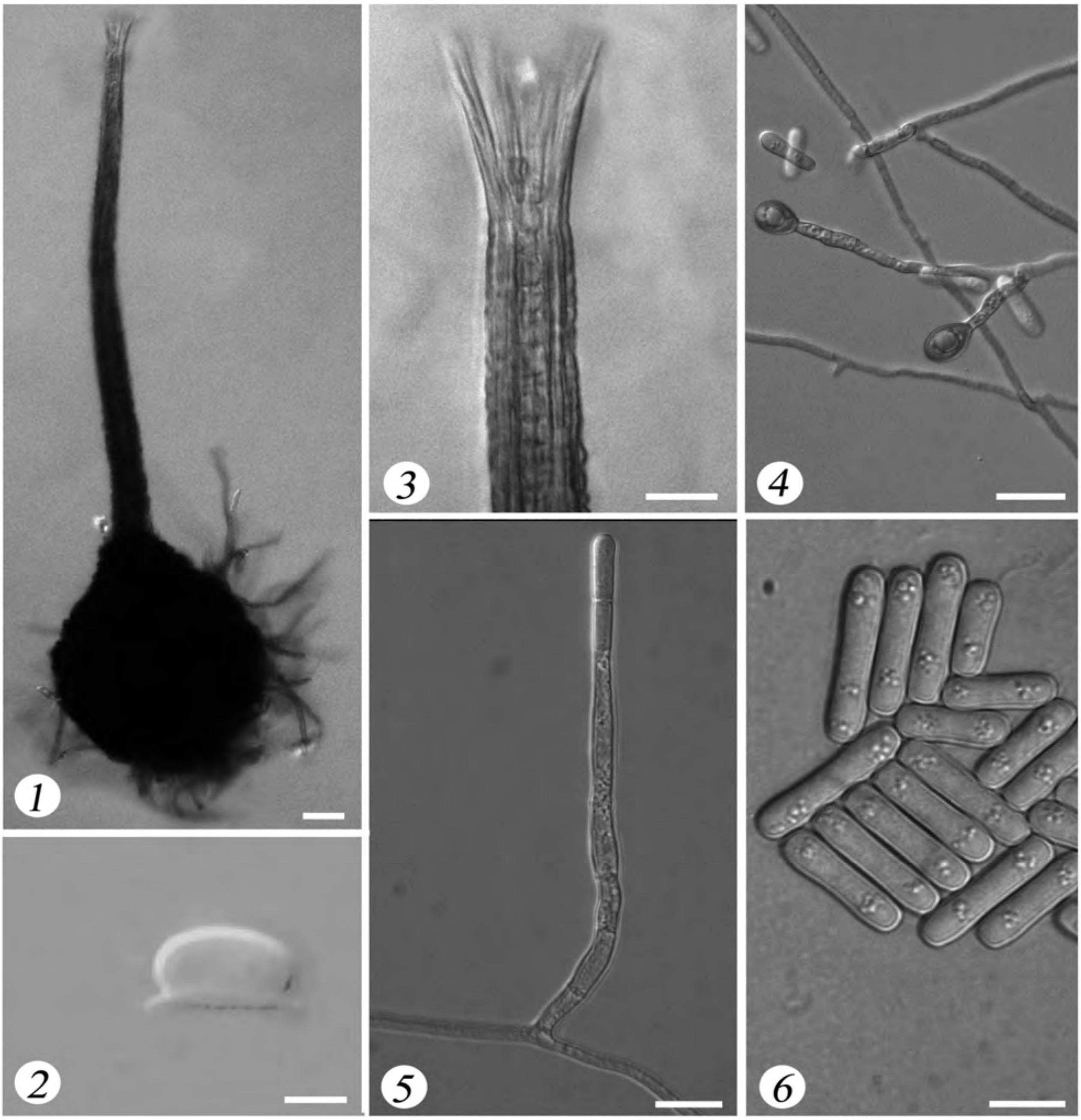

Figure 9: Morphological characteristics of *Ceratocystis tsitsikammensis prov. nom*. 1) Globose to obpyriform ascomatal base (scale bar = 10µm), 2) Hat-shaped ascospores in side view (scale bar = 5µm), 3) Divergent ostiolar hyphae (scale bar = 10µm), 4) Ovoid chlamydospores (scale bar = 5µm), 5) Phialidic conidiogenous cell with emerging bacilliform conidia (scale bar = 5µm), 6) Bacilliform shaped conidia (scale bar = 5µm).

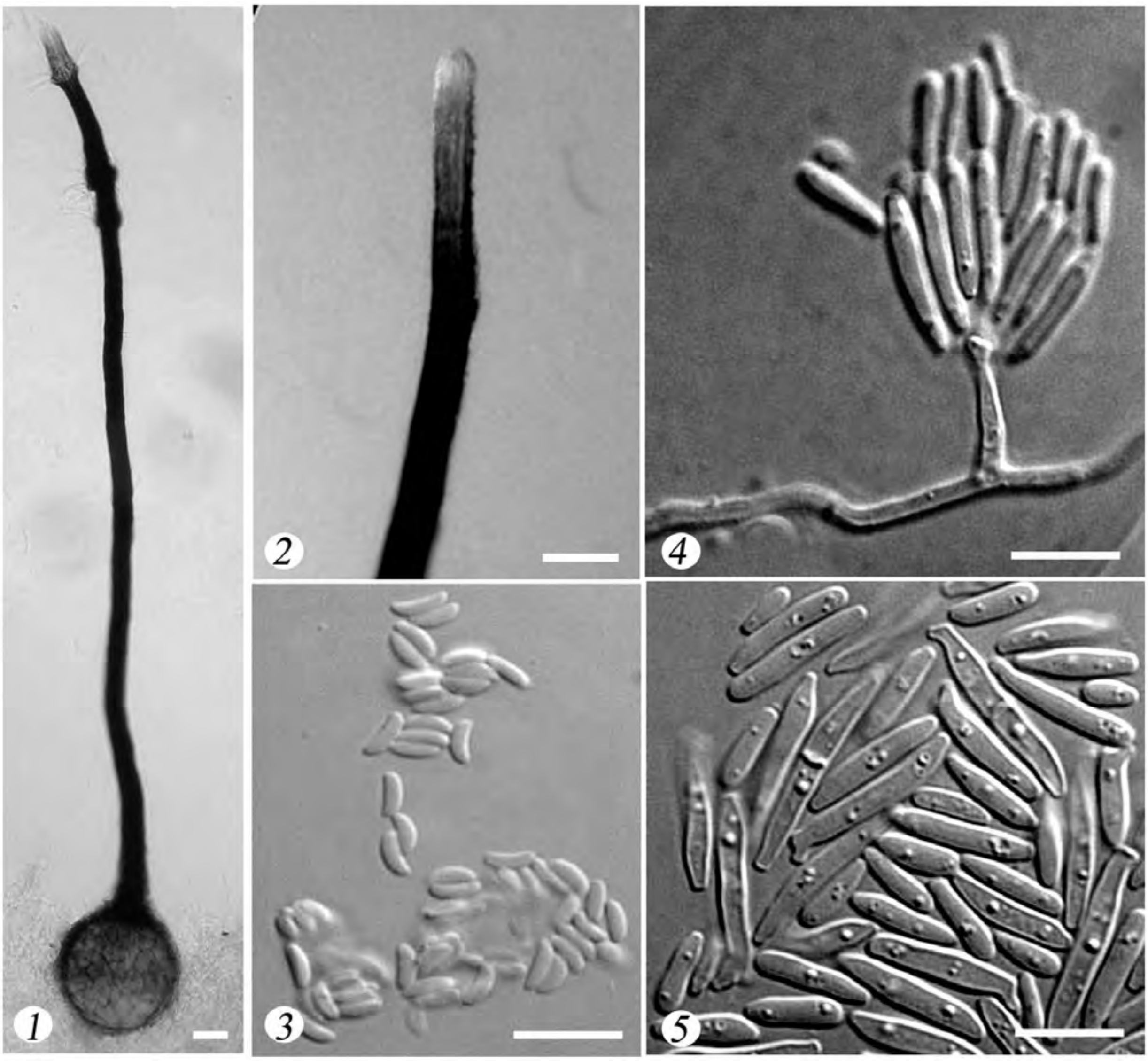

Figure 10: Morphological characteristics of *Ophiostoma longiconidiatum prov. nom*. 1) Globose ascomatal base (scale bar = 10µm), 2) Ostiolar hyphae absent (scale bar = 10µm), 3) Allantoid ascospores (scale bar = 5µm), 4) Conidiogenous cell with emerging conidia (scale bar = 5µm), 5) Conidia, oblong, acerose, proximal end distintly foot-shaped in some cases (scale bar = 5µm).

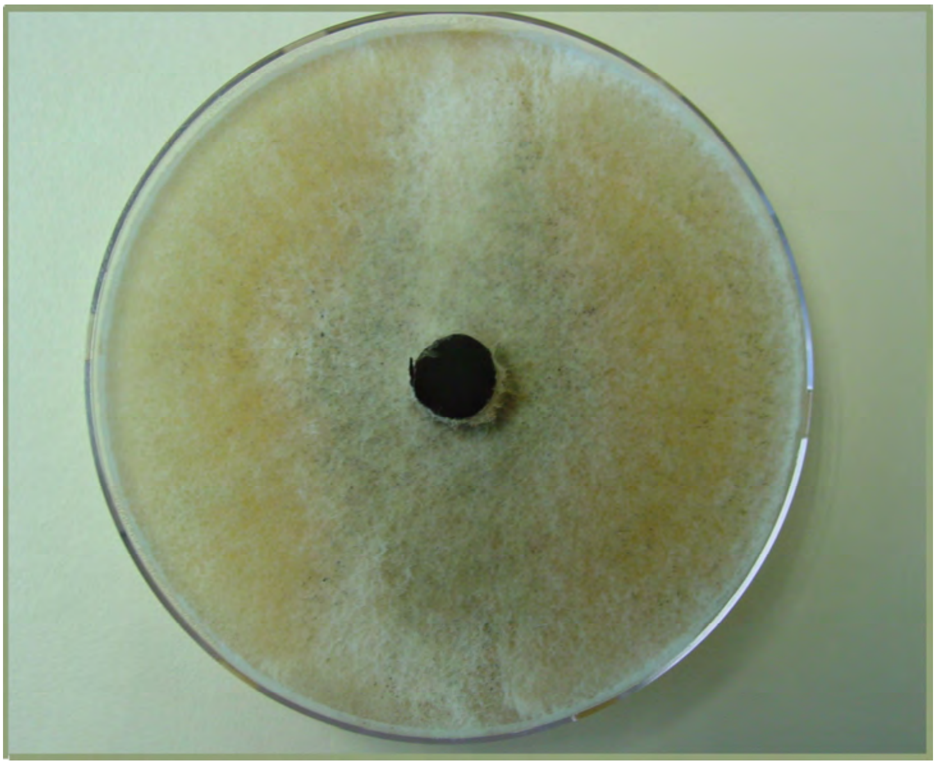

Figure 11. Cultural morphology of *Ceratocystis savannae prov. nom*. on MEA. *Colonies* smoke grey (21'''''), fluffy almost pale. Mycelium forming thick mat on agar.

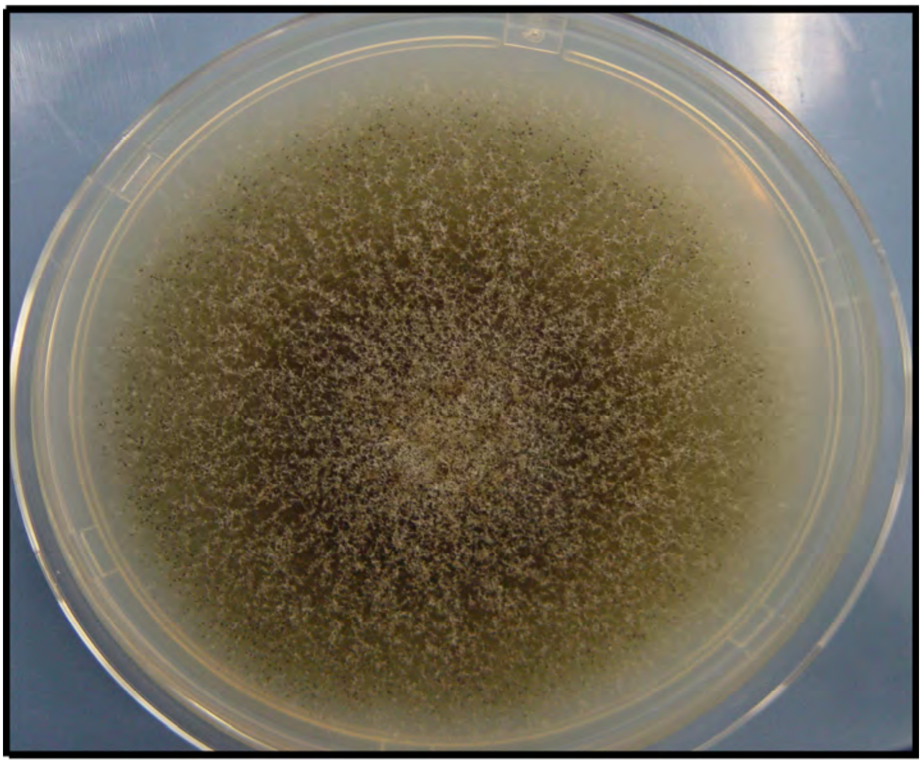

Figure 12. Cultural morphology of *Ceratocystis tsitsikammensis prov. nom*. on MEA. *Colonies* greenish olivaceous (23"'i) almost dark coloured. Colonies surfaces acattered with black coloured ascomata. Mycelium immersed and superficial.

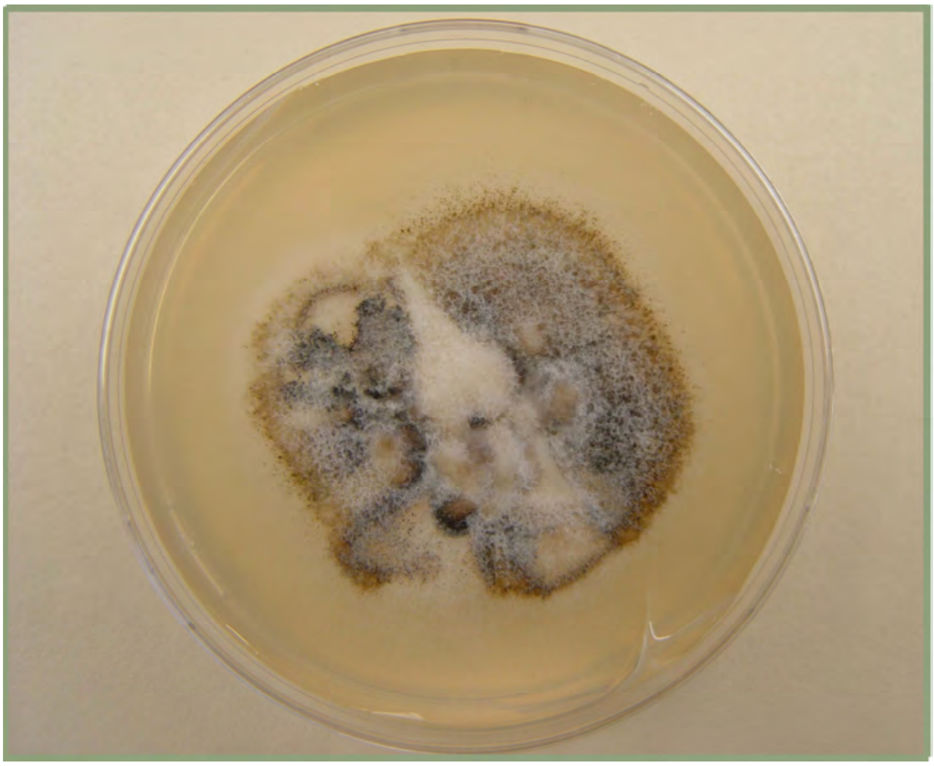

Figure 13. Cultural morphology of *Ophiostoma longiconidiatum prov. nom*. on MEA. *Colonies* pale mouse grey (15”′′’d) almost brown, fluffy.

CHAPTER 5

Ophiostoma species associated with native broad-leaved trees in Norway

1.0 ABSTRACT

Ophiostoma spp. include important pathogens of trees and agents of sap stain, especially in the Northern Hemisphere. These fungi infect wounds on trees and are typically carried by insects, especially bark beetles. *Ophiostoma* spp. on coniferous hosts in the Northern Hemisphere are well-known. However, other than for the serious pathogens *Ophiostoma ulmi* and *O. novo-ulmi*, very little research has been done on the occurrence of this group on native broad-leaved trees, especially in the Nordic countries. In this study, surveys were conducted in several areas of Norway to isolate *Ophiostoma* spp. associated with wounds on native broad-leaved trees belonging to the genera *Betula*, *Fagus*, *Populus*, *Quercus*, *Sorbus* and *Tilia*. Morphological studies and comparisons of DNA sequences from the ITS and 5.8S gene regions were used to confirm the identity of the fungi collected. The pathogenicity of selected isolates was also determined using artificial inoculation studies on young trees under field conditions. *Ophiostoma* spp., and especially their *Pesotum* anamorphs, were common on wounds on the trees sampled. In most cases, they were associated with wood stain. *Ophiostoma* spp., including *P. quercus*, *P. catonianum* and *P. pluriannulatum* were consistently isolated from the wounds. These species are here reported for the first time from wounds on broad-leaved trees in Norway. In pathogenicity trials, *O. quercus* produced lesions on *Betula* spp. and *P. tremula* trees, suggesting that it could play a role in tree decline and sap stain. The results of this study emphasise that the diversity of *Ophiostoma* spp. on broad-leaved trees is still incompletely understood in Norway and other European countries.

2.0 INTRODUCTION

The fungal genus *Ophiostoma sensu lato* and its asexual states include many important pathogens and agents of sap stain in lumber. Amongst the pathogens the Dutch elm disease fungi, *O. ulmi* (Buismann) Nannf and *O. novo-ulmi* Brasier are by far the best known. Most species of *Ophiostoma* are, however, agents of sap stain of lumber and can reduce its commercial value substancially (Seifert 1993). Amongst these fungi, *O. minus* (Hedgcock) H. & P. Sydow, *O. pluriannulatum* (Hedgcock) H. & P. Sydow and *O. piceae* (Munch) H & P Sydow are probably the most important sap stain species of lumber in the Northern Hemisphere (Seifert 1993).

Ophiostoma sensu lato is a generic aggregate including *Ophiostoma* H. & P. Sydow *sensu stricto* with *Pesotum* Crane and *Sporothrix* Hektoen & Perkins anamorphs, *Ceratocystiopsis* Upadhyay & Kendrick with *Hyalorhinocladiella* Upadhyay & Kendrick anamorphs and *Grosmannia* Goidanich with *Leptographium* Lagerberg & Melin anamorphs (Upadhyay 1981, Zipfel *et al.* 2006). In their sexual form, these fungi typically produce long-necked ascomata with sticky spores at their apices, that facilitate insect dispersal (Bakshi 1950). Asexual structures are typically erect conidiophores with sticky spores at their apices (*Hyalorhinocladiella*, *Pesotum* and *Leptographium*) or dry spores (*Sporothrix*) that can be wind-dispersed (Crane & Schoknecht 1973, Ingold 1971, Malloch & Blackwell 1993).

Ophiostoma spp. infect wounds on trees and are commonly vectored by insects. Bark beetles (Coleoptera: Scolatinae) are common vectors of these fungi (Grossmann 1931, 1932, Harrington 2005, Kirisits 2004, Six 2003). *Ophiostoma* spp. are also commonly vectored by casual insects such as nitidulid beetles and flies (Appel *et al*. 1990, Gibbs 1980, Juzwik & French 1983). Research done by Gibbs (1980) and Juzwik & French (1983), showed that insects will under certain circumstances carry a mixed inoculum of both sap stain and pathogenic fungi and eventually, the sap stain fungus could out-compete the pathogen and prevent infection of wounds (Gibbs 1980). This type of mutualism has been demonstrated between sap feeding beetles, the oak wilt fungus *Ceratocystis fagacearum* (Bretz) Hunt and *O. piceae*, a well-known sap stain fungus, and other members of the *O. piceae* complex (Gibbs 1980).

During the course of the past three decades, many *Ophiostoma* spp. have been reported from Norway and other European countries. These fungi have mostly been isolated from conifer infesting bark beetles. In Norway and Sweden for example, numerous *Ophiostoma* spp. have been reported to colonize *Picea abies* L. successively after beetle attack (Solheim 1992a, Solheim 1992b, Käärik 1975). However, the only records of an *Ophiostoma* sp. on hardwood trees in Norway are of *O. piceae*, which was reported from decayed *Betula pubescens* Ehrh. (Venn 1972) and the dutch elm disease fungus *O. ulmi* which was reported from *Ulmus glabra* Huds. (Roll-Hansen 1985, Venn 1986). For hardwood tree species, *Ophiostoma* spp. have been studied in more detail in Austria than in other European countries. *O. quercus* (Georgev.) Nannf. has been reported from *Quercus robur* L. and *Q. petraea* (Mattuschka) Liebl. (Halmschlager *et al*. 1994). It was also found associated with *Taphrorychus bicolor* Herbst infesting *Fagus sylvativa* L. (Kirisits *et al*. 2000, Lin 2003). *Ophiostoma piceae* has also been associated with *T. bicolor* infesting *F. sylvatica* (Lin 2003), while *O. fusiforme* Aghayeva & Wingfield was recently reported from *Q. petraea* (Aghayeva *et al*. 2004) and *O. lunatum* Aghayeva & Wingfield from *Carpinus betulus* L. (Aghayeva *et al*. 2004). The Dutch elm disease fungi have also been reported from many *Ulmus* spp. in Austria (Kirisits & Konrad 2004).

Knowledge regarding the geographic origin and host range of fungal pathogens or fungi that might be commercially relevant is important in developing effective quarantine and management strategies. As part of a collaborative project between research organizations in Norway and South Africa and funded by the Norwegian and South African Governments, a study was undertaken to survey for, and identify, possible *Ophiostoma* spp. that infect wounds on native broad-leaved tree species in Norway. This study aimed to address the current lack of detailed knowledge pertaining to this group of potentially important fungi in Norway and which could pose a possible threat to countries with which Norway trade, including countries in Africa. As a large number of isolates were obtained, and it is notoriously difficult to identify some *Ophiostoma* spp. based only on morphology, they were grouped according to culture morphology and selected isolates sequenced to obtain an indication of the species richness among the samples collected.

3.0 MATERIALS AND METHODS

3.1 Collection of isolates

Surveys of native broad-leaved trees in Norway were conducted during the summer months (between June and July) of 2004 and 2005. Sampling focused on wounds artificially induced on trees, freshly cut stumps of ***Betula*** and ***Populus*** spp. and log ends at saw mills and loading depots. In 2004, sampling was restricted to forests around the town of Ås situated in the Boreonemoral vegetation zone and a loading depot in South Western Sweden, near the town of Filipstad in the Middle Boreal Zone and not far from the Norwegian border (Moen 1999). In 2005, sampling was done in both the southern part of Norway in the Nemoral vegetation zone and in many municipalities in the Tromsø county, situated in the Middle Boreal vegetation zone (Moen 1999).

For the artificial wounds, an axe was used to remove sections of bark (10 X 20 cm^2) from living trees growing near lake Årungen in Ås, to expose the cambium. An uneven number of trees were wounded, depending on availability, including nine ***Betula*** spp., four ***Quercus*** spp., four ***Sorbus aucuparia*** L., four ***Salix*** spp. and one ***Populus tremula*** L. tree. These wounds were left for two months after which pieces of bark and wood containing cambial tissue were collected from all wounds that had signs of Ophiostomatoid fungi when examined with a 10X magnification hand lens. Samples collected from freshly cut stumps and log ends at saw mills and loading depots were obtained in a similar fashion by chopping pieces of infected tissues, especially where bark flaps protecting the wound from drought was visible, from them. Samples were stored in brown paper bags and transported to the laboratory. Dry samples were sprayed with water and sealed in plastic bags to induce sporulation of the fungi. Samples were examined daily for the development of fungal fruiting bodies characteristic of ***Ophiostoma*** spp. and their anamorphs.

Isolations of the fungi were made by lifting spore masses directly from fruiting structures with a sterile needle onto 2% malt extract agar [MEA: 20g malt extract, 15g agar, Biolab, Midrand, South Africa and 1000ml deionised water] containing 0.05g/l of the antibiotic streptomycin (SIGMA-ALDRICH, Steinheim, Germany). Cultures were grown at 24ºC for 7 days to obtain pure colonies. Replicates of each isolate were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the culture collection (NFRI) of the Norwegian Forestry and Landscape Institute (Skog og Landskap) in Ås.

3.2 Morphology of cultures

Isolates were grouped into morphotypes, based on culture morphology, after purification on MEA. From these morphotypes single drops of conidia or ascospores from selected isolates, or small pieces of mycelium were transferred from pure cultures to Oatmeal agar media (OMA: 30g Oats, 20g Biolab agar and 1Lt. deionised water) to promote sporulation and for comparison with previously published characteristics. Cultures were incubated at 24ºC until sporulation and then grouped into morphotypes according to differences in colony colour (Rayner 1970) and macro-morphology.

3.3 DNA isolation and amplification

Isolates, representing each of the different morphological groups, identified based on morphology, were selected for DNA sequence comparisons. Single spore drops from pure cultures were grown on 2% MEA for 10 days. Mycelium was then transferred to 1.5ml Ependorf tubes using a sterile scalpel. DNA was extracted using the protocol described by Möller *et al*. (1992), except that 10µl of RnaseA (Roche Diagnostics) was added at the final step and incubated overnight at room temperature to digest RNA. The presence of DNA was verified by separating an aliquot of 5µl on 1% agarose gels, containing ethidium bromide and visualized under Ultraviolet light. The internally transcribed spacer regions (ITS1 & 2) and 5.8S gene of the ribosomal RNA operon were amplified using an Eppendorf Mastercycler (Merck, Hamburg, Germany) and primers ITS1 and ITS4 (White *et al*. 1990). DNA template (60ng) was used to prepare 25µl PCR reactions. The reaction mix contained 2.5µl of 10X reaction buffer with $MgCl_2$ (25mM) (Roche Diagnostics, Mannheim, Germany), 2.5µl $MgCl_2$ (25mM) (Roche Diagnostics, Mannheim, Germany), 1U of Taq polymerase (Roche Diagnostics), 2.5µl of deoxynucleotide triphosphate mix (dNTP) (10mM) and 0.5µl of each primer (10mM). The conditions used for the thermal cycling were as follows: an initial denaturation of the DNA at 96ºC for 2min, followed by 35 cycles consisting of denaturation at 94ºC for 30s, annealing at 55ºC for 30s, primer extension at 72ºC for 1min and a final extension at 72ºC for 10min. An aliquot of 5µl of the PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized under UV light.

3.4 DNA sequencing

PCR products were purified using Sephadex G-50 Gel (SIGMA-ALDRICH, Steinheim, Germany), as recommended by the manufacturer. Purified products (1µl) were electrophoresed in a 1% agarose gel to estimate the concentration of DNA. Subsequently an accurate concentration of the purified PCR product was determined using the 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), according to the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Between 60-100ng PCR product was used to prepare a 10µl sequencing PCR that also contained 2µl of ready reaction mixture (Big dye), 2µl of 5X reaction buffer, 1µl of primer (10mM) and enough water to complete the volume of 10µl. The same primers were used as those described for the PCR amplifications. Both DNA strands were sequenced.

3.5 Sequence alignment and phylogenetic analyses

A preliminary identity for the sequences of isolates from Norway was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov). Sequences from both strands for each isolate were checked visually and combined using the programme Sequence Navigator version 1.01 (ABI PRISM, Perkin Elmer), by comparing the nucleotides and their corresponding peaks. Sequences were then aligned using MEGA version 3.0 (Kumar *et al*. 2004). Additional sequences of related *Ophiostoma* spp. were obtained from the GenBank database to fortify the comparisons. Phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford 1998). Heuristic searches using maximum parsimony with 10 random addition sequence replicates, branch swapping and Tree Bisection Reconstruction (TBR) were performed. Trees were rooted using *O. piliferum* (Fries) H. & P. Sydow as an outgroup. Confidence levels of the phylogenies were estimated with the bootstrap method (1000 replications) (Felsenstein 1985).

3.6 Pathogenicity tests

Pathogenicity tests were conducted with two isolates (CMW15566, CMW15748), selected from the two most common morphotypes (C and D) isolated from wounds in 2004. These tests were made near the town of Ås in field plots with natural regeneration of *P. tremula* and a mixture of *Betula pubescens* Ehrh and *B. pendula* which both are very common in the area. Ten trees, approximately five-years-old, of each *Betula* spp. and *P. tremula* were inoculated with the test fungi and two trees of the same age were inoculated with sterile agar discs to serve as controls.

Inoculations were done on stems of saplings 1-2cm in diameter. This was done by growing the two test fungi on MEA for ten days and inoculating MEA discs of 5mm diameter, overgrown with the fungi into bark wounds of equal size made on the trees using a sterile 5mm metal cork borer. Wounds were made in such a way that the sapwood on the stems of the trees to be inoculated was exposed. The wounds and agar discs were sealed with parafilm (Pechiney, Chicago, USA) to protect them from desiccation. Six weeks after inoculation (42 days), both bark and cambial lesions were examined and lesion lengths were recorded. Subsequently, re-isolations were made from the lesions to meet the requirements of Koch's postulates. Finally, data were analysed statistically using SAS/STAT in SAS (SAS Institute Inc. 1999).

4.0 RESULTS

4.1 Collection of isolates

Two hundred and thirty-two cultures (Table 1) were obtained from bark and cambial samples collected from more than 200 logs, stumps and stems of broad-leaved tree species spanning six different native tree genera in Norway (Table 1). Tree species from which *Ophiostoma* isolates were obtained included *Betula* spp. (Southern Norway), *B. pubescens* (Northern Norway), *P. tremula* (Southern Norway), *Quercus* spp. (Southern Norway), *S. aucuparia* (Southern Norway) and *Salix* spp. (Northern Norway) (Table 1). In many cases, the fungi were isolated from wood with obvious discolouration.

4.2 Morphology of cultures

Some of the cultures obtained produced ascomata typical of *Ophiostoma* spp. with very long necks bearing masses of ascospores while others produced only *Pesotum* anamorphs in culture (Table 2). A few isolates produced both ascomata and synnemata in culture. As a first line of identification, isolates were separated into those with sexual fruiting structures (*Ophiostoma* spp.) and those with only asexual fruiting structures (*Pesotum* spp.). Those producing ascomata were thus grouped into a single morphotype A) based on the fact that all produced similar long necked ascomata scattered over the petri plates. Morphotype A was further characterised by fluffy, dark coloured colonies. On OMA, the cultures producing only *Pesotum* fruiting structures were grouped into three morphotypes. Morphotype (B) was characterised by light coloured, circular and concentric colonies, with synnema bearing slimy spores becoming creamy toward the centers of the colonies. Morphotype (C) was comprised of cultures with ligth-brown to dark colonies that were fluffy toward the middle with a ring of synnemata bearing a mass of cream-colored conidia. Morphotype (D) was comprised of cultures with pale white to grey colonies with synnemata scattered over the plate and with conidia accumulating at their apices in white masses. Based on the cultural variation, a total of sixteen isolates including representative strains from different tree genera, different geographical locations sampled and different morpho-groups were selected for DNA sequencing (Table 5.3) to obtain an indication of the species diversity within the samples collected in Norway, but without having to sequence all 200 isolates collected.

4.3 DNA sequencing and phylogenetic analyses

All fifteen isolates from Norway, selected for DNA sequencing produced fragments of approximately 650 bp, using the primers ITS1 and ITS4. Preliminary blast searches suggested that the isolates resided in three groups. Comparison of isolates from Norway with those from GenBank in PAUP resulted in a total of 666 characters including gaps, with 399 constant characters, 25 parsimony-uninformative characters and 242 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option resulted in 708 trees. Eighteen best trees were retained, of which one was selected for representation (Figure 1). The consistency index (CI) and the retention index (RI) values were 0.807 and 0.94 respectively.

Isolates from four different morpho-groups in Norway could be separated into three distinct taxa (Table 2). The first group, including isolates from morpho-group B, C and D clustered with strains of *O. quercus*, supported by a bootstrap value in the Neighbor joining tree and the Maximum parsimony tree of 68% and 77%, respectively. The second group, including three isolates from Norway in morpho-groups B and D, and one collected from Austria in a previous study, clustered with strains of *O. catonianum* supported by a bootstrap value of 68% in the NJ tree and 53% in the MP tree. The last group from Norway, representing isolates in morpho-group A grouped with *O. pluriannulatum* supported by a bootstrap value of 97% in the NJ tree and 67% in the MP tree. Strains in the *O. pluriannulatum* group produced ascomata with long necks on MEA while strains in the *O. catonianum* and *O. quercus* groups produced only *Pesotum* anamorphs on OMA and these could not be separated based on culture morphology.

4.4 Pathogenicity tests

At the time when lesion lengths of inoculations were measured, all the trees inoculated appeared healthy externally. No disease symptoms such as cankers, wilting of the foliage or gum exudation could be observed. Wounds created during inoculations were covered by callus tissue for most of the trees, showing clear signs of tree recovery. However, for both tree species inoculated, bark lesions were not significant, but lesions on the cambial surface were very obvious. On *Betula* spp. these lesions had lengths ranging from 87mm to 118mm (Figure 2). Lesions in the xylem of *P. tremula* were smaller, but still differed from the controls ($P < 0.0001$) with average lesion lengths ranging from 20mm to 28mm (Figure 5.3). In all cases, re-isolations from lesions resulted in growth of the inoculated fungus.

5.0 DISCUSSION

In this study we provide the first data of a study to investigate the diversity and host range of *Ophiostoma* spp. in Norway. More than 200 isolates were obtained in this short study period, showing for the first time that *Ophiostoma* spp. are common on native hardwood trees in Norway. As so many isolates were obtained, and it is notoriously difficult to distinguish certain *Ophiostoma* spp. based only on morphology, for example the *O. piceae* complex, we did a rough screening of isolates into morphogroups, based mainly on culture morphology. From these groups we selected some isolates from each group, attempting to also select them from different hosts and areas, for further characterization. This lead us to identify three *Ophiostoma* spp. from this initial study. They include *O. catonianum*, *O. pluriannulatum* and *O. quercus*. Of these fungi *O. quercus* was most common among the isolates sequenced. These fungi are reported here for the first time from Norway. We also identified isolates collected during previous studies from Austria and Sweden, thus reporting *O. quercus* for the first time from Sweden, and *O. catonianum* from Austria.

Finding *O. quercus* on hardwood trees in this study was not unusual. The fungus is well-known to have a wide distribution in the northern hemisphere on deciduous timber, although many reports have been obscured by taxonomic confusion. Many early reports of the fungus have been as *O. piceae* (Hunt 1956, Przybyl & de Hoog 1989, Upadhyay 1981) and it is only relatively recently that isolates of *O. piceae* from hardwoods have been recognized as most probably representing the distinct fungus, *O. quercus* (De Beer *et al*. 2003a, Halmschlager *et al*. 1994, Harrington *et al*. 2001, Kim *et al*. 1999, Pipe *et al*. 1995). Some authors have also referred to the fungus as *O. querci* which might have confused the literature pertaining to it (De Beer *et al*. 2003b). Interestingly, the fungus has a cosmopolitan distribution on hardwoods and is found both in the northern and southern hemisphere (Brasier & Kirk 1993, De Beer *et al*. 2003a, Halmschlager *et al*. 1994, Harrington *et al*. 2001, Kim *et al*. 1999, Morelet 1992, Pipe *et al*. 1995). While its common occurrence on wounds in this study, nine out of a total of fifteen isolates sequenced, and occurring in two vegetation zones, suggests that it is native to the areas of investigation, this is an intriguing question that deserves critical study.

It has been hypothesized that *O. quercus* is native to the Northern Hemisphere (Harrington *et al*. 2001). It is thus not strange to find the fungus commonly associated with wounds on broad-leaved trees in Norway and also from Austria and Sweden. However, it has also been suggested that the fungus might be native to the Southern Hemisphere, owing to its ability to grow at high temperatures (32^{o}C) (Brasier & Stephens 1993). Furthermore, the fact that *O. quercus* is common on various native trees in the Southern Hemisphere might also suggest that it could be native to that part of the world (De Beer *et al*. 2003a). Research involving population studies will be useful to resolve speculation regarding the origin of *O. quercus* and such studies will benefit from the inclusion of isolates from this investigation.

Results of this study showed that isolates of *O. quercus* could result in lesions on inoculated *Betula* and *Populus* trees. However, no trees died during the inoculation study and only a limited number of trees of suitable size for inoculation could be obtained. The fungus is clearly not a virulent pathogen and its ecological role on fresh wounds is unknown. However, it may play a role in protecting wounds from infection by pathogens, as has been shown in oaks by Gibbs (1980) who showed that the fungus (reported as *O. piceae*) could protect wounds from infection by the oak wilt pathogen *C. fagacearum* (Bretz) Hunt. Alternatively, based on our inoculation studies it could cause disease under conditions unfavorable for the tree, or at least contribute considerably to sapwood stain of trees. Further studies, with more isolates and more trees are required to expand on our results.

Isolation of *O. catonianum* from hardwood trees in this study was an interesting result. This is because the fungus has not previously been found in Norway or Austria and it is also a taxonomically interesting fungus. *O. catonianum* was first described in Italy from infected plant tissues on pear trees (*Pyrus communis* L.) based on a single isolate (Goidanich 1935). The fungus was not recorded again, until the current study. Given the difficulty in distinguishing between *O. catonianum* and other *Ophiostoma* anamorphs that produce synnemata, it is possible that it has been recorded in Europe but under the name *O. piceae*. For example, *O. piceae* was reported from *Betula* sp. in Norway by Venn (1972) and this report could have included *O. catonianum* or other species in the *O. piceae* complex. At least nine species of *Ophiostoma*, are recognized as part of the *O. piceae* complex including *O. catonianum* (Harrington *et al*. 2001). Species in the complex are very similar to each other based on cultural characteristics and DNA sequence comparison are currently needed to identify species with confidence. The re-discovery of *O. catonianum* after so much years, now provides us with the opportunity to study this enigmatic fungus in more detail, as no preserved material is available from the original description anymore and only a single culture existed in the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands (CBS). The fact that we isolated it from Northern Norway, as well as identified it from cultures from Austria, shows that it has a much broader distribution and host range than previously indicated.

The occurrence of *O. plurianulatum* on hardwood trees in this study is not unusual. The fungus is widely distributed in the northern hemisphere on both hardwood and conifer timber where it can cause sap stain (Davidson 1935, Eslyn & Davidson 1976, Hedgcock 1906, Hunt 1956, Lagerberg *et al*. 1927) and is vectored by nitidulid beetles (Jewell 1956). The occurrence of the fungus in Norway on wounds from broad-leaved trees in two tree genera and in two vegetation zones indicate that the diversity of this fungus is not yet well understood in Norway and that similar to other countries, it is most likely common on a number of tree genera.

This study has improved our knowledge about the occurrence, host range and geographic distribution of *Ophiostoma* spp. in Norway. *O. quercus* is without doubt a widespread species occurring on a wide range of substrates as found in this study. It is also culturally very diverse in morphology, grouping in three culture morphogroups in this study. *O. catonianum* and *O. pluriannulatum*, although only representing three of the sequenced isolates each in this study, could be more widespread than indicated, due to the rough screening used in the study. More research is required on the remaining isolates collected from Norway to obtain a clear picture of the exact species diversity on native broad-leaved trees in the country. However, this study has clearly showed that information is still lacking regarding the occurrence of *Ophiostoma* spp. in Norway, despite the fact that this is one of the best study fungal genera in the world. This lack of information regarding a well-known genus of pathogens in a European country serves to illustrate the potential threat to other countries, such as South Africa.

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Table 1: List of Isolates collected from Norway and Sweden during surveys in 2004 and 2005.

CMW	Host Tree	Location	Years	ID	Number of Isolates	Vegetation Zone	Collector
15699, 15558, 15715, 15523, 15710, 15601, 15559, 15540, 15702, 15748, 15603, 15568, 15604, 15605, 15606, 15569, 15709, 15754, 15706, 15607, 15608, 15549	*Betula* spp	Filpstad	2004	*Pesotum* spp.	22	Boreonemoral	J. Roux
15591, 15705, 15756, 15563, 15509, 15564, 15599, 15521	*Betula* spp.	Årungen	2004	*Pesotum* spp.	8	Boreonemoral	J. Roux
15752	*Betula* spp.	Trysil	2004	*Pesotum* spp.	1	Boreonemoral	J. Roux
15751	*Populus tremula*	Årungen	2004	*Pesotum* spp.	1	Boreonemoral	J. Roux
15592	*P. tremula*	Svenneby	2004	*Pesotum* spp.	1	Boreonemoral	J. Roux
15593, 15546, 15750, 15746, 15714, 15704, 15744, 15707, 15757, 15570, 15600, 15539, 15701	*Quercus* spp.	Årungen	2004	*Pesotum* spp.	13	Boreonemoral	J. Roux
15535, 15527, 15560, 15566, 15544	*Sorbus* spp.	Årungen	2004	*Pesotum* spp.	5	Boreonemoral	J. Roux
15565	*Salix* spp.	Årungen	2004	*Pesotum* spp.	1	Boreonemoral	J. Roux
15536, 15561, 15725, 15755	Hardwood	Svenneby	2004	*Pesotum* spp.	4	Boreonemoral	J. Roux
19176, 19185, 22066, 22068, 22070, 22072	*Betula* spp.	Tresnes	2005	*Pesotum* spp.	6	Nemoral	G. Kamgan & H. Solheim
19181	*Populus* spp.	Tresnes	2005	*Ophiostoma* spp.	1	Nemoral	G. Kamgan & H. Solheim
19175, 19178, 19182, 19183, 19184, 22071	*Populus* spp.	Tresnes	2005	*Pesotum* spp.	6	Nemoral	G. Kamgan & H. Solheim
19192, 19193, 19192, 19193, 22078	*Populus* spp.	Øydna	2005	*Pesotum* spp.	5	Nemoral	G. Kamgan & H. Solheim
19186, 19187, 19188, 19197, 19198, 19199, 19200, 19201, 19202, 19203, 19204, 19205, 19206, 19207, 19208, 19209, 19211, 19212, 19213, 19214, 19215, 19216, 19217, 19218, 19219, 19220, 19221, 19222, 19223, 19224, 19225, 19227, 19229, 22080, 22081, 22087	Quercus spp.	Øydna	2005	*Pesotum* spp.	36	Nemoral	G. Kamgan & H. Solheim
19230, 19231, 19232, 19233, 19234, 22088	*Quercus* spp.	Salthaug	2005	*Pesotum* spp.	6	Nemoral	G. Kamgan & H. Solheim
19235, 19236, 19237, 19238, 19239, 19240, 19241, 19242, 19243, 19244, 19245, 19246, 19247, 19248, 19249, 19250, 19251, 19252, 19253, 22089, 22090	*Quercus* spp.	Salthaug	2005	*Pesotum* spp.	21	Nemoral	G. Kamgan & H. Solheim
19255, 19256, 19257, 19261	*Quercus* spp.	Lyngdal	2005	*Pesotum* spp.	4	Nemoral	G. Kamgan & H. Solheim
19254	*Populus* spp.	Salthaug	2005	*Pesotum* spp.	1	Nemoral	G. Kamgan & H. Solheim
19258	*Populus* spp.	Lyngdal	2005	*Pesotum* spp.	1	Nemoral	G. Kamgan & H. Solheim
19259	*Betula* spp.	Lyngdal	2005	*Pesotum* spp.	1	Nemoral	G. Kamgan & H. Solheim
19260	*Salix* spp.	Lyngdal	2005	*Pesotum* spp.	1	Nemoral	G. Kamgan & H. Solheim
19313, 19315, 19316, 19328, 19330, 18962	*Betula* spp	Målselv	2005	*Ophiostoma* spp	6	Middle boreal	G. Kamgan & H. Solheim
18919, 18930, 18934, 18939, 18941, 18942, 18949, 18950, 18953, 18955, 18956, 18961, 18966, 18968, 18969, 19263, 19299, 19300, 19301, 19302, 19303, 19305, 19306, 19311, 19312, 19314, 19317, 19318, 19319, 19320, 19324, 19323,	*Betula* spp.	Målselv	2005	*Pesotum* spp.	39	Middle boreal	G. Kamgan & H. Solheim

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19325, 19326, 19329, 19331, 22060, 22062, 22064, 22061	*Salix* spp.	Målselv	2005	*Pesotum* spp.	1	Middle boreal	G. Kamgan & H. Solheim
19293	*Betula* spp.	Salangen	2005	*Ophiostoma* spp	1	Middle boreal	G. Kamgan & H. Solheim
18876, 22055, 18878, 18879, 18881, 18883, 18870, 18887, 18889, 18891, 18893, 18895, 18896, 18908, 18910, 19265, 19266, 19267, 19268, 19270, 19272, 19275, 19276, 19277, 19278, 19279, 19280, 19281, 19283, 19284, 19285, 19286, 19287, 19289, 19292, 19296, 22058.	*Betula* spp.	Salangen	2005	*Pesotum* spp	37	Middle boreal	G. Kamgan & H. Solheim
19264	*Salix* spp	Bardu	2005	*Pesotum* spp.	1	Middle boreal	G. Kamgan & H. Solheim
18917, 19297	*Populus* spp.	Sørreisa	2005	*Pesotum* spp.	2	Middle boreal	G. Kamgan & H. Solheim

Table 2: *Ophiostoma* species and their morphogroups identified in this study.

Species	Isolate number	Hosts	Area	Vegetation zone	Collectors
O. catonianum	CMW18919	*Betula pubescens*	Sørreisa	Middle boreal	G. Kamgan, H. Solheim
(Morphotype B, D)	CMW18966	*B. pubescens*	Målselv	Middle boreal	G. Kamgan, H. Solheim
	CMW19320	*B. pubescens*	Målselv	Middle boreal	G. Kamgan, H. Solheim
O. pluriannulatum	CMW19181	*Populus tremulae*	Tresnes	Nemoral	G. Kamgan, H. Solheim
(Morphotype A)	CMW19313	*B. pubescens*	Målselv	Middle boreal	G. Kamgan, H. Solheim
	CMW19329	*B. pubescens*	Målselv	Middle boreal	G. Kamgan, H. Solheim
O. quercus	CMW22066	*Betula* spp.	Tresnes	Nemoral	G. Kamgan, H. Solheim
(Morphotype B, C, D)	CMW19187	*Quercus* spp.	Øydna	Nemoral	G. Kamgan, H. Solheim
	CMW15750	*Quercus* spp.	Årungen	Boreonemoral	J. Roux
	CMW15746	*Quercus* spp.	Årungen	Boreonemoral	J. Roux
	CMW15704	*Quercus* spp.	Årungen	Boreonemoral	J. Roux
	CMW15528	*Betula* spp.	Svenneby	Boreonemoral	J. Roux
	CMW15566	*Sorbus aucuparia*	Årungen	Boreonemoral	J. Roux
	CMW15544	*Sorbus aucuparia*	Årungen	Boreonemoral	J. Roux
	CMW15560	*Sorbus aucuparia*	Årungen	Boreonemoral	J. Roux

Table 3: *Ophiostoma* reference strains and their accession numbers used in this study for DNA sequence comparison.

Species	Isolate number	Genbank accession number	Other numbers	Hosts	Collectors	Origin
O. catonianum	C1084	AF198243	CBS263.35	*Pyrus communis*	G. Goidanich	Italy
	[a]CMW18919	EF408592	NA	*Betula pubescens*	G. Kamgan, H. Solheim	Norway
	[a]CMW18966	EF408593	NA	*B. pubescens*	G. Kamgan, H. Solheim	Norway
	[a]CMW19320	EF408591	NA	*B. pubescens*	G. Kamgan, H. Solheim	Norway
	[a]CMW17860	EF408594	NA	*Tilia cordata*	T. Kirisits	Austria
O. floccosum	C1086	AF198231	CBS799.73		A. Käärik	Sweden
	CMW7661	AF493253		*Pinus elliottii*	ZW. de Beer	South Africa
O. himal-ulmi	C1183	AF198233	CBS374.67; ATCC36176; ATCC36204	*Ulmus* sp.	HM. Heybroek	India
	C1306	AF198234	HP27	*Ulmus* sp.	CM. Brasier	India
O. kryptum		AY304434	DAOM229702 (IFFFBW/1)	*Larix decidua*	T. Kirisits & MJ. Wingfield	Austria
		AY304437	IFFFHasd/1	*L. decidua*	T. Kirisits & MJ. Wingfield	Austria
O. multiannulatum		AY934512	CBS124.39	NA	NA	NA
O. novo-ulmi	C510	AF198236		*Ulmus* sp.	NA	Iowa, USA
	C1185	AF198235	CBS298.87; WCS637	*Ulmus* sp.	HM. Heybroek	Russia
O. perfectum	C1104	DQ062970	CBS636.66	NA	NA	NA
O. piceae		AF198226	C1087; CBS108.21	*Abies or Picea*	E. Münch	Germany
	CMW7648	AF493249	C967; H2181	*Picea sitchensis*	DB. Redfern & JF. Webber	United Kingdom
O. piliferum		AF221070	CBS129.32	*Pinus sylvestris*	H. Diddens	United Kingdom
		AF221071	NA	NA	NA	
O. pluriannulatum		AY934517	MUCL18372	NA	NA	USA
	C1033	DQ062971	NZ-150	*P. radiata*	Farrell	New Zealand
	C1567	DQ062972	UAMH9559; WIN(M)869	*Podocarpus* sp.	Reid	New Zealand
	[a]CMW19181	EF408597	NA	*Populus tremulae*	G. Kamgan, H. Solheim	Norway
	[a]CMW19313	EF408595	NA	*B. pubescens*	G. Kamgan, H. Solheim	Norway
	[a]CMW19329	EF408596	NA	*B. pubescens*	G. Kamgan, H. Solheim	Norway
O. quercus	C970	AF198239	CBS102353, H1039	*Quercus* sp.	PT. Scard & JF. Webber	United Kingdom
	CMW7656	AF493250		*Q. robur*	MJ. Wingfield	South Africa
	CMW2463	AF493239	0.96	*Fagus sylvatica*	M. Morelet	France
	CMW7650	AF198238	C969; CBS102352; H1042	*Quercus* sp.	PT. Scard & JF. Webber	United Kingdom
	CMW7645	AF493246	W3; HA367	*Q. robur*	T. Kirisits & E. Halmschlager	Austria
	[a]CMW22066	EF408582	NA	*Betula* sp.	G. Kamgan, H. Solheim	Norway
	[a]CMW19187	NA	NA	*Quercus* sp.	G. Kamgan, H. Solheim	Norway
	[a]CMW15750	EF408584	NA	*Quercus* sp.	J. Roux	Norway
	[a]CMW15746	EF408585	NA	*Quercus* sp.	J. Roux	Norway
	[a]CMW15704	NA	NA	*Quercus* sp.	J. Roux	Norway
	[a]CMW15528	EF408579	NA	*Betula* sp.	J. Roux	Norway
	[a]CMW15608	NA	NA	*Betula* sp.	J. Roux & H. Solheim	Sweden
	[*]CMW15748	NA	NA	*Betula* sp.	J. Roux & H. Solheim	Sweden

	*[a]CMW15566	EF408583	NA	*Sorbus aucuparia*	J. Roux	Norway
	[a]CMW15544	EF408581	NA	*S. aucuparia*	J. Roux	Norway
	[a]CMW15560	EF408580	NA	*S. aucuparia*	J. Roux	Norway
	CMW17866	NA	NA	*Q. robur*	T. Kirisits	Austria
	CMW17892	EF408586	NA	*Q. robur*	T. Kirisits	Austria
	CMW17828	EF408587	NA	*Q. robur*	T. Kirisits	Austria
	CMW17864	NA	NA	*Q. robur*	T. Kirisits	Austria
	CMW17811	NA	NA	*Q. robur*	T. Kirisits	Austria
	CMW17841	NA	NA	*Q. robur*	T. Kirisits	Austria
	CMW17894	EF408589	NA	*T. cordata*	T. Kirisits	Austria
	CMW17877	EF408588	NA	*T. cordata*	T. Kirisits	Austria
	CMW17893	EF408590	NA	*Fagus sylvatica*	T. Kirisits	Austria
O. setosum	AU16053	AF128927	NA	NA	NA	Canada
	AU16038	AF128929	NA	NA	NA	Canada
O. subannulatum		AY934522	CBS188.86	NA	NA	NA
O. tetropii		AY194507	CBS428.94	*P. abies*	T. Kirisits	Austria
	DAOM229566 (C01-015)	AY194493	NA	*P. glauca*	G. Alexander	McNabs Island, Canada
O. ulmi	C1182	AF198232	CBS102.63; IMI101223; JCM9303	*Ulmus* sp.	WF. Holmes & HM. Heybroek	Netherlands

[a] Isolates sequenced in this study.

* Isolates used for inoculations in this study.

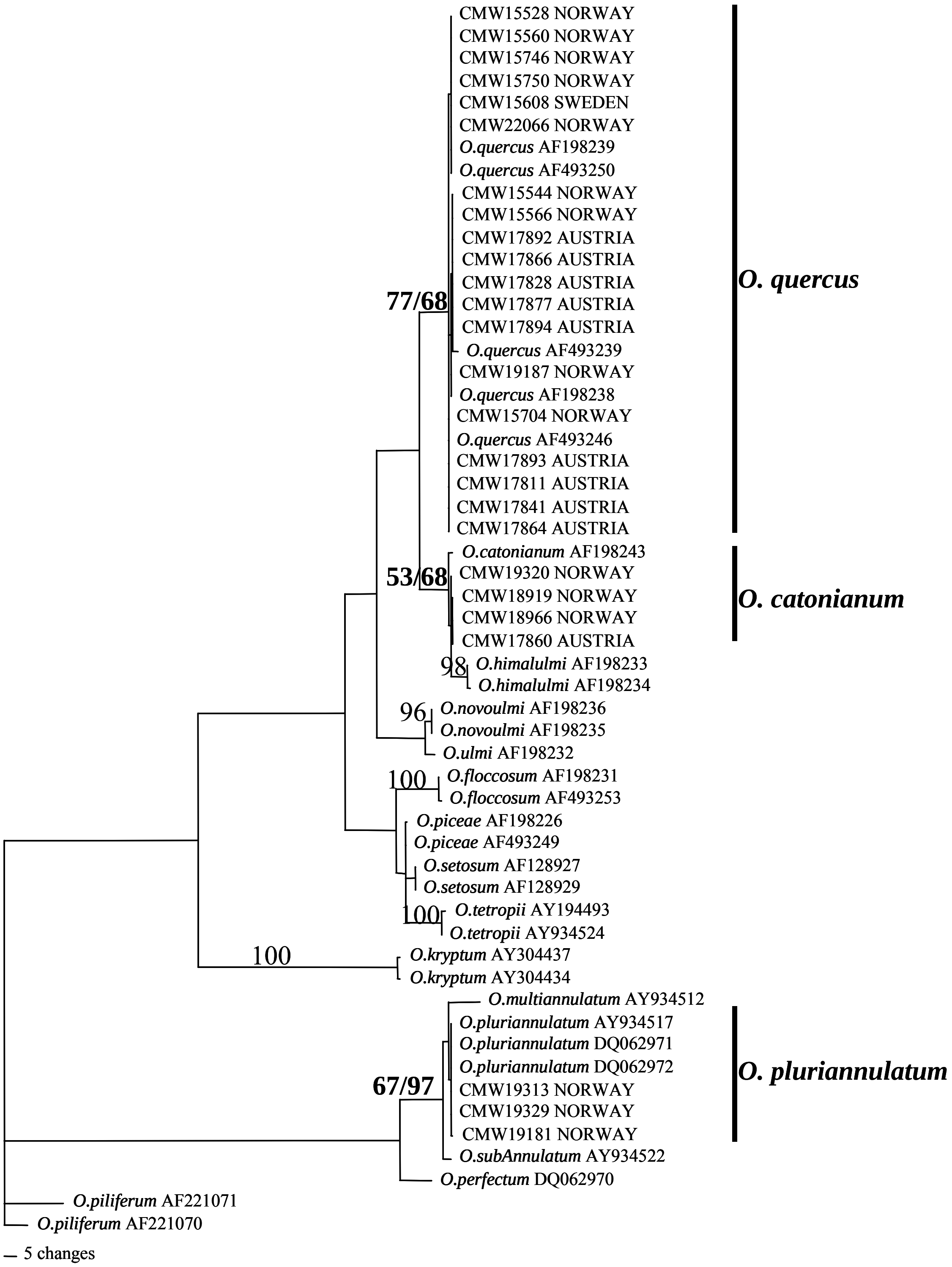

Figure 1: Phylogenetic tree produced by a heuristic search of the ITS sequence data. *Ophiostoma piliferum* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated behind each clade.

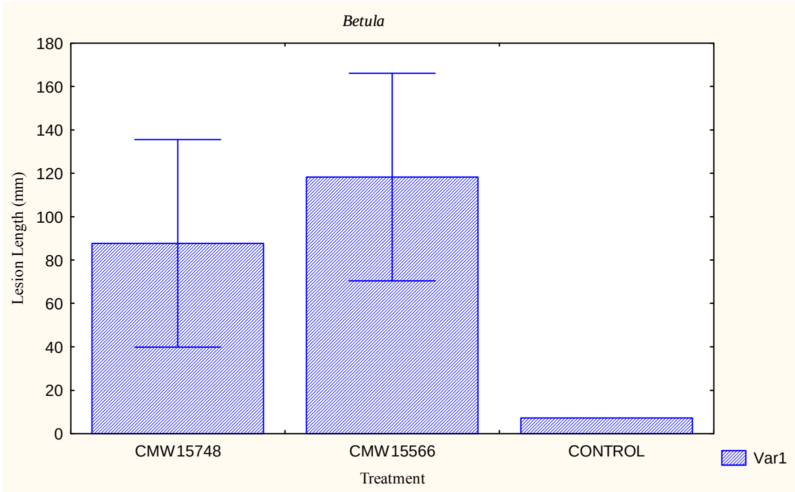

Figure 2: Histogram showing results of field inoculation trials with *O. quercus* (CMW15748, 15566) on *Betula* spp., Lsmean = 87, R = 0.24, CV = 83.54, P<0.0554, Confidence limit = 95%, Average lesion lengths (87.7 – 118.2) mm.

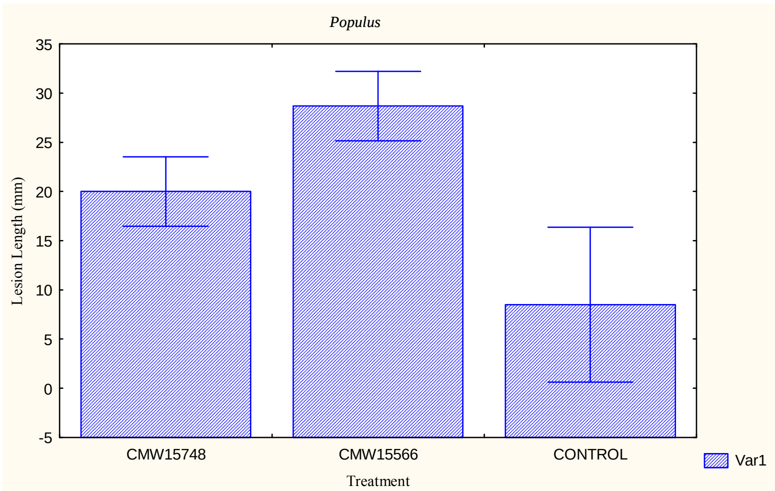

Figure 3: Histogram showing results of field inoculation trials with *O. quercus* (CMW15748, 15566) on *Populus tremula*, Lsmean = 22.9, R = 0.608, CV = 23.24, P<0.0001, Confidence limit = 95%, Average lesion lengths (20 – 28.5) mm.

SUMMARY

This thesis concerns the study of selected *Ceratocystis* species and *Ophiostoma* species infecting wounds on broad-leaved trees, particularly those occurring in Africa. However, two chapters also deal with these fungi from Australia, Norway, Sweden and Austria. The dissertation is comprised of a literature review, followed by four research chapters, addressing the occurrence of *Ceratocystis* spp. and *Ophiostoma* spp. in Africa, Australia and Norway.

The first chapter of the dissertation is a review of *Ceratocystis* spp. and *Ophiostoma* spp. with particular reference to Africa, hardwood tree species and wound infections. The review highlights the importance of wood and trees, especially on the African continent, and discusses the threat of deforestation. This is despite efforts by many African countries to establish forests of non-native tree species to address the demand for wood and wood products on the continent. Reforestation is associated with increased risks of the introduction of pests and pathogens, including species of *Ceratocystis* and *Ophiostoma*. The taxonomic history of these two fungal genera is summarized, and the review further focuses on the economically important species in these genera, particularly those infecting hardwood tree species on the African continent. The lack of information regarding *Ceratocystis* spp. and *Ophiostoma* spp. on hardwood trees in Africa is thus highlighted. Furthermore, the review summarised the dispersal mechanisms of these pathogens, highlighting dispersal too and infection of wounds.

Ceratocystis pirilliformis was described in 2003 and it is the only species in the *Ceratocystis fimbriata* species complex that has pear-shaped ascomatal bases. This fungus was first described from Australia where *Eucalyptus* spp. are endemic. It was later reported from South Africa on *Eucalyptus grandis* trees. Chapter two of this dissertation attempts to address questions regarding the geographic distribution, impact and origin of *C. pirilliformis* in South Africa. This was in line with the fact that it has been suggested that the fungus is likely native to Australia. To address this question, surveys were conducted in many Eucalyptus planting areas in South Africa and the genetic diversity of the fungus in the country was investigated using microsatellite markers previously developed for *C. fimbriata*. *C. pirilliformis* was found in three Eucalyptus-growing areas of South Africa, which has considerably increased the known geographic range of the fungus in South Africa. The gene diversity as well as the genotypic diversity for the fungus was found to be

very low in the country and the population is apparently clonal. Results thus support the view that *C. pirilliformis* was accidentally introduced into South Africa.

In chapter three of this dissertation, *O. quercus* is reported for the first time from wounds on non-native *Acacia mearnsii* in Uganda. In addition a new *Pesotum* sp., *P. australi prov. nom.* is described from wounds on native *A. mearnsii* in Australia. This fungus resembles other *Pesotum* anamorphs of *Ophiostoma* in many ways, especially species of the *O. piceae* complex. However, it can be distinguished from these species by many morphological traits and also based on phylogenetic inference. The closest phylogenetic neighbor of *P. australi prov. nom.* is *O. quercus*. The fact that it was isolated from *A. mearnsii* in Australia indicates that it is probably a native fungus in that area.

In chapter four, two *Ceratocystis* spp. and one *Ophiostoma* sp. are described as new to science, from wounds on native broad-leaved tree in South Africa. Three other *Ophiostoma* spp. are also reported in this study. Until recently, very little research has been done with regard to *Ceratocystis* spp. and *Ophiostoma* spp. occurring on native tree species in Africa. However, results presented in this chapter strongly suggest that these fungi are common on native trees in Africa and many other species, including potential pathogens await discovery.

Chapter five of the dissertation reports, for the first time, *Ophiostoma catonianum*, *O. pluriannulatum* and *O. quercus* from native broad-leaved trees in Norway. It also reports *O. catonianum* for the first time from Austria and *O. quercus* for the first time from Sweden. In the past, very little research has been undertaken to explore the diversity of these fungi on hardwood trees in the Nordic countries or other parts of Europe, where most research has been focused on *Ceratocystis* spp. and *Ophiostoma* spp. associated with conifer-infesting bark beetles. This chapter represents a preliminary study with important discoveries. It indicates that these fungi are common on wounds on hardwood trees in Europe and emphasizes the importance of expanding these studies in the Nordic countries, to include more hosts and geographic areas. Such studies will almost certainly reveal more species and possibly new species of *Ceratocystis* and *Ophiostoma*.