BIODIVERSITY AND ECOLOGY OF OPHIOSTOMATOID FUNGI ASSOCIATED WITH TREES IN THE CAPE FLORIST REGION OF SOUTH AFRICA

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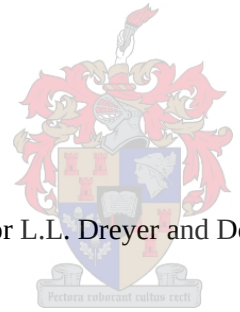

Promoters: Professor L.L. Dreyer and Doctor Francois Roets

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DECLARATION

I, the undersigned hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or part been submitted at any university for a degree.

Tendai Musvuugwa Date: 31/01/2014

SUMMARY

Very little is known about the diversity of fungi associated with Afromontane forests of the Cape Floristic Region (CFR) of South Africa. The ophiostomatoid fungi include many species, some known as pathogens in the CFR, while others are well-known saprophytes important in wood degradation. This study focused on the biodiversity and ecology of tree-associated ophiostomatoid fungi (Ophiostomatales) in the CFR. In addition to this, mites and subcortical beetles associated with the CFR trees were collected, regardless of whether they were associated with ophiostomatoid fungi or not. A relatively high diversity of ophiostomatoid fungi were collected from native trees, ten of which were newly described here. Three further fungal species, two of which are probably new to science, were also collected from exotic *Pinus* species growing in these forests. Four Ophiostomatales species (including three newly described species) were associated with subcortical beetles on *Rapanea melanophloeos* and *Olea capensis ssp. macrocarpa*. These were *Sporothrix pallida*, *Sporothrix aemuluphilus*, *Raffaelea scabbardiae* and *Raffaelea rapaneae, associated* with the beetles *Lanurgus* sp. 1, *Ctonoxylon* sp. 1, *Xyleborinus aemuluphilu*s and a Platypodinae species. This represents a first study to explore the associations between subcortical beetles and ophiostomatoid fungi on native trees in the CFR. In addition to fungi associated with subcortical beetles, several members of the Ophiostomatales associated with wounds on *Rapanea melanophloes* trees were also collected. These included *Ophiostoma stenoceras*, *Sporothrix reniformis*, *S. rapaneae*, *S. lunateae* and *S. noisomeae*. All but *O. stenoceras* were new to science, and were formally described here. All of these wound-associated species from *R. melanophloeos* belong to the *Sporothrix schenckii – O. stenoceras* complex, except for *S. noisomeae* that was provisionally placed in the *S. lignivora* complex. Besides fungal taxa collected from wounds on *Rapanea melanophloeos*, other fungi were also collected from wounds on other host trees species. Three more previously undescribed ophiostomatoid fungal species were collected from this niche. They included *Sporothix capensis* collected from *O. capensis* ssp. *macrocarpa*, *Graphilbum roseus* collected from many different, unrelated host trees and *Graphium ilexiense* (Microascales), isolated from wounds on *Ilex mitis*. The latter represented the first isolation of an ophiostomatoid fungus from this host tree species. Two possibly new fungal species (*Sporothrix* sp. 1, *Ceratocystiopsis* sp. 1) and *Ophiostoma ips*, associated with three bark beetles (*Orthotomicus erosus*, *Hylurgus ligniperda* and *Hylastes angustatus*), were collected from *Pinus*. Several fungal species were collected from both native trees and non-native trees. These included *Sporothrix fusiforme* from *Brabejum stellatifolium* and *Acacia mearnsii*, *O. quercus* and *O. pluriannulatum*-like fungus from several native trees and from *A. mearnsii*. This suggests a possibility for host

shifting of some of these fungi between native and non-native hosts or even between different native hosts. Eight non-ophiostomatoid fungi associated subcortical beetles taxa were found also to infest native trees in the Afromontane forests and in total more than 4500 beetle individuals were collected. Some species of ophiostomatoid fungi collected in this study were found to be associated with other arthropods such as mites. Four phoretic mites species associated with ophiostomatoid fungi (*Dendrolaelaps quadrisetus*, *Histiogaster* sp. 3, *Elattoma* sp. 1 & 2) were collected. In addition, sixteen species of tree wound-associated mites were collected from 12 native trees. Of these, nine were associated with several ophiostomatoid fungi (*Graphilbum roseus*, *O. pluriannulatum*-like, *O. quercus*) that were isolated from several different host trees. This suggests that they may aid in the transport of these fungi from one host species to another.

The possible consequences of transfers of Ophiostomatales species between hosts were tested using pathogenicity tests, which highlighted that some fungi are pathogenic on several different trees. Transfers seemed most likely in fungal species isolated from wounds, especially those associated with mites, because the mites may aid in the vectoring of these. When phoretic mites were tested for their specificity to their vector beetles, they proved to be highly specific. Although some of the fungi associated with these mites and their sub-cortical beetles were also pathogenic, it is less likely for these fungi to be transferred to other host tree species due to the high specificity of their arthropod associates.

This study represents one of a few studies that focused on ophiostomatoid fungi, subcortical beetles and mites associated with trees in the Afromontane forests of South Africa. Although we collected a high diversity of Ophiostomatales members, many more still await discovery. It is recommended that future studies focus on the complex inter-organismal interactions in many of the systems uncovered in this study.

OPSOMMING

Baie min is bekend oor die diversiteit van fungi wat met die Afromontane woude van die Kaapse Floristiese Streek (KFS) van Suid Afrika geassosieer is. Die ophiostomatoïde fungi sluit baie spesies in, sommiges bekend as patogene in die KFS, terwyl ander bekende en belangrike saprofiete in houtdegradasie is. Hierdie studie het op die biodiversiteit en ekologie van die boom-geassosieerde ophiostomatoïde fungi (Ophiostomatales) in die KFS gefokus. Daarbenewens is myte en subkortikale kewers wat met die KFS bome geassosieer word ook versamel, ongeag of hulle geassosieerd was met ophiostomatoïde fungi of nie. ‘n Relatief hoë diversiteit van ophiostomatoïde fungi is van inheemse bome versamel, tien waarvan hier nuut beskryf is. Drie verdere fungi spesies, twee waarvan ook waarskynlik nuut is tot die wetenskap, is ook vanaf *Pinus* spesies versamel wat in hierdie woude gegroei het. Vier Ophiostomatales spesies (insluitend drie nuut beskryfde spesies) wat met subkortikale kewers op *Rapanea melanophloeos* en *Olea capensis* L. *ssp. macrocarpa* geassosieer is, is ook versamel. Hulle was *Sporothrix pallida*, *Sporothrix aemuluphilus*, *Raffaelea scabbardiae* en *Raffaelea rapaneae*, geassosieer met die kewers *Lanurgus* sp. 1, *Ctonoxylon* sp. 1, *Xyleborinus aemuluphilu*s en ‘n Platypodinae spesie. Hierdie verteenwoordig die eerste studie wat die assosiasies tussen subkortikale kewers en ophiostomatoïde fungi op inheemse bome in die KFS ondersoek. Addisioneel tot fungi geassosieer met die subkortikale kewers, is verskeie lede van die Ophiostomatales vanaf wonde op *Rapanea melanophloes* bome versamel. Hulle sluit in *Ophiostoma stenoceras*, *Sporothrix reniformis*, *S. rapaneae*, *S. lunateae* en *S. noisomeae*. Almal behalwe *O. stenoceras* was nuut tot die wetenskap, en is hier formeel beskryf. Al hierdie wond-geassosieerde spesies vanaf *R. melanophloeos* behoort aan die *Sporothrix schenckii – O. stenoceras* kompleks, behalwe vir *S. noisomeae* wat voorlopig in die *S. lignivora* kompleks geplaas is. Benewens fungi taxa wat van die wonde op *Rapanea melanophloes* versamel is, is ander fungi ook vanaf die wonde op ander gasheer boom spesies versamel. Drie verdere ophiostomatoïde fungus spesies is in hierdie nis versamel. Hulle sluit in *Sporothix capensis* wat vanaf *O. capensis* ssp. *macrocarpa* versamel is, *Graphilbum roseus* wat vanaf baie verskillende, onverwante gasheer bome versamel is en *Graphium ilexiense* (Microascales), wat vanaf wonde op *Ilex mitis* versamel is. Laasgenoemde verteenwoordig die eerste isolasie van ‘n ophiostomatoïde fungus vanaf hierdie gasheer boom spesie. Twee moontlik nuwe fungus spesies (*Sporothrix* sp. 1, *Ceratocystiopsis* sp. 1) en *Ophiostoma ips*, geassosieer met drie baskewers (*Orthotomicus erosus*, *Hylurgus ligniperda* en *Hylastes angustatus*) is vanaf *Pinus* versamel. Verskeie fungi spesies is van beide inheemse en nie-inheemse bome versamel. Hulle het *Sporothrix fusiforme* vanaf *Brabejum stellatifolium* en *Acacia mearnsii*, *O. quercus* en *O. pluriannulatum*-like fungus vanaf verskeie inheemse bome

en vanaf *A. mearnsii* ingesluit. Dit suggereer die moontlikheid van gasheer-skuiwing van sommige van hierdie fungi tussen inheemse en uitheemse gashere of selfs tussen verskillende inheemse gashere. Agt nie- ophiostomatoïde geassosieerde subkortikale kewers was ook versamel en in totaal is meer as 4500 kewer indiwidue versamel. Sommige ophiostomatoïde fungus spesies wat in hierdie studie versamel is, was met ander geleedpotiges soos myte geassosieer. Vier foretiese myt spesies wat met ophiostomatoïde fungi geassosieer is (*Dendrolaelaps quadrisetus, Histiogaster* sp. 3, *Elattoma* sp. 1 & 2), is versamel. Nege addisioneële myt spesies was met verskeie ophiostomatoïde spesies vanaf verskeie boomspesies geassosieer (*Graphilbum roseus, O. pluriannulatum*-like, *O. quercus*). Dit suggereer dat myte die vervoer van hierdie fungi van een gasheer spesie na die ander mag bewerkstellig.

Die moontlike gevolge van die oordrag van Ophiostomatales spesies tussen gashere is getoets deur patogeniteitstoetse. Dit het beklemtoon dat sommige fungi patogenies is op verskeie onverwante boomspesies. Oordraag van spesies is mees waarskynlik in fungi spesies wat vanaf wonde geisoleer is, veral dié wat met myte geassosieer is, want die myte mag hierdie fungi help vervoer. Toe foretiese myte getoets is vir hulle spesifisiteit tot hulle vektore, is hulle hoogs spesifiek bevind. Alhoewel sommige fungi wat met hierdie myte en hulle geassosieerde kewers geassosieer word wel patogenies is, is dit minder waarskylik dat hulle na ander gasheer bome sal verskuif as gevolg van die hoë spesifisiteit van hulle geleedpotige assosiate.

Hierdie studie verteenwoordig een van net enkele studies gefokus op ophiostomatoïde fungi, subkortikale kewers en myte wat met bome van die Afromontane woude van Suid-Afrika geassosieer is. Alhoewel ons ‘n hoë diversiteit van Ophiotomatale lede versamel het, wag baie meer fungi spesies waarskynlik nog op ontdekking. Daar word voorgestel dat toekomstige studies fokus op die komplekse inter-organismiese interaksies in baie van die sisteme wat in hierdie studie blootgelê is.

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Chapter 1: Introduction

1.1. Fungal diversity

There is a conservative estimate of about 1.5 million fungal species worldwide, but less than 10% of species are known (Hawksworth 1991). Other estimates range from 0.5- 9.9 million fungal species, but the 1.5 million estimate has been widely cited and accepted as a working hypothesis (Hawksworth 2004; 2001). The number of fungal species that have been discovered so far is thought to be a small proportion of the number that actually exists due to little or no research done in most habitats (Guarro *et al*. 1999). The kingdom Fungi includes smuts, rust, mushrooms, mildews, yeasts, molds and toadstools and is divided into the phyla Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota (Guarro *et al*. 1999). The phylum Ascomycota is the largest in the kingdom, including about half of all the known fungal species and approximately 80% of all known pathogenic and opportunistic fungi (Guarro *et al*. 1999). Basic morphological features shared by all ascomycetes are the presence of asci inside the ascomata and the presence of bilayered hyphal walls with a thin electron-dense outer layer and a relatively electron-transparent inner layer (Hawksworth *et al*. 1995). Traditionally ascomycetes have been grouped into six classes, namely Hemiascomycetes, Plectomycetes, Pyrenomycetes, Discomycetes, Laboulbeniomycetes and Loculoascomycetes (Müller & von Arx 1973). Over the years other taxonomic schemes have been established for ascomycetes, which were accepted or rejected in varying degrees (Guarro *et al*. 1999; Hawksworth *et al*. 1995). Molecular systematics, however, brought an improved understanding of the systematic affinities of different ascomycetes groups. For example one of the first ascomycete phylogenetic trees recognised three main groups, which are the basal ascomycetes, the true yeasts and the filamentous ascomycetes with fruiting bodies (Berbee & Taylor 1992). Today this classification is widely accepted.

In southern Africa, mycology was formally initiated in 1905 by Pole Evans who established a national collection of fungi in Pretoria (Crous *et al*. 2006). Initially emphasis was placed on the collection of fungal specimens, taxonomic work and the description of new species (Doidge 1950). Over time, the focus shifted to the study of important fungi that proved pathogenic to plants (Baxter 1994). This led to the study of various fungi that cause diseases on crop plants such as *Botryosphaeria* Ces. & De Not. (Denman *et al*. 2003; 2000), Xylariaceae and Valsaceae (Adams *et al*. 2005). Many new pathogenic species were also described during these studies. The ophiostomatoid fungi represent the best-studied

ascomycetous fungal group in South Africa. This group started to receive attention from plant and forest pathologists in this region in the 1990s (Agricultural News 1990), with a main focus on the genera *Ophiostoma* Syd. and *Ceratocystis* Ellis & Halst. (Marais & Wingfield 2001; Roux *et al*. 2001a). Considerable attention was also given to the ascomycetous genus *Mycosphaerella* Johanson (Crous & Braun 2003; Crous 1998), which has been studied more than most other ascomycetes genera (Crous *et al*. 2006) and the pathogenic ascomycetes on Proteaceae and Restionaceae. This culminated in the discovery and description of many new species and genera (Lee *et al*. 2003; Taylor & Crous 2000).

Although the biodiversity of South African plants (Cowling & Hilton-Taylor 1994; Davis *et al*. 1994), birds (Stattersfield *et al*. 1998) and mammals (Kerley *et al*. 2003; Brooks *et al*. 2001) have been well-studied, almost nothing is known about the microorganisms associated with native plants in the country. In fact, fungal diversity has been under-studied worldwide compared to plants (Hawksworth 2004). More than 200 000 fungal species are thought to be associated with South African plants, but of these only approximately 800 endemic fungal species have been described (Crous *et al*. 2006).

The southern tip of the African continent is floristically exceptionally rich and has been described as one of 6 global Floral Kingdoms (Takhtajan 1986). The Cape Floral Kingdom represents the only floral kingdom confined to a single country and occupies an area of only approximately 90 000 km^2 (Cowling & Heijnis 2001). It was redefined as Cape Floristic Region (CFR) by Goldblatt & Manning (2002). They reported very high floral diversity within the CFR, including about 9000 vascular plant species, 70% of which are endemic to the region (Goldblatt & Manning 2002). The Proteaceae and Restionaceae are the defining plant families in the CFR, with 96% of Proteaceae species and 94% of Restionaceae species endemic to the region (Goldblatt & Manning 2002). Some of the plant species within the CFR (e.g. the genus *Protea*) are iconic, with substantial value linked to biodiversity, agriculture and ecotourism.

The emphasis of research on CFR fungi has been on surveying plant pathogenic and saprobic species (Lee *et al*. 2004; Crous *et al*. 2004; Swart 1999; Knox-Davies *et al*. 1986). With the possible exception of mycorrhizae, virtually no studies on CFR fungi have evaluated symbiotic interactions between fungi and other organisms. The deficiency in knowledge of CFR fungal biodiversity has resulted in an overwhelming lack of research on the ecology of CFR fungi. This is surprising, as symbioses are extremely important in shaping biological systems at various trophic levels and includes, amongst others, the role of mutualists and

pathogens in regulating plant diversity and abundance (Boucher *et al*. 1982). The role of mutualisms is of particular interest, as these often allow organisms to take advantage of new, otherwise unsuitable, niches (Boucher *et al*. 1982). Therefore there is clearly an urgent need to document fungal diversity within the CFR and to assess the ecological function of both native and exotic species.

1.2. Ecological importance of fungi

Fungi play a key role in ecosystem functioning. Their presence in the ecosystem can be beneficial, but may also lead to devastating consequences to other organisms and the environment. Some of the important ecological roles include being pathogens and/or mutualists of plants and animals, decomposers of organic matter, animal food and biocontrol agents for some pests (Sace 2010).

1.2.1. Importance of fungi as Mycorrhizae

Fungi may influence plant health positively or negatively. Mychorrhizal fungi form mutualistic relationships with about 75-80% of vascular plants (Smith & Read 1997; Hawksworth 1991), which involve associations between fungal hyphae and plant roots. There are different types of mychorrhizal fungi, including endomycorrhizae and ectomycorrhizae. The fungal hyphae of endomycorrhizae enter plant root cells, while ectomycorrhizal fungi associate with plant roots externally (Cairney 2000). Arbuscular mycorrhiza is the best-known type of endomycorrhizal fungi and forms associations with about 80% of the world's plant species. Ectomycorrhiza are common in woodlands and mostly form associations with trees such as oaks, spruces, firs and pines (Cairney 2000). In some cases they form rhizomorphs, which are thick hyphal strands that conduct water and nutrients over long distances. Fungi benefit from the mutualistic relationship by obtaining sugars as food source and in turn the fungi supply nutrients, mostly phosphorus and water, to their associated plant's roots through their network of hyphae in the soil. Mychorrhizae also produce a protein that helps to glue small soil particles together, resulting in good soil structure that provides more air spaces for soil organisms and plant roots, as well as allowing easy movement of water (Smith & Read 1997).

1.2.2. Importance of fungi as biocontrol agents

Fungi also play a very important role as parasites in the natural biocontrol of other organisms (Hawksworth 1991). Compared to using chemical pesticides, this method is cheaper and less damaging to the environment. For example, the Chinese caterpillar fungus is useful for controlling insect pests on crops, as it parasitizes insects. Spores of the fungi are sprayed on the insect pests (Pegler *et al*. 1994). Colorado potato beetles that can lead to huge losses are also controlled by fungi (Storch & Dill 1987). Other pests that have been controlled using fungi include leaf hoppers, citrus rust mites and spittlebugs (Bobick *et al*. 2004; Kendrick 1992; Cooke 1977).

1.2.3. Importance of fungi in fungus farming

Fungi are an important food source for different organisms, including nematodes, small mammals, insects and molluscs. They are also important mutualists of wood boring insects and therefore important in the breakdown and nutrient cycling of dead plant material (Hawksworth 1991). For example, members of the Microascales and Ophiostomales are the only food source of approximately 2 000 Scolytinae ambrosia beetles species. For this reason these fungi are actively cultivated by the beetles (Jordal & Cognato 2012). The active care and maintenance of the fungal crops by the beetles is referred to as fungus farming (De Fine Licht & Biedermann 2012). Ambrosia beetles have specialised pockets (mycangia) on their bodies in which fungal spores are vectored. The female beetles seek out recently dead trees, bore into their xylem and excavate new tunnels. Whilst doing this, mutualistic fungal spores are inoculated in the wood from mycangia or via beetle faeces (De Fine Licht & Biedermann 2012; Jordal & Cognato 2012). Fine mycelia grow into the wood from these spores. As soon as the beetle eggs hatch the larvae feed on the fungal structures that cover the tunnel walls. In some species the galleries are further expanded at this stage (Jordal & Cognato 2012; Beaver 1989). The primary mutualistic ambrosia fungi are usually species of the ascomycete genus *Raffaelea* Arx & Hennebert. Other secondary symbionts include other filamentous fungi such as *Graphium* Corda, *Ophiostoma* Syd., *Paecilomyces* Bainier and *Penicillium* Link (Biedermann 2012; De Fine Licht & Biedermann 2012; Kajimura & Hijiii 1993). Initially the primary mutualistic fungi dominate the gallery microbial flora, while eggs are laid and the larvae develop. It is only after the first offspring have matured that other saprobic fungi such as *Paecilomyces* and *Penicillium* appear and increase in frequency (Kajimura & Hijii 1993). Besides being a food source for the ambrosia beetles, the mutualistic fungi provide steroids

required by the beetles for physiological processes such as hormone production, moulting and metamorphosis (Jordal & Cognato 2012; Kok *et al*. 1970). Bark beetles also supplement their diet with fungi, mostly fungi belonging to the Ophiostomales, but this association is believed to be facultative in most cases. This means that the life cycle of the beetles can be completed even in the absence of fungi in their diets (Jordal & Cognato 2012). Addition of fungi as a supplement to their diet can, however, increase the fitness of the beetles, as was shown to be the case for *Dendroctonus* Erichson species (Six & Paine 1998).

Besides the ambrosia beetles, two other insect lineages, fungus-growing ants and fungus-growing termites, are regarded as true fungus farmers (De Fine Licht & Biedermann 2012). The agricultural mutualistic symbiosis between fungi and fungus-growing termites and leafcutter ants are among the most impressive animal phenomena in the world (De Fine Licht & Biedermann 2012). Leafcutter ants are endemic to South and Central America, Mexico and some parts of the southern United States. They are restricted to the two genera *Atta* Fabricius and *Acromyrmex* Mayr, which cut and process fresh plant material to serve as a nutritional substrate for their fungal mutualist partners (Schultz & Brady 2008). Different species of leafcutter ants use different species of fungi, although all the fungi used by the ants belong to the Lepiotaceae family. The fungi benefit from the ants by feeding on freshly cut plant material, which is kept free from moulds and pests by the ants. In cases where a leaf type is toxic to the fungus, the fungus release chemical signals that the ants can detect. Ants then stop collecting such toxic leaves. The ants in turn benefit by feeding their larvae on the cultivated fungi (Sunijian 2012). This agricultural mutualistic symbiosis between the ants and fungi has allowed the leafcutter ants to occupy previously inaccessible niches that have abundant resources (Waller 1988).

Fungus-growing termites (Isoptera; subfamily Macrotermitinae,) also have a spectacular and sophisticated agricultural mutualistic symbiosis with fungal species in the genus *Termitomyces* R. Heim, phylum Basidiomycota. The fungus-growing termites are ecologically dominant in savannas, although they also occur in some African rain forests (Wood & Sands 1978). Fungal crops are cultivated on combs constructed from faecal material found within the colonies of these fungus-growing termites (Aanen & Eggleton 2005; Darlington 1994). The fungus (*Termitomyces*) is a white-rot fungus and it benefits from the relationship by being continuously provided with plant substrates. The termites also provide a highly regulated and constant growth environment for their mutualist fungal partners (Nobre *et al.* 2011). The fungus is among the few organisms that can digest lignin (Bignell 2000). The termites benefit by feeding on older plant parts, which would have been well-

decomposed by the fungi (Aanen & Boomsma 2005), therefore allowing the termites to exploit complex plant substrates (Nobre *et al*. 2011). Cultivation of fungi by fungus-growing termites has led to them become one of the most important decomposer groups in the Old World Tropics (tropical Africa and parts of Arabia and Indomalaya) (Aanen & Eggleton 2005). For example, they are the predominant decomposers in dry savannas, accounting for up to about 20% of all C-mineralization (Wood & Sands 1978). Just as in the case of leafcutter ants, this agricultural mutualistic symbiosis with fungi has allowed the termites to occupy previously inaccessible niches that have abundant resources (Waller 1988).

1.2.4. Importance of fungi as plant pathogens

Compared to viruses and bacteria, fungi are considered to be the biggest killers on the planet (Jones 2013), responsible for over 70% of recorded regional and global extinctions (Fisher *et al*. 2012). Pathogenic fungi can lead to devastating declines in plant species, ultimately leading to changes in forest composition and landscapes (Douglas 2008). Before the 1900s the American chestnut was one of the most dominant and important hardwood tree species in the Eastern United States. It was used commercially as source of pulpwood, lumber, tannins, poles, edible nuts and railroad ties. The introduction of the non-native fungus *Cryphonectria parasitica* (Murrill) Barr into the United States from Eastern Asia led to the infection of chestnut trees with the chestnut blight fungus, which almost eradicated the tree species from the forests (Douglas 2008; Heiniger & Rigling 1994). The fungal pathogen enters trees through wounds and cracks. It grows in and under the bark, killing the area around the tree twig, branch or trunk. Large cankers are formed that eventually spread over the entire tree surface, ultimately killing the tree. Most of the sprouts that continue to grow from the stumps today still usually succumb to the disease and die (Douglas 2008; Heiniger & Rigling1994).

The woodwasp, *Sirex noctilio* Fabricus, along with its symbiotic fungi *Arnylostereurn areolatum* (Fr.) Boid. and *A. chailletii* (Pers.: Fr.) Boid, are responsible for major losses of *Pinus* plantations where they have established in the Southern Hemisphere (Hurley *et al*. 2007; Madden 1988). The woodwasp has become a significant pest of exotic Pine plantations in countries such as South Africa, New Zealand, Australia and some countries in South America, where it was accidentally introduced on *Pinus radiata* D. Don and other *Pinus* species during the 20th century (Hurley *et al*. 2007; Carnegie *et al*. 2005; Tribe & Cillié 2004; Klasmer *et al*. 1998; Madden 1988; Neumann *et al*. 1987; Madden 1975; Rawlings & Wilson 1949). The woodwasp and its associated fungi originate from temperate regions of the

Northern Hemisphere and of Eurasian (Spradbery & Kirk 1978). It is also native to northern Africa and it is not an important pathogen in its native range (Spradbery & Kirk 1978). *Arnylostereurn areolatum* is spread through asexual arthrospores, which are vectored in the mycangia of female *S. noctilio* (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999). The female *S. noctilio* lays eggs into stressed trees and at the same time deposits its associated fungus (*A. areolatum*, along with a phototoxic mucus) (Morgan & Stewart 1996; Neumann & Minko 1981; Taylor 1981). The combination of *A. areolatum* and the mucus eventually leads to death of trees drilled by *S. noctilio* (Carnegie *et al*. 2006). The host range of *S. noctilio* in *Pinus* is wide, but the most susceptible species are *P. radiata, P. taeda* Blanco and *P. patula* Schltdl. & Cham. (Carnegie *et al*. 2005; Tribe & Cillié 2004; Spradbery & Kirk 1978). In southern Australia over 5 million trees, with a value of between A$10-12 million, were killed between 1987 and 1989 due to attack by *S. noctilio* and its associated fungus (Haugen *et al*. 1990). *Sirex noctilio* was first detected in the Western Cape Province of South Africa in 1994 (Tribe 1995). By 2002 it had spread to the Eastern Cape Province, followed by KwaZulu-Natal, where extensive damage in *P. patula* plantations have been recorded (Tribe & Cillié 2004).

In South Africa many additional plant species are threatened by native micro-organisms, as well as by those that have yet to reach South Africa's shores. For example, just recently a new and serious stem canker disease on the native *Rapanea melanophloeos* Mez. has been discovered in a botanical garden in Western Cape Province of South Africa (Chen *et al*. 2013). *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf. & Jol. Roux is the fungal pathogen responsible for this canker disease and poses a major threat to the survival of *R. melanophloeos*. At the moment the origin of the pathogen is unknown, although it is suspected that it might have been introduced (Chen *et al*. 2013). Similarly, numerous ophiostomatoid fungi are known as pathogens of native and introduced trees in South Africa, some of which have been introduced (Kamgan 2008; Roux *et al* 2004a & b; Roux & Wingfield 1997). The group not only includes serious plant pathogens, but also pathogens of humans and saprophytes that are important in degradation (De Meyer *et al*. 2008; Marimon *et al*. 2008; 2007; Aghayeva *et al*. 2004; Zhou *et al*. 2004; Hektoen & Perkins 1900). This group of fungi therefore provides an excellent model system on which to base a study of the importance and role of micro-organisms in the Cape Floristic Region of South Africa.

1.3. Taxonomy of ophiostomatoid fungi

The ophiostomatoid fungi represent a polyphyletic group of morphologically similar genera that are artificially grouped together (De Beer *et al*. 2013a; Spatafora & Blackwell 1994). The morphological similarities of these fungi, as well their structural evolutionary convergence due to adaptation to insect dispersal, have led to these taxa being studied as a collective (Wingfield *et al*. 1993). Phylogenetically many constituent taxa are, however, not closely related despite the similarities in their morphology (De Beer *et al*. 2013a; Viljoen *et al*. 1999). Ophiostomatoid fungi belong to genera that are located in two orders, namely the Ophiostomatales and Microascales. Genera found in the Ophiostomatales include *Ophiostoma*, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Leptographium* Lagerb. & Melin, *Raffaelea*, *Fragosphaera* Shear and *Graphilbum* H.P. Upadhyay & W.B. Kendr. (De Beer *et al*. 2013a & b; Zipfel *et al* 2006; Upadhyay 1981). *Ophiostoma sensu stricto* is central to the Ophiostomatales and includes several species complexes such as the *Ophiostoma ulmi* complex, *Ophiostoma pluriannulatum* complex and the *Ophiostoma ips* complex. *Ophiostoma s. str.* is part of *Ophiostoma sensu lato*, a larger contingent that is not well-defined at this stage, and includes groups such as the *Sporothrix schenckii – O. stenoceras* complex (De Beer *et al*. 2013a & b). Although De Beer *et al*. (2013a) showed that the *S. schenckii – O. stenoceras* complex forms a lineage distinct from *Ophiostoma s. str*., and may represent a distinct genus, at present they treat the complex as part of *Ophiostoma s. l. Leptographium s. l.* is another major clade found in the Ophiostomatales, but it is non-monophyletic. Several species complexes and lineages found in *Ophiostoma s. l.* and *Leptographium s. l.* still need their generic status reconsidered and reassessed (De Beer *et al*. 2013a). Since the delineation of the genera *Ceratocystiopsis* and *Graphilbum*, their close relationship with *Ophiostoma* has been clear (Upadhyay & Kendrick 1975), while *Raffaelea* and *Fragosphaera* were initially classified in other orders (De Beer *et al*. 2013a).

The order Microascales include the families Ceratocystidaceae, Gondwanamycetaceae and Graphiaceae. Genera belonging to these families include *Ceratocystis* Ellis & Halst., *Cornuvesica* Viljoen, M.J. Wingf. & Jacobs, *Custingophora* Stolk, Hennebert & Klopotek, *Graphium*, *Sphaeronaemella* P. Karst. and *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas (De Beer *et al*. 2013b). *Ceratocystis sensu lato* contains some well-defined species complexes, which form around *C. fimbriata* Ellis & Halst. and *C. caerulescens* (De Beer *et al.* 2013b). De Beer *et al*. (2013b) placed the genus *Cornuvesica* in the Ceratocystidaceae based on phylogenetic analyses, its beetle-associated ecology and anamorphs that are similar to those of *Ceratocystis* (Hutchinson & Reid 1988). Its separation from *Ceratocystis* is

supported by phylogenetic distance (De Beer *et al*. 2013b). The genus *Custingophora* is currently known only from its anamorphs, with only one species, *Custingophora olivacea* Stolk, Hennebert & Klopotek, belonging to this genus (De Beer *et al*. 2013b). Previously the anamorphs of *Knoxdaviesia* were treated in *Custingophora* (Reblova & Winka 2000), but based on phylogenetic distance, De Beer *et al*. (2013b) treated *Custingophora* as distinct from *Knoxdaviesia*. Species belonging to *Graphium* are only known as anamorphs and belong to the Graphiaceae. The description of the Graphiaceae was justified by the phylogenetic distance between *Graphium* and other families in the Microascales (De Beer *et al*. 2013b). Currently the status of *Sphaeronaemella* in the Microascales is uncertain, as the presence of gaps in the sequences of some species belonging to this genus complicates the alignment and analysis of DNA sequences with other groups. This reduces the confidence in its phylogenetic placement (De Beer *et al*. 2013b).

1.4. Dispersal ecology of ophiostomatoid fungi

Regular vectors of ophiostomatoid fungi include various wood- and sap-feeding beetles (Jacobs & Wingfield 2001; Bridges & Moser 1983; Upadhyay 1981; Barras & Perry 1975). In the Northern Hemisphere, ophiostomatoid fungi are best known as associates of bark beetles (Coleoptera: Curculionidae, Scolytinae) on conifers (Kirisits 2004; Paine *et al* 1997; Wingfield *et al*. 1993). Ophiostomatoid fungi have developed several adaptations to enable them to be dispersed by arthropods (Malloch & Blackwell 1993). In most cases these fungi have a sexual form that produces long-necked ascomata. Ascospores, which are mostly produced in evanescent asci within the bases of the ascomata, are exuded through the necks and are presented in sticky masses on the apices of the necks, which facilitate dispersal by arthropods (Malloch & Blackwell 1993). The spores have adhesive coats that allow them not to be washed away by water. This ensures that they are only released from their vectors when an appropriate substrate such as a tree host is encountered (Whitney 1982). Beetle-associated fungi benefit from their hosts (beetles) by being transported from one tree to the other (Dowding 1969). In addition to transportation to new substrates, the fungi also benefit from this association by being actively cared for by the beetles (notably ambrosia beetles), which protect the symbiotic fungi from antagonistic fungal species (Beaver 1989; Francke-Grosman 1967).

Many of the vector beetles have evolved specialised spore carrying structures known as mycangia (Batra 1963). A broader definition of mycangium regards them as any structure that

consistently transports fungi, regardless of its form or the presence of secretory cells (Furniss *et al*. 1987; Beaver 1986). This means shallow pits, setae and deeper pockets that act as fungal repositories and are not associated with any glands, can also be viewed as mycangial (Klepzig & Six 2004). Six (2004) has gone a step further to define mycangia based on three morphological forms. She recognizes pit mycangia, sac mycangia and setal brush mycangia. The pit mycangium has all its fungal repositories formed by shallow depressions of the exoskeleton, with or without setae. The sac mycangium consists of complex invaginations, which form deep pockets, tubes or cavities in the exoskeleton. The setal brush mycangium has dense brushes of setae, which may or may not arise from depressions in the exoskeleton (Six 2004). These types can be further subdivided based on the presence or absence of glands (Six 2004). However a specialist view of a mycangium defines it as an invagination of the integument lined with glands or secretory cells that is specialised for the acquisition and transport of fungi (Levieux *et al*. 1991; Batra 1963). Besides the transport of fungi, the mycangia also help to maintain the bark beetle-fungus symbioses (Klepzig & Six 2004) by protecting the fungi from ultra violet light and prevent desiccation in transit (Klepzig & Six 2004). Mycangial glands may also produce secretions that help to protect and support the growth of fungal propagules and may also selectively act against non-symbiotic fungi (Paine & Birch 1983; Barras & Perry 1972; Happ *et al*. 1971).

The relationships between these vectors and their fungal partners are often mutualistic and the system thus serves as a model for the study of multi-organism interactions and evolution (e.g. Ayres *et al*. 2000; Six & Paine 1998; Paine *et al*. 1997). Beetles benefit from this mutualism in that the fungal symbionts supplement beetle nutrition (Eckhardt *et al*. 2004; Baker & Norris 1968; Six & Paine 1998) by concentrating nitrogen (Ayres *et al*. 2000) in the nutritionally poor woody environment. A good example is that of the southern pine beetle, *Dendroctonus frontalis* Zimmermann, which is helped by its mycangial fungal symbionts to meet its nitrogen needs (Klepzig *et al*. 2001a). Aryes *et al*. (2000) showed that these mycangial fungal symbionts could concentrate nitrogen better than an antagonistic non-mycangial associate. Due to this, the southern pine beetle consumes less phloem than beetle species not associated with mutualistic fungi or lacking mycangia (Klepzig & Six 2004). Beetles feed on fungi at both the larval and adult stages (Klepzig & Six 2004). In beetles, sterols are essential to provide elements for cellular structures, important for hormone synthesis and also needed for viable egg production. In most plant tissues sterol concentrations are very low and in most cases sterols produced by plants cannot be utilised by insects (Clayton 1964). It is most likely that fungi provide a source of sterols to their host

beetles, for example ergosterol, which is a major sterol produced by fungi and also mostly used by insects for their nutrition (Svoboda *et al*. 1978; Clayton 1964). To support this, Fox *et al*. (1993) and Six & Paine (1998) have shown that levels of pupation and oviposition in a number of bark beetle species are reduced in the absence of their symbiotic fungi. In some cases bark beetle-associated fungi exerts competition against antagonistic fungi, therefore helping to protect beetle brood from contact with these antagonistic fungi (Klepzig & Six 2004). This helps to ensure that the bark beetle larvae comes into contact with, and only ingests, the right fungi needed for their development and survival (Klepzig & Six 2004; Klepzig & Wilkens 1997).

In addition to beetles, mites also play a significant role in the dispersal of ophiostomatoid fungi (Lombardero *et al*. 2003; Klepzig *et al*. 2001a; 2001b; Moser 1985; Bridges & Moser 1983). Just as in the case of beetles, mites have also developed specialised flap-like integument structures known as sporothecae in which spores of their mutualistic fungi are carried (Roets *et al*. 2007). For example, ascospores of *Ophiostoma minus* (Hedgcock) H. & P. Sydow and *Ceratocystiopsis ranaculosus* Perry & Bridges are frequently contained in such flap-like structures on the mite species *Tarsonemus ips* Lindquist, *T. krantzii* Smiley & Moser and *T. furasii* Cooreman (Acarina: Tarsonemidae) (Moser 1985; Bridges & Moser 1983). Again the relationship between the mites and their phoretic fungi are often mutualistic (Moser 1985; Bridges & Moser 1983). In some cases the mites feed and reproduce exclusively on their fungal partners, confirming that the association is mutualistic (Roets *et al*. 2007). For example mites that feed exclusively on the *Ophiostoma* species found in *Protea* infructescences, in turn vector these fungi (Roets *et al*. 2007). There is evidence that mites that are phoretic on wood-inhabiting beetles (Lombardero *et al*. 2003; Klepzig *et al*. 2001a; 2001b) and their fungal symbionts may interfere with normal beetle development. For example, it has been demonstrated that *O. minus*, a mutualistic associate of *Tarsonemus* species, limits the reproductive success of the bark beetle *D. frontalis* on which these mites are phoretic (Lombardero *et al*. 2003).

1.5. Ecological and anthropogenic importance of ophiostomatoid fungi

Ophiostomatoid fungi include important plant pathogens, including species that have led to significant worldwide losses in the agricultural and forestry sectors. Many species also cause plant diseases and sapstain on lumber, logs and pulpwood (Zhou *et al*. 2001), leading to high economic losses. In agriculture, many ophiostomatoid fungi that are regarded as important

pathogens belong to the genus *Ceratocystis*. For example, *C. fimbriata s.s.* is a well-known pathogen responsible for causing black rot of sweet potatoes and canker on coffee (Kile 1993). *Ceratocystis paradoxa* (Dade) Moreau is responsible for causing the rotting of pawpaw and pineapple leaves and pineapple fruit (Kile 1993).

Examples of well-known ophiostomatoid fungi that are virulent tree pathogens include *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier. These were responsible for the Dutch elm disease pandemics in North America and Europe due to the movement of *Ophiostoma* species and their vectors from Europe to North America (Brasier & Buck 2001; Heybroek 1993; Lamb 1979). The fungi were introduced into North America through the importation of infested elm timber (Brasier 1990; Peace 1960). *Ophiostoma ulmi* caused the original Dutch elm disease epidemic in the mid-1900s (Brasier 1990; Peace 1960). A more aggressive pathogen, *O. novo-ulmi*, replaced *O. ulmi* during the second half of the 20th century (Heybroek 1993; Lamb 1979). The outbreak of *O. novo-ulmi* led to catastrophic losses in Europe and had an even greater impact in North America, which resulted in millions of dead elm trees (Brasier & Buck 2001).

In Brazil, Oman and Pakistan, two ophiostomatoid fungal species have been responsible for causing serious wilt disease in Mango trees (*Mangifera indica* L.). The disease is commonly known as mango blight or seca in Brazil, where it was first reported in the 1930s. It is characterised by wilting of the leaves, flowers and stems of mango trees (Viégas 1960). *Ceratocystis fimbriata s.l.* was identified as the pathogen responsible for this disease in Brazil (Ribeiro 1980; Viégas 1960) in association with the wood-boring beetle *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytinae), which is native to Asia and is believed to be the vector of the fungus (Ploetz 2003; Wood 1982). In Oman and Pakistan, *Ceratocystis manginecans* M. van Wyk causes a similar disease to mango blight and the same wood-boring beetle, *H. mangiferae*, is also associated with this fungus (Al Adawi *et al*. 2006; Van Wyk *et al.* 2005). These two fungal species are virulent pathogens that can rapidly kill mango trees and therefore pose a very serious threat to the mango cultivation industry in these countries (Woolhouse *et al*. 2005). Similarly, the catastrophic oak die-back and mass mortality of oak trees in Japan has been attributed to the fungus *Raffaelea quercivora* Kubono et Shin. and its associated ambrosia beetle *Platypus quercivorus* (Murayama) (Kubono & Ito 2002).

In South Africa, the fungus *Ceratocystis albifundus* De Beer, Wingfield & Morris is responsible for the death of the introduced *Acacia mearnsii* De Wild (Roux & Wingfield 1997). This virulent fungus causes a disease known as Ceratocystis wilt or wattle wilt, which

has led to significant economic losses in the *A. mearnsii* forestry industry of South Africa (Roux *et al*. 1999). Some of the disease symptoms include wood discolouration, red to black discolouration of the bark, formation of cankers as well as wilting and die-back of the trees (Roux *et al*. 1999; De Beer 1994; Morris *et al*. 1993; Wingfield & Kemp 1993). Some species have been recorded as sapstaining fungi, leading to degradation of the quality of pine logs that are exported to Asian countries, resulting in great financial losses in the South African forestry industry (Zhou *et al*. 2001).

1.6. Diversity of ophiostomatoid fungi in Africa including South Africa

Compared to the rest of the world, only a few reports of ophiostomatoid fungi have come from Africa, and most of the species that have been reported belong to the genera *Ceratocystis* and *Ophiostoma* (Kamgan *et al*. 2008). Most of these reports have come from South Africa, (Table 1) and involve associations with non-native tree taxa. For example, *Ceratocystis pirilliformis* I. Barnes & M.J. Wingf. and *C. moniliformis* were both reported from *Eucalyptus* species (Roux *et al*. 2004b). *Ceratocystis fimbriata* which was also reported from a *Eucalyptus* species, causes serious wilting and dieback (Roux *et al* 2004a). *Graphium pseudormiticum* M. Mouton & M.J. Wingf., an associate of a pine-infesting bark beetle (Mouton *et al*. 1994), was isolated from *Pinus* species in South Africa.

It is interesting that a number of the reports of tree-associated *Ophiostoma* species infesting *Pinus* spp. in South Africa also involve non-native bark-beetle species. These *Ophiostoma* species are associated with three exotic pine infesting bark beetles, *Hylastes angustatus* Herbst, *Hylurgus ligniperda* Fabricus and *Orthotomicus erosus* Wollaston (Zhou *et al*. 2001; Tribe 1992). The fungal species that these exotic bark beetles are associated with include *Pesotum fragrans* (Math.-Käärik) G. Okada & Seifert, *O. piliferum* (Fr.) Syd. & P. Syd., *O. floccosum* Math.-Käärik, *O. stenoceras* (Robak) Nannf, *O. abietinum* Marm. & Butin and *O. pluriannulatum* (Hedgc.) Syd. & P. Syd. (Zhou *et al*. 2006). *Ophiostoma quercus* (Georgévitch) Nannf., which is quite a common fungus globally, has also been recorded on some exotic hosts in South Africa, including amongst others *Eucalyptus grandis* W. Hill (De Beer *et al*. 1995).

Ceratocystis albifundus was first isolated from *Acacia mearnsii* on which it was pathogenic (Wingfield *et al*. 1996; Morris *et al*. 1993). It was also isolated from this host in other African countries such as Uganda (Roux *et al*. 2001b), Kenya (Roux *et al*. 2005) and Tanzania (Roux

et al. 2005). However, it was also isolated from several native hosts in South Africa such as *Protea* species (Roux *et al*. 2004a; Wingfield *et al*. 1996; Gorter 1977), *Acacia caffra* Willd, *Burkea africana* Hook, *Combretum molle* R.Br. ex G. Don, *Faurea saligna* Harv, *Ozoroa paniculosa* (Sond.) R. Fern. & A. Fern. and *Terminalia sericea* Cambess (Roux *et al*. 2007). The discovery of this fungus on native tree hosts strengthened the view that this species is native to South Africa (Barnes *et al*. 2005; Roux *et al*. 2001a). This in turn, provided motivation to determine whether other ophiostomatoid fungi occur on native trees in South Africa. This led to the discovery of several other, often pathogenic, species (Kagman *et al*. 2008). Examples of these species include *C. tsitsikammensis* Kamgan & Jol. Roux, which was isolated from *Rapanea melanophloeos* and *Ocotea bullata* (Burch.) Baill, and was pathogenic on *Rapanea* trees. Another example was *C. savannae* that was isolated from *Acacia nigrescens* Oliv., *Combretum zeyherii* and *T. sericea* (Kamgan *et al*. 2008). *Ophiostoma quercus* (Georgev.) Nannf., which has a wide host range in both the Southern and Northern Hemisphere, has also been reported from South Africa on native hosts including *R. melanophloeos* and *Terminalia sericea* (Kamgan *et al*. 2008; De Beer *et al*. 1995), while a recently described *Graphium* species, *G. adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux, was isolated from *Adansonia digitata* L. (Cruywagen *et. al* 2010).

Various ophiostomatoid fungi have been reported from an unusual habitat, the seed cones of native *Protea* species in South Africa. Examples include *Ophiostoma phasma* Roets, Z.W. de Beer & M.J. Wingf., *O. africanum* G.J. Marais & M.J. Wingf., *O. protearum* G.J. Marais & M.J. Wingf., *O. splendens* G.J. Marais & M.J. Wingf., *O. gemellus* Roets, Z.W. de Beer & Crous, *O. palmiculminatum* Roets, Z.W. de Beer & M.J. Wingf. and the anamorphic *Sporothrix variecibatus* Roets, Z.W. de Beer & Crous (Roets *et al*. 2008; 2006; Marais & Wingfield 2001; 1997; 1994). These fungi are thought to be confined to the infructescences of various *Protea* species and have developed a symbiotic relationship with insects and mites that associate with these plants (Roets *et al*. 2007). In this system mites are the primary vectors of *Ophiostoma* species and also feed and reproduce exclusively on their fungal partners, confirming that the association is mutualistic (Roets *et al*. 2007). The mites have evolved specialized structures that frequently contain spores of *Ophiostoma* (Roets *et al*. 2007). Long distance dispersal of these mites is achieved by phoresy on various beetle species that frequent *Protea* flowers (Roets *et al*. 2009a). Although focussing on a single plant genus, these studies have resulted in the identification of at least seven *Ophiostoma* species new to science and have provided data for the first molecular study on fungal radiation patterns in the CFR (Roets *et al*. 2009b).

Besides South Africa, the only other country from where *Protea*-associated members of *Ophiostoma* have been described is Zambia. Roets *et al*. (2010) described *Ophiostoma protea-sedis* Roets, M.J. Wingf. & Z.W. de Beer and *O. zambiensis* Roets, M.J. Wingf. & Z.W. de Beer from *Protea* species in Zambia, which brought the number of known *Ophiostoma* species associated with *Protea* infructescences to nine. In addition to *C. albifundus*, *C. fimbriata* and *O. zambiensis*, only a few other ophiostomatoid fungi have been recorded from African countries other than South Africa. Examples include *Leptographium eucalyptophilum* K. Jacobs, M.J. Wingf, & Jol. Roux reported from *Eucalyptus* trees in the Democratic Republic of the Congo (Jacobs *et al*. 1999), *Graphium madagascariense* Cruywagen & Z.W. de Beer and *G. fabiforme* Cruywagen & Z.W. de Beer that were isolated from *Adansonia rubrostipa* Jum. & H. Perrier in Madagascar. These presented the first *Graphium* species to be reported from angiosperms in the tropics or subtropics (Cruywagen *et. al*. 2010).

Table 1: Summary of ophiostomatoid fungi recorded from South Africa, including their hosts, collection location and vector associates (Roets *et al*. 2010; Kamgan *et al* 2008; Zhou *et al*. 2001; Cruywagen *et al*. 2010; Zhou *et al*. 2006; Doidge 1950; Gorter 1977; Talbot 1956; Marasas *et al*. 1966; Scott & Du Toit 1970; Eicker 1974; Jooste 1978; Wingfield & Marasas 1980; Wingfield & Marasas 1983; Wingfield *et al*. 1988; Wingfield *et al.* 1993; Wingfield & Van Wyk 1993; De Beer *et al*. 1995; 2003; Linde & Smit 1999; Marais & Wingfield 2001; Barnes *et al*. 2003; Lee *et al*. 2004; Roux *et al*. 2004b)

Species	**Host**	**Collection location**	**Associated arthropods**
Ophiostoma abietinum	*Pinus* spp.	Mpumalanga	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*
Ophiostoma africanum	*Protea dracomontana*	KwaZulu-Natal	
	Protea caffra	Gauteng	
	Protea gaguedi	Unknown	
Ophiostoma aurorae	*Pinus* spp.	Mpumalanga	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*

Ophiostoma floccosum	*Pinus elliottii*	Mpumalanga	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*
Ophiostoma galeiforme	*Pinus pinaster* *Pinus elliottii* *Pinus patula*	KwaZulu-Natal, Mpumalanga	*Hylurgus ligniperda*
O. gemellus	*Protea caffra*	Gauteng	*Tarsonemus* sp.
Ophiostoma ips	*Pinus elliottii* *Pinus patula* *Pinus radiata*	KwaZulu-Natal, Mpumalanga Western Cape	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*
Ophiostoma longiconidiatum	*Terminalia sericea* *Faurea saligna*	Leeuwfontein collaborative nature reserve, Mpumalanga	
Ophiostoma. palmiculminatum	*Protea repens*	Western Cape	*Trichouropoda* sp.
Ophiostoma phasma	*Protea laurifolia*	Western Cape	
Ophiostoma piliferum	*Pinus spp.*	Mpumalanga	*Orthotomicus erosus* *Hylastes angustatus* *Hylurgus ligniperda*
Ophiostoma piceae	*Pinus patula* *Pinus elliottii*	KwaZulu-Natal, Mpumalanga	*Hylurgus ligniperda*
Ophiostoma pluriannulatum	*Pinus patula* *Pinus elliottii* *Pinus radiata* *Pinus pinaster*	KZ-Natal, Mpumalanga Western Cape	*Orthotomicus erosus* *Hylastes angustatus* *Hylurgus ligniperda*
Ophiostoma	*Protea caffra*	Gauteng	

protearum			
Ophiostoma quercus	*Olinia* sp.	Unknown	
Ophiostoma quercus	*Eucalyptus grandis* *Quercus robur* *Terminalia sericea* *Rapanea melanophloeos* *Pinus*	Leeuwfontein collaborative nature reserve, Mpumalanga Groenkloof Forest, Tsitsikamma, Western Cape Mpumalanga	*Orthotomicus erosus* *Hylastes angustatus* *Hylurgus ligniperda*
Ophiostoma splendens	*Protea repens* *Protea neriifolia*	Western Cape	Beetle associate
O. stenoceras	*Pinus patula* *Pinus elliottii* *Pinus radiata* *Pinus pinaster* *Acacia mearnsii* *Eucalyptus* spp. *Malus* sp.	KwaZulu-Natal, Mpumalanga Western Cape	*Hylastes angustatus* *Hylurgus ligniperda*
Sporothrix schenckii	Human		
Sporothrix variecibatus	*Protea longifolia* *Protea repens* *Eucalyptus* leaf litter	Western Cape	*Trichouropoda* sp.
Sporothrix sp.	*Pinus* spp.		*Orthotomicus erosus,* *Hylastes angustatus,* *Hylurgus ligniperda*
Ceratocystis adiposa	Shoots of *Pinus* sp.		
Ceratocystis albifundus	*Terminalia sericea*	Kruger National Park, Mpumalanga	

	Acacia mearnsii	Western Cape	
	Combretum zeyherii		
	Protea sp.		
Ceratocystis fimbriata	Rhodocoma gigantea		
Ceratocystiopsis minuta	*Pinus elliottii*	KwaZulu-Natal, Mpumalanga	*Hylastes angustatus, Hylurgus ligniperda*
	Pinus patula	Western Cape	*Orthotomicus erosus*
	Pinus radiata		
Ceratocystis moniliformis	*Erythrina sp.*		
	Eucalyptus grandis		
Ceratocystis radicicola	*Phoenix dactylifera*		
Ceratocystis pirilliformis	*Eucalyptus grandis*		
Ceratocystis savannae	*Acacia nigrescens*	Kruger National Park, Mpumalanga	
	Combretum zeyherii	Leeuwfontein collaborative nature reserve, Mpumalanga	
	Terminalia sericea		
	Sclerocarya birrea		
	Burkea africana		
Ceratocystis tsitsikammensis	*Rapanea melanophloeos*	Groenkloof Forest, Tsitsikamma, Western Cape Province	
	Ocotea bullata		
Pesotum fragrans-like	*Rapanea melanophloeos*	Groenkloof Forest, Tsitsikamma	*Orthotomicus erosus*
	Rhus chirindensis	Mpumalanga	*Hylastes angustatus*
	Pinus spp.		*Hylurgus ligniperda*
Leptographium lundbergii	*Pinus patula*	KwaZulu-Natal, Mpumalanga	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*
	Pinus radiata	Western Cape	

Leptographium procerum	*Pinus patula* *Pinus radiata*	Western Cape	*H. angustatus*
Leptographium reconditum	*Triticum rhizosphere*		
Leptographium serpens	*Pinus elliottii* *Pinus patula* *Pinus radiata* *Pinus pinaster*	KwaZulu-Natal, Mpumalanga Western Cape	*Orthotomicus erosus, Hylastes angustatus, Hylurgus ligniperda*
Leptographium procerum	*Pinus* sp.		*Hylastes angustatus*
Leptographium truncatum	Roots of *Pinus taeda*		
Graphium adansoniae	*Adansonia digitata*	Musina, Limpopo Kruger National Park, Mpumalanga	
Graphium putredinis	Soil		
Graphium pseudormiticum	*Pinus sp.*		*Orthotomicus erosus*
Thielaviopsis basicola	*Nicotiana tabacum*		
Thielaviopsis paradoxa	*Saccharum officinarum*		
Raffaelea albimanens	*Ficus sycamorus*		*Platypus externedentatus*
Raffaelea hennebertii	*F. sycamorus*		*Platypus externedentatus*
Raffaelea arxii	*Cussonia umbellifera*		*Xyleborus torquatus*
Knoxdaviesia capensis	*Protea* spp.		
Knoxdaviesia proteae	*Protea repens*		
Grosmannia serpens	*P. pinaster, P. radiata*		

Hyalorhinocladiella sp	*Pinus spp*	*Ohortomicus erosus, Hylastes angustatus, Hylurgus. ligniperda*

1.7. Successful utilisation of newly encountered hosts

There is a worldwide increase in the spread of fungi, mainly due to anthropogenic movements (Anderson *et al*. 2004). In a number of cases these movements have resulted in host switching by the fungi, which in some cases has been aided by their vectors (Woolhouse *et al*. 2005; Wingfield 2003). This has caused significant disease epidemics, which have threatened some new hosts with extinction (Slippers *et al*. 2005). In Europe there is currently a spread of invasive pathogens on pines, horse chestnuts, alders, oaks and cypresses, and in some of these instances these pathogens are very aggressive (Brasier 2008). Events of host switching seem to be accelerated by the increasing globalisation of trade in plants, as well as flaws in the international protocols of plant biosecurity (Brasier & Webber 2010). Introduction of a pathogen beyond its normal range provides a sudden exposure to new biotic and abiotic influences such as different climates, different vectors, new hosts and new biological competitors. There is usually limited resistance by the new host population and excessive aggressiveness by the introduced pathogen due to lack of prior co-evolution, which may lead to devastating outbreaks of disease (Brasier 2008; Brasier & Buck 2001).

One of the well-known examples of host switching is the chestnut blight disease caused by the pathogenic fungus *Cryphonectria parasitica* (Murrill) Barr on the American chestnut trees *Castanea dentate* (Heiniger & Rigling1994). The fungus was introduced into North America through the importation of Japanese chestnut trees *Castanea crenata* Siebold and Zuccarini, which hosted the fungus (Brasier 2008). The American chestnut that had never been exposed to this fungus before was highly susceptible, especially the larger trees. The American chestnut forests were destroyed by the disease throughout most of their natural range in the USA within thirty years (Brasier 2008). The fungus, which can easily be vectored from one tree to the other by a variety of agents such as insects, small animals, wind and rain, was responsible for nearly eradicating this tree species in its native range. Other examples include *Puccinia psidii* Winter that switched hosts from native Myrtaceae to introduced *Eucalyptus* trees in South America and Brazil (Coutinho *et al*. 1998) *Fusarium circinatum* Nirenberg & O'Donnell that was introduced from native Mexican pines and switched to *Pinus radiata* in

California U.S.A (Gordon *et al*. 2001) and *Phytophthora ramorum* Werres, De Cock & Man in't Veld that switched hosts from *Rhododendron* onto evergreen oak and tanoak trees in Coastal California and Oregon, killing several million trees in the process (Grünwald *et al*. 2008). The latter species also switched hosts from *Rhododendron* to larch trees in Europe (Brasier & Webber 2010).

A number of fungal species have also switched hosts in South Africa. *Armillaria mellea*, (Vahl. Fr.) Kummer is an alien fungus that has become invasive in Cape Town and is posing a threat to the sensitive native forests found on the foothills of this city (Wingfield *et al*. 2010). The fungus is believed to have been introduced into South Africa from Europe by the early Dutch settlers in the mid to late 1600s (Coetzee *et al*. 2001). A couple of years after its discovery in Cape Town, *A. mellea* was also found to have spread and caused the death of native *Protea* plants in Kirstenbosch Botanical Garden in Cape Town (Wingfield *et al*. 2010). Another fungus, the notorious soilborne root rot pathogen *Phytophthora cinnamomi* Rands (Zentmyer 1980) is believed to have originated from either Papua New Guinea or Sumatra (Linde and Smit 1999; Old *et al.* 1984). It was first recorded in South Africa in 1931 (Doidge & Bottomley 1931), and is now responsible for causing diseases to the native Fynbos vegetation, a vegetation type unique to the southwestern Cape region. It has also led to some economic losses by causing root diseases of *Eucalyptus* species, *Pinus* species (Linde *et al* 1994; Wingfield & Knox-Davies 1980), grapevines (Van der Merwe *et al*. 1972) and commercially cultivated *Protea* species (Von Broembsen & Brits 1985).

Host switching has also been recorded in ophiostomatoid fungi. A well-known example is that of the Dutch elm disease pandemics, which resulted from movement of *Ophiostoma* species and their vectors from Europe to North America (Douglas 2008; Brasier & Buck 2001; Lamb 1979). The fungal pathogens causing this disease (*O. ulmi* and *O. novo-ulmi*), along with their vector European elm bark beetle *Scolytus multistriatus* Marsham, were introduced through logs imported from Europe in the 1920s (Brasier 1990; Webber 1990; Peace 1960). The American elms quickly succumbed to infection by the fungal pathogens as they were highly susceptible to the exotic pests. This resulted in hundreds of millions of dead elm trees (Brasier & Buck 2001). It was also easy for the pathogens to spread from tree to tree through root grafts and beetle feeding activities, as the trees were planted in rows along parks and streets (Douglas 2008; Brasier & Buck 2001; Heybroek 1993; Lamb 1979).

Laurel wilt disease is another widely known recent example of a disease that resulted due to host switching of ophiostomatoid fungi. This disease kills members of the Lauraceae, including avocado trees, and has invaded much of the southeastern USA. It is caused by a

pathogenic fungus *Raffaelela lauricola* T.C. Harr., Fraedrich & Aghayeva that was introduced from southern Asia, probably through the mycangia of the invasive exotic red bay ambrosia beetle *Xyleborus glabratus* Eichhoff (Harrington *et al*. 2008). The fungus, along with its vector beetle, was introduced into USA on solid wood packing material (Harrington *et al*. 2008).

In South Africa, *C. albifundus* successfully utilises many hosts. The fungus, which was originally isolated from *Protea* sp. (Morris *et al*. 1993; Gorter 1977), is native to South Africa and has moved onto introduced *A. mearnsii* (Roux & Wingfield 1997). It occurs naturally on a relatively large number of native tree species from five different families (Roux *et al*. 2007). The fungus is extremely virulent and threatens the cultivation of *A. mearnsii* in South Africa (De Beer 1994) and has led to significant economic losses in the *A. mearnsii* plantation industry (Roux *et al*. 1999). Other ophiostomatoid fungi known from native and non-native hosts in South Africa include *O. quercus*, *Sporothrix variecibatus* and *Pesotum fragrans*-like (Table 1). The possible ecological and economic effect of the movement of these fungi between native and non-native hosts is largely unknown.

1.8. Aims and objectives of this study

The main aim of this study was to investigate the ophiostomatoid fungal diversity in the CFR (focussing on members of the Ophiostomatales) and some of the determinants that enable these species to infect new hosts. Specific objectives included:

1. Document the diversity of ophiostomatoid fungi associated with trees in the CFR.
2. Document the diversity of wood- and sap-feeding beetles and mites as associates of ophiostomatoid fungi on trees in the CFR.
3. Determine inter-organism specificity between ophiostomatoid fungi, their arthropod vectors and their host plants.
4. Determine the pathogenicity of key fungal species isolated in this study on various important native and exotic trees.
5. Determine the role of mutualisms between mites, their vectors and ophiostomatoid fungi in shaping fungal host ranges.

Outline of thesis chapters:

CHAPTER 2: Ophiostomatoid fungi, including three new species associated with bark and ambrosia beetles, from native trees in the Cape Floristic Region of South Africa.

This chapter sets out to document the diversity of ophiostomatoid fungi associated with bark and ambrosia beetles from native trees in the CFR. Identification of the ophiostomatoid fungal species collected is based on sequence data obtained from the ITS, BT, LSU and CAL regions of the fungal spp. collected. All new fungal species discovered from this niche are described in this chapter and are given provisional names.

CHAPTER 3: Ophiostomatoid fungi from wounds on *Rapanea melanophloeos* in Afromontane forests

At several Afromontane forests sites, *Rapanea melanophloeos* trees have wounds caused by storm damage, and in many instances were observed to be dying, suggesting that this species is under considerable threat. In this chapter the diversity of ophiostomatoid fungi associated with wounds on *R. melanophloeos* is documented. Fungal species were identified through DNA sequencing of the ITS, BT and CAL regions, and all newly encountered species were provisionally named and described in this chapter.

CHAPTER 4: Ophiostomatoid fungi from wounds on storm-damaged trees in Afromontane forests of the Cape Floristic Region.

The aim of this chapter was to identify and document ophiostomatoid fungal species specifically associated with wounds of native trees (except *R. melanophloeos*) in the CFR. New species isolated in this study are described and named here.

CHAPTER 5: Association between sub-cortical beetles (Coleoptera: Curculionidae), mites (Acari) and ophiostomatoid fungi (Ascomycota: Ophiostomatales) on Afromontane forest trees in the CFR.

The aim of this chapter was to build on previous studies (Chapters 2, 3 and 4) and document the total diversity of ophiostomatoid fungi and their associated arthropods on CFR trees, focussing on those associated with wounds, sub-cortical beetles and mites.

CHAPTER 6: The danger posed by ophiostomatoid fungi when encountering new hosts.

In this chapter the pathogenicity of some of the collected fungi was tested on three important native hosts and three important exotic plantation species. This was done to assess the

potential threat these fungal species could pose to new hosts in the region. The likelihood of fungi moving to new hosts was also investigated by identifying known host ranges of the fungi and their vector organisms and by testing the specificity of various mites towards their natural beetle vectors and those that they would not normally associate with.

CHAPTER 7: Conclusion

The main collective conclusions of the entire study are summarized in this chapter, and recommendations for future research are made.

1.7. References

Aanen, D.K. and Boomsma, J.J. 2005. Evolutionary dynamics of the mutualistic symbiosis between fungus-growing termites and *Termitomyces* fungi. In *Insect-Fungal Associations: Ecology and Evolution*, (Vega, F. and Blackwell, M. eds.), Oxford: Oxford University Press, pp. 191–210.

Aanen, D.K. and Eggleton, P. 2005. Fungus-growing termites originated in African rain forest. *Current Biology* 15: 851-855.

Adams, G.C., Wingfield, M.J., Common, R. and Roux, J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs (Ascomycota, Diaporthales, Valsaceae) from *Eucalyptus*. *Studies in Mycology* 52: 1–147.

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W. and Kirisits, T. 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* 96:866–878.

Agricultural News 1990. Numbers 23 (11 June), 29 (23 July), 37 (17 Sept.), 39 (1 Oct.) & 42 (22 Oct.) Directorate of Agricultural Information, Pretoria.

Al Adawi, A.O., Deadman, M.L., Al Rawahi, A.K., Al Maqbali, Y.M., Al Jahwari, A.A., Al Saadi, B.A., Al Amri, I.S. and Wingfield, M.J. 2006. Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology*116: 247–254.

Anderson, P.K., Cunningham, A.A., Patel, N.G., Morales, F.J., Epstein, P.R. and Daszak, P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution* 19: 535–544.

Ayres, M.P., Wilkens, R.T., Ruel, J.J. and Vallery, E. 2000. Fungal relationships and the nitrogen budget of phloem-feeding bark beetles (Coleoptera: Scolytidae). *Ecology* 81: 2198–2210.

Baker, J.M. and Norris, D.M. 1968. A complex of fungi mutualistically involved in the nutrition of the ambrosia beetle *Xeleborus ferrugineus*. *Journal of Invertebrate Pathology* 11: 246–250.

Barnes, I., Nakabonge, G., Roux, J., Wingfield, B.D. and Wingfield, M.J. 2005. Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* 54: 189-195.

Barnes, I., Roux, J., Wingfield, B.D., Dudzinski, M.J., Old, K.M. and Wingfield, M.J. 2003. *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* 95: 865–871.

Barras, S.J. and Perry, T.J. 1975. Interrelationships among microorganisms, bark or ambrosia beetles, and woody host tissue: an annotated bibliography, 1956–1974. U.S. Department of Agriculture Forest Service General Technical Report SO-10. Southern Forest Experiment Station, New Orleans, U.S.A.

Barras, S.J. and Perry, T.J. 1972. Fungal symbionts in the Prothoracic mycangium of *Dendroctonus frontalis*. *Zeitschrift fiir angwandefe Enfomologie* 71: 95-104.

Batra L.R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science* 66: 213-236.

Baxter, A.P. 1994. Past, present and prospective activities: Mycology Unit, PPRI. In: Proceedings of the 13th Plenary Meeting of AETFAT, Malawi. (Seyani, J.H. and Chikuni, A.C. eds), pp. 687–695.

Beaver, R.A. 1989. Insect-fungus relationships in the bark and ambrosia beetles. In *Insect-fungus interactions*, (Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. eds.) Academic Press, London, United Kingdom, pp. 121–143.

Beaver, R.A. 1986. The taxonomy, mycangia, and biology of *Hypothenemus curtipennis* (Schedl), the first known cryphaline ambrosia beetle (Coleoptera: Scolytidae). *Enfomologica Scandinavic*a 17: 131-135.

Berbee, M. L., and Taylor, J.W. 1992. Detecting the morphological convergence in true fungi using 18S RNA sequence data. *BioSystems* 28:117–125.

Biedermann, P.H.W. 2012. Evolution of cooperation in ambrosia beetles. PhD thesis. University of Bern, Institute of Ecology and Evolution.

Bignell, D.E. 2000. Introduction to symbiosis. In *Termites: Evolution, Sociality, Symbioses, Ecology*, (Abe, T. Bignell, D.E. and Higashi, M. eds.) (Dordrecht: Kluwer Academic Publishers), pp. 189–208.

Bobick, J.E., Balaban, N., Bobick, S. and Roberts, L. 2004. The Handy Biology Answer Book. Visible Ink Press. 43311, Joy Road, Canton, MI 48187 – 2070.

Boucher, D.H., James, S. and Keeler, K.H. 1982. The ecology of mutualism. *Annual Review of Ecology and Systematics* 13: 315-347.

Brasier, C.M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57: 792–808.

Brasier, C.M. 1990. China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* 39: 5–16.

Brasier, C. and Webber, J. 2010. Sudden larch death. An aggressive and unpredictable fungal pathogen is devastating larch plantations in Britain. Its remarkably broad host range, and the possibility of further geographical spread, give heightened cause for concern. *Nature* 466: 824-825.

Brasier, C.M. & Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Bridges, J.R. and Moser, J.C. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* 8: 9–12.

Brooks, T., Balmford, A., Burgess, N., Fjeldsa, J., Hansen, L.A., Moore, J., Rahbek, C. and Williams, P. 2001. Towards a blueprint for conservation. Africa. *BioScience* 51: 613–624.

Cairney, J.W.G. 2000. Evolution of mycorrhiza systems. *Naturwissenschaften* 87: 467–475.

Carnegie, A.J., Matsuki, M., Haugen, D.A., Hurley, B.P., Ahumadam R., Klasmer, P., Sun, J. and Iede, E. 2006. Predicting the potential distribution of *Sirex noctilio* (Hymenoptera: Siricidae), a significant exotic pest of *Pinus* plantations. *Annals of Forest Science* 63: 119–128.

Carnegie, A.J., Eldridge, R.H. and Waterson, D.G. 2005. The history and management of sirex wood wasp, *Sirex noctilio* (Hymenopter a: Siricidae), in pine plantations in New South Wales, Australia. *New Zeealand Journal of Forest Science* 35: 3–24.

Chen, S.F., Wingfield, M.J., Roets, F., and Roux, J. 2013. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea* in South Africa. *Plant Pathology* 62: 667-678.

Clayton, R.B. 1964. The utilisation of sterols by insects. *Journal of Lipid Research* 5: 3-19.

Coetzee, M.P.A., Wingfield, B.D., Harrington, T.C., Steimel, J., Coutinho, T.A., and Wingfield, M.J. 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* 10: 387–396.

Cooke, R. 1977. The Biology of Symbiotic Fungi. John Wiley & Sons, New York.

Coutinho, T.A., Wingfield, M.J., Alfenas, A.C. and Crous P.W. 1998. *Eucalyptus* rust: a disease with the potential for serious international implications. *Plant Disease* 82: 819–825.

Cowling, R.M. and Heijnis, C.E. 2001. The identification of broad habitat units as biodiversity entities for a systematic conservation planning in the Cape Floristic Region. *South African Journal of Botany* 67: 15–38.

Cowling, R.M. and Hilton-Taylor, C. 1994. Plant diversity and endemism in southern Africa: an overview. In: *Botanical Diversity in Southern Africa*, (Huntley, B.J. ed.), National Botanical Institute, Kirstenbosch, pp. 31–52.

Crous, P.W. 1998. *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir* 21: 1–170.

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., de Beer, Z.W., Wingfield, M.J. and Hawksworth, D.L. 2006. How many species of fungi are there at the tip of Africa? *Studies in Mycology* 55: 13–33.

Crous, P.W., Denman, S., Taylor, J.E., Swart, L. and Palm, E. 2004. Cultivation and diseases of Proteaceae: *Leucadendron*, *Leucospermum* and *Protea*. CBS Biodiversity Series 2, Centraalbureau voor Schimmelcultures, Utrecht.

Crous, P.W. and Braun, U. 2003. *Mycosphaerella* and its anamorphs. 1. Names published in *Cercospora* and *Passalora*. *CBS Biodiversity Series* 1: 1–571.

Cruywagen, E.M., de Beer Z.W., Roux, J. and Wingfield M.J. 2010. Three new *Graphium* species from baobab trees in South Africa and Madagascar. *Persoonia* 25: 61–71.

Darlington, J. 1994. Nutrition and evolution in fungus-growing termites. In: *Nourishment and Evolution in Insect Societies* (Hunt J.H. and Nalepa C.A. eds.), Westview Press, Boulder, CO, pp. 105–130.

Davis, S.D., Heywood, V.H., Hamilton, A.C. (Eds.), 1994. Centres of Plant Diversity. A Guide and Strategy for their Conservation. Vol. 1. Europe, Africa, South West Asia and The Middle East. WWF and IUCN, IUCN Publications Unit, Cambridge.

De Beer, C. 1994. *Ceratocystis frimbiata* with special reference to its occurrence as a pathogen on *Acacia Mearnsii* in South Africa. M.Sc. Thesis. University of the Orange Free State. Bloemfontein. South Africa, pp 42-88.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013a. The ophiostomatoid fungi: their dual position in the Sordarimycetes. In: *Ophiostomatoid fungi: expanding frontiers* (Seifert, K.A., De Beer, Z.W., Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 1-19.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013b. A nomenclator for ophiostomatoid fungi genera and species in the Ophiostomatales and Microascales. In: *Ophiostomatoid fungi: expanding frontiers* (Seifert, K.A., De Beer, Z.W., Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 245-322.

De Beer, Z.W., Wingfield, M.J. and Kemp, G.H.J. 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* 91: 6.

De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2003. The *Ophiostoma piceae* complex in the southern hemisphere: a phylogenetic study. *Mycological Research* 107: 469-476.

De Fine Licht, H.H. and Biedermann, P.H.W. 2012. Patterns of functional enzyme activity in fungus farming ambrosia beetles. *Frontiers in Zoology* 9:13.

De Meyer, E.M., De Beer, Z.W., Summerbell, R.C., Moharram, A.M., De Hoog, G.S., Vismer, H.F. and Wingfield, M.J. 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting

Sporothrix species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* 100:647–661.

Denman, S., Crous, P.W., Groenewald, J.G., Slippers, B., Wingfield, B.D. and Wingfield, M.J. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. *Mycologia* 95: 294–307.

Denman, S., Crous, P.W., Taylor, J.E., Kang, J-C., Pascoe, I. and Wingfield, M.J. 2000. An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology* 45: 129–140.

Doidge, E.M. 1950. The South African fungi and lichens to the end of 1945. *Bothalia* 5: 1–1094.

Doidge, E.M. and Bottomley, A. M. 1931. A revised list of plant diseases in South Africa. *South African Journal of Botany* 11:1-78.

Douglas, S.M. 2008. Plant health problems. Department of Plant Pathology and Ecology. The Connecticut Agricultural Experiment Station, 123 Huntington Street, P. O. Box 1106. New Haven, CT 06504.

Dowding, P. 1969. The dispersal and survival of spores of fungi causing blue stain in pine. *Transactions of the British Mycological Society* 52: 125-137.

Eckhardt, L.G., Goyer, R.A. Klepzig, K.D. and Jones, J.P. 2004. Interactions of *Hylastes* species (Coleoptera: Scolytidae) with *Leptographium* species associated with Loblolly pine decline. *Journal of Economic Entomology* 97:468–474.

Eicker, A. 1974. The mycoflora of an alkaline soil of the open savannah of the Transvaal. *Transactions of British Mycological* Society 63: 281–288.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., MacCraw, S.L. and Gurr, S.J. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484: 186-194.

Fox, J.W, Wood, D.L. and Ackers, R.P. 1993. Survival and development of *Ips paraconfusus* Lanier (Coleoptera: Scolytidae) reared axenically and with tree-pathogenic fungi vectored by co-habiting *Dendroctonus* species. *Canadian Entomologist* 97: 527-536.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects. In *Symbiosis*, Vol II., (Henry, S.M. ed.), Academic Press, New York, U.S.A, pp. 171–180.

Furniss, M.M., Woo, Y., Deyrup, M.A. and Atkinson, T.H. 1987. Prothoracic mycangium on pine-infesting *Pityoborus* spp. (Coleoptera: Scolytidae). *Annals of the Entomological Society of America* 83: 692-696.

Goldblatt, P. and Manning, J. 2002. Plant diversity of the Cape Region of South Africa. *Annals of the Missouri Botanical Garden* 89: 281–302.

Gordon, T.R., Storer, A.J. and Wood, D.L. 2001. The pitch canker epidemic in California. *Plant Disease* 85: 1128– 1138.

Gorter, G.J.M.A. 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Science Bulletin no. 392. Pretoria, South Africa: Plant Protection Research Institute, Department of Agricultural Technical Services.

Grünwald, N.J., Goss, E. M. and Press, C. M. 2008. *Molecular Plant Pathology* 6:729–740.

Guarro, J., Gené J. and Stchigel, A.M. 1999. Developments in fungal taxonomy. *Clinical Microbiology Reviews* 12: 454.

Happ, G.M., Happ, C.M., and Barras, S.J. 1971. Fine structure of the Prothoracic mycangium, a chamber for the culture of symbiotic fungi, in the southern pine beetle, *Dendroctonus frontalis*. *Tissue and Cell* 3: 295-308.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D.N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon* 104: 399-404.

Haugen, D.A., Bedding. R.A., Underdown, M.G. and Neumann, F.G. 1990. National strategy for control of *Sirex noctilio* in Australia. Australian Forest Grower 13. *Special Liftout Section No*. 13: 8.

Hausner, G. Reid, J. and Klassen, G.R. 1993. On the subdivision of *Ceratocystis s.l*., based on partial ribosomal DNA sequences. *Canadian Journal of Botany* 71: 52-63.

Hawksworth, D.L., 2004. Fungal diversity and its implications for genetic resource collections. *Studies in Mycology* 50: 9–18.

Hawksworth, D.L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research* 105: 1422-1432.

Hawksworth, D.L. 1991. The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycological Research* 95: 641-655.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. 1995. Ainsworth and Bisby's dictionary of the fungi, 8th ed. International Mycological Institute, Egham,United Kingdom.

Heiniger, U. and Rigling, D. 1994. Biological control of chestnut blight in Europe. *Annual Review of Phytopathology* 32: 581-599.

Hektoen, L. and Perkins, C.F. 1900. Refractory subcutaneous abscesses caused by *Sporothrix schenckii*, a new pathogenic fungus. *Journal of Experimental Medicine* 5: 77–89.

Heybroek, H.M. 1993. Why bother about the elm? In: *Dutch Elm Disease Research. Cellular and Molecular Approaches*, (Sticklen, M.B. and Sherald, J.L. eds.), Springer-Verlag, New York, pp. 1–8.

Hurley, B.P., Slippers, B. and Wingfield, M.I. 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. *Agricultural and Forest Entomology* 9:159-171.

Hutchinson, L.J. and Reid, J. 1988. Taxonomy of some potential wood-staining fungi from New Zealand. 2. Pyrenomycetes, Coelomycetes and Hyphomycetes. *New Zealand Journal of Botany* 26: 83-98

Jacobs, K. and Wingfield, M.J. 2001. *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. APS press, St Paul, Minnesota, U.S.A.

Jacobs, K., Wingfield, M.J. and Roux, J. 1999. *Leptographium eucalyptophilum*, a new species from *Eucalyptus* in the Congo. *South African Journal of Botany* 65: 388-391.

Jooste, W.J. 1978. *Leptographium reconditum* sp. nov. and observations on conidiogenesis in Verticicladiella. *Transactions of the British Mycological Society* 70: 152–155.

Jones, N. 2013. It could happen one night. Death by fungus. *Nature* 493: 155-156.

Jordal, B.H. and Cognato, A.I. 2012. Molecular phylogeny of bark and ambrosia beetles reveals multiple origins of fungus farming during periods of global warming. *BMC Evolutionary Biology* 12:133.

Kajimura, H. and Hijii, N. 1992. Dymamics of the fungal symbionts in the gallery system and the mycangia of the ambrosia beetle, *Xylosandrus mutilatus* (Blandford) (Coleoptera, Scolytidae). *Ecological Research* 7:107–117.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Kendrick, B. 1992. The fifth kingdom, 2nd ed. Mycologue Publications, Waterloo, Canada.

Kerley, G.I.H., Pressey, R.L., Cowling, R.M., Boshohof, A.F. and SimsCastley, R. 2003. Options for the conservation of large and medium-sized mammals in the Cape Floristic Region. *Biological Conservation* 112: 169–190.

Kile, G.A. 1993. Plant diseases caused by species of Ceratocystis sensu stricto and Chalara. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A., Webber, J. eds). America Phytopathological Society Press, St. Paul, MN, USA, pp. 173-183.

Kirisits, T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: *Bark and wood boring insects in living trees in Europe, a synthesis*, (Lieutier, F. ed.), Kluwer Academic Press, The Netherlands, pp. 181-235.

Klasmer, P., Fritz, G., Corley, J. and Botto, E. 1988. Current status of research on *Sirex noctilio* F. in the Andean-Patagonian region in Argentina. In: Training in the control of *Sirex noctilio* by use of natural enemies, (Iede, E., Shaitza, E., Penteado, S., Reardon, R., Murphy, T. eds.), Proceedings of a Conference, USDA Forest Service (FHTET 98-13), pp. 89–90.

Klepzig, K.D and Six, D.L. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis* 37: 189-205.

Klepzig, K.D., Moser, J.C., Lombardero, M.J., Ayres, M.P., Hofstetter, R.W. and Walkinshaw, C.J. 2001a. Mutualism and antagonism: Ecological interactions among bark beetles, mites and fungi. In: *Biotic Interactions In Plant-Pathogen Associations*, (Jeger, M.J. and Spence, N.J. eds), CAB International, New York, U.S.A, pp. 237–267.

Klepzig, K.D., Moser, J.C., Lombardero, F.J, Hofstetter, R.W. and Ayres, M.P. 2001b. Symbiosis and competition: Complex interactions among beetles, fungi and mites. *Symbiosis* 30: 83–96.

Klepzig, K.D. and Wilkens, R.T. 1997. Competitive interactions among symbiotic fungi of the southern pine beetle. *Applied and Environmental Microbiology* 63: 621-627.

Kok, L.T., Norris, D.M. and Chu H.M 1970. Sterol metabolism as a basis for a mutualistic symbiosis. *Nature* 225: 661–662.

Knox-Davies, P.S., van Wyk, P.S. and Marasas, W.F.O. 1986. Diseases of proteas and their control in the South-western Cape. *Acta Horticulturae* 185: 189–200.

Kubono, T. and Ito, S. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Lamb, R .1979. World without Trees. Wildwood House, London.

Lee, S., Mel'nik, V., Taylor, J.E. and Crous, P.W. 2004. Diversity of saprobic hyphomycetes on Proteaceae and Restionaceae from South Africa. *Fungal Diversity* 17: 91–114.

Lee, S., Groenewald, J.Z., Taylor, J.E., Roets, F. and Crous, P.W. 2003. Rhynchostomatoid fungi occurring on Proteaceae. *Mycologia* 95: 902–910.

Levieux, J., Cassier, P., Guillaumin, D. and Roques, A. 1991. Structures implicated in the transportation of pathogenic fungi by the European bark beetle, *Ips sexdentatus* Boemer: Ultrastructure of a mycangium. *Canadian Entomologist* 123: 245-254.

Linde, C. and Smit, W.A. 1999. First report of rhizosis caused by *Ceratocystis radicicola* on date palms in South Africa. *Plant Disease* 83: 880.

Linde, C., Kemp, G. H. J. and Wingfield, M. J. 1994. *Pythium* and *Phytophthora* species associated with eucalyptus and pines in South Africa. *European Journal of Plant Pathology*. 24:1006-1009.

Lombardero, M.J., Ayres, M.P., Hofstetter, J.W., Moser, J.C. and Klepzig, K.D. 2003. Strong indirect interactions of Tarsonemus mites (Acarina: Tarsonemidae) and *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Oikos* 102: 243–252.

Madden, J.L. 1988. Sirex in Australasia. In: *Dynamics of forest insect populations*, (Berryman A.A. ed.), Plenum Press, New York, pp 407-429.

Madden, J.L. 1975. An analysis of an outbreak of the woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae), in *Pinus radiate*. *Bulletin of Entomological Research* 65: 491–500.

Malloch, D. and Blackwell, M. 1993. Dispersal biology of the ophiostomatoid fungi. In: *Ceratocystis and Ophiostoma: taxonomy, ecology and pathogenicity*, (Wingfield, M.J., Seifert, K.A. and Webber, J.F. eds.) APS Press, St. Paul, U.S.A., pp. 195–206.

Marais, G.J. and Wingfield, M.J. 2001. *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. *Mycological Research* 105: 240-246.

Marais, G.J. and Wingfield, M.J. 1997. *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. *Canadian Journal of Botany* 75: 362-367.

Marais, G.J. and Wingfield, M.J. 1994. Fungi associated with infructescences of *Protea* species in South Africa, including a new species of *Ophiostoma*. *Mycological Research* 98: 374-396.

Marasas, W.F.O., Van der Westhuizen, G.C.A., Van Warmelo, K.T. and Papendorf, M.C. 1966. New and interesting records of South African fungi. Part V. *Bothalia* 9: 229–243.

Marimon, R., Gené, J., Cano, J. and Guarro, J. 2008. *Sporothrix luriei*: a rare fungus from clinical origin. *Medical Mycology* 46: 621–625.

Marimon, R., Cano, J., Gene, J., Sutton, D.A., Kawasaki, M. and Guarro, J. 2007. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. *Journal of Clinical Microbiology* 45:3198–3206.

Morgan, F.D. and Stewart, N.C. 1996. The biology and behaviour of the woodwasp *Sirex noctilio* (F.) in New Zealand. *Transactions of Royal Society of New Zealand* 7: 195–204.

Morris, M.J., Wingfield, M.J. and De Beer, C. 1993. Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* 42: 814-817.

Moser, J.C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. *Transactions of the British Mycological Society* 84: 750–753.

Mouton, M., Wingfield, M.J., Wyk, P.S. van and Wyk, W.J. van. 1994. *Graphium pseudormiticum* sp. nov.: a new hyphomycete with unusual conidiogenesis. *Mycological Research* 98: 1272–1276.

Mu¨ller, E. and von Arx, J.A. 1973. Pyrenomycetes: Meliolales, Coronophorales, Sphaeriales. In: *The fungi: an advanced treatise*, vol. 4A, (Ainsworth, G.C., Sparrow, F.K. and Sussman, A.S. eds.), Academic Press, Inc., New York, N.Y, pp. 87–132.

Neumann, F.G., Morey, J.L. and McKimm, R.J. 1987. The sirex wasp in Victoria Department of Conservation, Forests and Lands, Victoria. *Bulletin Number* 29: 44.

Neumann, F.G. and Minko, G. 1981. The sirex wood wasp in Australian radiata pine plantations. *Australian Journal of Forestry* 44: 46–63.

Nobre, T., Kone, N.A., Konate, S., Linsenmair, K.E. and Aanen, D.K. 2011. Dating the fungus-growing termites' mutualism shows a mixture between ancient codiversification and recent symbiont dispersal across divergent hosts. *Molecular Ecology* 20: 2619-2627.

Old, K.M., Moran, G.F. and Bell, J.C. 1984. Isozyme variability among isolates of *Phytophthora cinnamomi* for Australia and Papua New Guinea. *Canadian Journal of Botany* 62: 2016–2022.

Paine, T.D., Raffa, K.F. and Harrington, T.C. 1997. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* 42: 179–206.

Paine, T.D. and Birch, M.C. 1983. Acquisition and maintenance of mycangial fungi by *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae). *Environmental Entomology* 12: 1384-1386.

Peace, T. 1960. The status and development of elm disease in Britain. *Forestry Commission Bulletin* 33: 1–44.

Pegler, D. N., Yao, Y. J. and Li, Y. 1994. The Chinese 'caterpillar fungus'. *Mycologist* 8: 3-5.

Ploetz, R.C. 2003. Diseases of mango. In: *Diseases of Tropical Fruit*, (Ploetz, R.C. ed.), CABI Publishing: Wallingford Oxford (UK), pp. 327–363.

Rawlings, G.B. and Wilson, N.M. 1949. *Sirex noctilio* as a beneficial and destructive pest to *Pinus radiata* forests. *New Zealand Journaal of Forestry* 6: 1–11.

Reblova, M. And Winka, K. 2000. Phylogeny of *Chaetosphaeria* and its anamorphs based on morphological and molecular data. *Mycologia* 92: 939-954.

Ribeiro, I.J.A. 1980 Seca da mangueira. Agentes causais e estudo da molestia. In: *Anais do I Simposio Brasiliero Sobre a Cultura de Mangueira*. Sociedade Brasileirade Fruticultura, Jaboticabal, pp. 123–130.

Roets, F., Wingfield, B.D, De Beer, Z.W., Wingfield, M.J. and Dreyer, L.L. 2010. Two new *Ophiostoma* species from *Protea caffra* in Zambia. *Persoonia* 24: 18-28.

Roets, F., Dreyer, L.L., Crous, P.W. and Wingfield, M.J. 2009a. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* 38: 143-152.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2009b. Fungal Radiation in the Cape Floristic Region: an analysis based on *Gondwanamyces* and *Ophiostoma*. *Molecular Phylogenetics and Evolution* 51: 111-119.

Roets, F., de Beer, Z.W., Wingfield, M.J., Crous, P.W and Dreyer, L.L. 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* 100: 496-510.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Roets, F., de Beer, W., Dreyer, L.L., Zipfel, R., Crous, P.W. and Wingfield, M.J. 2006. Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. *Studies in Mycology* 55: 199-212.

Roux, J., Harrington, T.C., Steimel, J.P. and Wingfield, M.J. 2001a. Genetic variation in the wattle pathogen *Ceratocystis albifundus*. *Mycoscience* 42: 327-332.

Roux, J., Wingfield, M. J. and Byabashaija, D. M. 2001b. First report of *Ceratocystis* wilt of *Acacia mearnsii* in Uganda. *Plant Disease* 85: 1029.

Roux, J., Heath, R.N., Labuschagne, L., Kamgan Nkuekam, G. and Wingfield, M.J. 2007. Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* 37: 292-302.

Roux, J., Meke, G., Kanyi, B., Mwangi, A., Hunter, G.C., Nakabonge, G., Heath R.N. and Wingfield, M.J. 2005. Diseases of plantation forestry tree species in Eastern and Southern Africa. *South African Journal of Science* 101: 409–413.

Roux, J., Labuschagne, L., Heath, R.N., Nkuekam, G.K. and Wingfield, M.J. 2004a. The occurrence of the wilt pathogen, *Ceratocystis albifundus* on native South African trees. Proceedings of the American Phytopathological Society Meeting, July 31-August 4, Anaheim, California. *Phytopathology* 94: 89.

Roux, J., VanWyk, M., Hatting, H. and Wingfield, M. J. 2004b. *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* 53: 414–421.

Roux, J., Dunlop, R. and Wingfield, M.J. 1999. Susceptibility of elite *Acacia mearnsii* families to *Ceratocystis* wilt in South Africa. *Journal of Forestry Research* 4: 187–190.

Roux, J. and Wingfield, M.J. 1997. Survey and virulence of fungi occurring on diseased *Acacia Mearnsii* in South Africa. *Forest Ecology and Management* 99: 327-336.

Sace, J. 2010. The role of fungi in the ecosystem. (Scudder, R. ed.), Bright Hub.

Schultz, T.R. and Brady, S. G. 2008. Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America* 105: 5435–5440.

Scott, D.B. and Du Toit, J.W. 1970. Three new *Raffaelea* species. *Transactions of the British Mycological Society* 55: 181–186.

Six, D.L. 2004. Bark beetle-fungus symbioses. In *Insect Symbioses*, (Bourtzis, K. and Miller, T. eds.), CRS press, Boca Raton, FL, USA, pp. 97-114.

Six, D.L. and Paine, T.D. 1998. Effects of mycangial fungi on host tree species progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology* 27: 1393–1401.

Slippers, B., Stenlid, J. and Wingfield, M.J. 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. *Trends in Ecology and Evolution* 20: 420-421.

Smith, S.E. and Read, D.J. 1997. Mycorrhizal symbiosis. Academic Press, London.

Spatafora, J.W. and Blackwell, M. 1994. The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* 98: 1-9.

Spradbery, J.P. and Kirk, A.A. 1978. Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* 68: 341-359.

Stattersfield, A.J., Crosby, M.J., Long, A.J. and Wedge, D.C. 1998. Endemic Bird Areas of the World. BirdLife Conservation Series 7. BirdLife International, Cambridge.

Storch, R.H. and Dill, J.F. 1987. *Beauveria bassiana* for control of colorado potato beetle (Coleoptera: Chrysomelidae) in Maine. Maine Agricultural Experiment Station University of Maine. *Techinical Bulletin* 128.

Sunjian, A. 2012. What are leafcutter ants? The Lurker's Guide to Leafcutter Ants. blueboard.com. Retrieved 2012-01-30.

Svoboda, J.A., Thompson, M.J., Robbins, W.E. and Kaplains, J.N. 1978. Insect sterol metabolism. *Lipids* 13: 742-753.

Swart, L. 1999. Pathogens associated with diseases of *Protea*, *Leucospermum* and *Leucadendron* spp. MSc thesis, Department of Plant Pathology, University of Stellenbosch, Stellenbosch.

Takhtajan, A. 1986. Floristic regions of the world. University of California Press, Berkeley, U.S.A.

Talbot, P.H.B 1956. New and interesting records of South African fungi. Part II. *Bothalia* 6: 489–500.

Taylor, J.E. and Crous, P.W. 2000. Fungi occurring on Proteaceae. New anamorphs for *Teratosphaeria*, *Mycosphaerella* and *Lembosia*,and other fungi associated with leaf spots and cankers of Proteaceous hosts. *Mycological Research* 104: 618–636.

Taylor, K.L. 1981. The sirex woodwasp: ecology and control of an introduced forest insect. In: *The Ecology of Pests*, (Kitching, R.L. and Jones, R.E. eds.), CSIRO, Australia, pp. 231–248.

Thomsen, I.M. and Koch, J. 1999. Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* 103: 817-823.

Tribe, G.D. and Cillié. J.J. 2004. The spread of *Sirex noctilio* Fabricus (Hymenoptera: Siricidae) in South African pine plantations and the introduction and establishment of its biological control agents. *African Entomology* 12: 9–17.

Tribe, G.D. 1995. The woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), a pest of *Pinus* species, now established in South Africa. *African Entomology* 3: 216–217.

Tribe, G.D. 1992. Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera: Scolytidae). *Journal of Entomological Research Society* 1: 77-84.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, U.S.A.

Upadhyay H.P. and Kendrick, W.B. 1975. Prodromus for a revision of *Ceratocystis* `(Microascales, Ascomycetes) and its conidial states. *Mycologia* 67: 798-805.

Van der Merwe, J. J. H., Joubert, D. J. and Matthee, F. N. 1972. *Phytophthora cinnamomi* root rot of grapevines in the Western Cape. *Phytophylactica* 4:133-136.

Van Wyk, M., Al Adawi, A.O., Wingfield, B.D., Al Subhi, A.M., Deadman, M.L. and Wingfield, M.J. 2005. DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* 34: 587–590.

Vasiliauskas, R. and Stenlid, J. 1999. Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research* 103: 824-829.

Viegas, A.P. 1960. Mango blight. *Bragantia* 19: 163–182.

Viljoen, C.D., Wingfield, B.D. and Wingfield, M.J. 1999. Relatedness of *Custingophora olivaceae* to *Gondwanamyces* spp. from *Protea*. *Mycological Research*. 103: 497-500.

Von Broembsen, S. L. and Brits, G. J. 1985. *Phytophthora* root rot on commercially cultivated proteas in South Africa. Plant Disease. 69: 211-213.

Waller, D. A. 1988. In: *Advances in Myrmecology*, (Trager, J. C. and Brill, E.J. eds.), Leiden, U.K., pp. 337–345.

Webber, J.F. 1990. The relative effectiveness of *Scolytus scolytus, S. multistriatus* and *S. kirschii* as vectors of Dutch elm disease. *European Journal of Forest Pathology* 20: 184–192.

Whitney, H.S. 1982. Relationships in bark beetles and symbiotic organisms. In: *Bark beetles in North America conifers* (Mitton, J.B. & Sturgeon, K.B. eds.), University of Texas, Austin, pp. 183-211.

Wingfield, M.J. 2003. Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from *Cryphonectria* canker. *Australian Plant Pathology* 32:133–139.

Wingfield, M.J. 1993. Problems in delineating the genus *Ceratocystiopsis*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*, (Wingfield, M.J, Seifert, K.A. and Webber, J. eds.). American Phytopathological Society, St. Paul. Minnesota.

Wingfield, M.J., Coetzee, M. P.A., Crous, P.W., Six, D. and Wingfield, B.D. 2010. Fungal phoenix rising from the ashes? *International Mycological Association Fungus* 1: 149–153.

Wingfield, M.J., De Beer, C., Visser, C. and Wingfield, B.D. 1996. A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* 19: 191-202.

Wingfield, M.J. and Kemp, G.H.J. 1993. Diseases of Pines, Eucalyptus and Wattles. In: *South African Forestry Handbook*, (Van der Sidje, H.A. ed.), The South African Association of Forestry. Pretoria, South Africa, pp. 231-248.

Wingfield, M.J., Seifert, K.A. and Webber, J. 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. APS Press: St. Paul, Minnesota.

Wingfield, M.J. and Van Wyk, P.S. 1993. A new species of *Ophiostoma* from *Protea* infructescences in South Africa. *Mycological Research* 97: 709–716.

Wingfield, M.J., Van Wyk, P.S. and Marasas, W.F.O. 1988. *Ceratocystiopsis proteae* sp. nov., with a new anamorph genus. *Mycologia* 80: 23–30.

Wingfield, M.J. and Marasas, W.F.O. 1983. Some *Verticicladiella* species, including *V. truncata* sp. nov., associated with root diseases of pine in New Zealand and South Africa. *Transactions of the British Mycological Society* 80: 231–236.

Wingfield, M.J. and Knox-Davies, P.S. 1980. Observations on disease in pine and eucalyptus plantations in South Africa. *Phytophylactica* 12: 57-60.

Wingfield, M.J. and Marasas, W.F.O. 1980. Verticicladiella alacris sp. nov., associated with a root disease of pines in the South Africa. *Transactions of the British Mycological Society* 75: 21–28.

Wood SL. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Naturalist, Memoirs* 6: 1–1356.

Wood, T.G., and Sands, W.A. 1978. The role of termites in ecosystems. In: *Production Ecology of Ants and Termites*, (Brian, M.V. ed.), Cambridge: Cambridge Univ. Press, pp. 245–292.

Woolhouse, M.E.J., Haydon, D.T and Antia, R. 2005. Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology and Evolution* 20: 238-244.

Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. The American Phytopathological Society, St. Paul, MN.

Zhou, X.D., De Beer, Z.W. and Wingfield, M.J. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.

Zhou, X.D., De Beer, Z.W., Ahumada, R., Wingfield, B.D. and Wingfield, M.J. 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* 15: 261-274.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* 53: 290-300.

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D. and Wingfield, M.J. 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* 55: 75-97.

Chapter 2: Three new species of Ophiostomatales associated with bark and ambrosia beetles, from native trees in the Cape Floristic Region of South Africa.

Abstract

Olea capensis ssp. *macrocarpa* and *Rapanea melanophloeos* are important canopy trees in the Afromontane forests of South Africa. Despite their importance in these forests, not much is known regarding their associated organisms. During recent surveys in these forests, many dying or recently dead individuals of these trees were found to be infested by bark and ambrosia beetles. This study documents the fungi associated with these insects from the two tree taxa in Afromontane forests of the Cape Floristic Region (CFR) of South Africa. Bark and wood samples were collected from *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* trees showing signs of subcortical beetle activity. Fungi were isolated from the surfaces of subcortical beetles emerging from collected bark and wood samples and from beetle galleries. Based on micro-morphological and phylogenetic analyses four fungal species belonging to two genera in the Ophiostomatales were identified from four species of subcortical beetles collected. These were *Sporothrix pallida* and three taxa here newly described as *S. aemuluphilus* sp. nov., *Raffaelea scabbardiae* sp. nov. and *Raffaelea rapaneae* sp. nov. This study represents the first collection of *S. pallida*, a species known from many environmental samples from across the world, from Scolytinae beetles. *Sporothrix aemuluphilus* sp. nov. represents the only member of the *S. schenckii-O. stenoceras* complex that is currently known exclusively as a Scolytinae beetle-associate. *Raffaelea scabbardiae* sp. nov. and *R. rapaneae* sp. nov. were associated with a Platypodinae beetle and a *Lanurgus* sp. beetle, respectively and represent the first species in the genus reported from the CFR. This study therefore highlights the need for more inclusive studies on the diversity and ecology of members of the Ophiostomatales associated with native plants in this biologically exceptionally rich region. Considering the immense diversity of native plants in the CFR, it is reasonable to assume that many taxa still await discovery.

Key words: ambrosia-beetle, bark-beetle, Microascales, *Ophiostoma*

2.1. Introduction

Olea capensis L. ssp. *macrocarpa* (C. H. Wright) I. Verd. (Oleaceae) and *Rapanea melanophloeos* Mez. (Myrsinaceae) are important canopy trees in the evergreen Afromontane forests of Africa. In southern Africa both species are distributed from Zambia in the north to around Cape Town in the south (Van Wyk & Van Wyk 1997). Although both trees are renowned for their attractive wood utilised for making furniture, not much is known regarding their associated organisms. The recent description of a serious stem canker disease of *R. melanophloeos* from the Cape Floristic Region (CFR) in South Africa, caused by the pathogenic fungus *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf. & Jol. Roux (Chen *et al*. 2013), highlighted the importance of understanding such relationships and led to the research reported here.

Apart from pathogens, wood boring insects, such as bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae and Platypodinae), may also represent a significant, but grossly understudied threat to native trees in these forests. These beetles form part of a diverse group that bore into a large diversity of trees, shrubs and other woody plants (Atkinson & Peck 1994; Atkinson & Equihua 1986). They are rated as some of the globally most important forest pests (Avtzis *et al*. 2012; Harrington 2005; Paine *et al*. 1997), including more than 6000 species in 225 genera (Avtzis *et al*. 2012; Six 2012; Knižek & Beaver 2004). They are usually found infesting conifers and hardwood trees (Avtzis *et al*. 2012). The Platypodinae includes about 1500 described species of ambrosia beetles (Beaver & Liu 2013; Wood & Bright 1992), most of which are found in tropical and sub-tropical regions (Schedl 1972). There they are of economic im portance, often penetrating felled timber along with their associated fungi, causing wood staining (Browne 1968). The majority of the Platypodinae species are non-host specific and usually breed in any suitable host of the right size that offers favourable conditions (Beaver 1977; Hulcr *et al.* 2007). However, there are exceptions where certain species are associated with particular families of trees (Beaver & Liu 2013). The ecology of only a small number of about 3400 Scolytinae and Platypodinae beetle species has been well-studied (Farrell *et al*. 2001; Batra 1963). In Africa, their diversity and effects on tree hosts, especially on native trees, is virtually unknown (Beaver *et al.* 2005; Jordal *et al.* 2001).

Generally most of these beetles are not very aggressive to their tree hosts, and do not significantly impact healthy trees (Paine *et al*. 1997; Wood 1982). However, in extreme cases under favourable conditions, beetle populations may rapidly increase to outbreak levels, posing a primary threat to their hosts (Avtzis *et al*. 2012; Six & Wingfield 2011; Hudgins *et al*. 2004; Paine *et al*. 1997). Under these circumstances they may become aggressive and

attack and cause death of healthy trees (Holsten 2001). An example is that of the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann, a primary beetle which is very aggressive and is capable of killing healthy pine trees (Payne 1980) and leads to large losses in pine production (Price *et al*. 1992). In some cases beetles pose a secondary threat to their host trees and attack only stressed trees, aiding in their rapid death and leading to decreased value of wood products (Six & Wingfield 2011; Haberkern *et al*. 2002; Werner & Holsten 1984). For example, drought stressed *Pinus edulis* Engelmann forests in the southwestern United States were destroyed by the beetle *Ips confusus* LeConte (Breshears *et al*. 2005). Saprophytic taxa, which comprise the largest group of bark and ambrosia beetles, exclusively colonize dead hosts and unlike the primary and secondary beetles, they rarely cause economic losses. For this reason they have remained relatively understudied world-wide (Avtzis *et al*. 2012; Six & Wingfield 2011; Hudgins *et al*. 2004; Paine *et al*. 1997).

For part of their life cycles, scolytine bark beetles reside in galleries under bark, whereas the galleries of scolytine- and platypodinae ambrosia beetles are found within the wood of their hosts. As they penetrate these tissues they can introduce a diverse array of fungi that colonise the wood from within the gallery system (Whitney 1982; Batra 1966). Ambrosia beetles depend on their fungal symbionts for nutrition (Batra 1966), while bark beetles mainly feed on the phloem of trees, with nutritional supplementation of their diets by some of their fungal associates (Harrington 2005; Six 2003). In some cases the relationship between these two organism groups are therefore mutualistic. The fungi provide nutrients for use during reproduction and hormone synthesis, to help overcome tree defence mechanisms and to protect beetles against antagonistic fungi (Klepzig & Six 2004; Klepzig *et al*. 2001; Paine *et al*. 1997; Wingfield *et al*. 1995; Harrington 1993; Berryman 1972). In turn, the fungi are vectored by the beetles from one host tree to the next under favourable conditions (Klepzig & Six 2004; Klepzig *et al*. 2001). In many cases the interaction is so strong that specialised spore-carrying structures (mycangia) have evolved in the beetles, but spores may also be transported on the exoskeletons and within the alimentary canal of some taxa (Klepzig & Six 2004; Six 2003; Paine *et al*. 1997; Beaver 1989; Batra 1963).

Fungi from the Ophiostomatales are some of the most common fungal associates of bark and ambrosia beetles (Alamouti *et al*. 2009; Harrington 2005; Klepzig & Six 2004, Six 2003; Jacobs & Wingfield 2001; Klepzig *et al*. 2001; Bridges & Moser 1983; Barras & Perry 1975). The order includes genera such as *Ceratocystiopsis* Upadhyay & Kendrick, *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Leptographium* Lagerb. & Melin, *Ophiostoma* Syd. and *Raffaelea* Arx & Hennebert (De Beer *et al*. 2013; Zipfel *et al* 2006; Upadhyay 1981), some of which

contain important tree pathogens (Brasier & Buck 2001; Zhou *et al*. 2001; Heybroek 1993). A well-documented example is that of *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier that are vectored by the European elm bark beetle (*Scolytus multistriatus* Marsham) (Webber 1990) and the American elm bark beetle (*Hylurgopinus rufipes* Eichhoff). These two pathogens were responsible for causing the deadly Dutch elm disease in Europe and U.S.A. (Brasier 2000; Pipe *et al*. 2000). *Raffaelela lauricola* T.C. Harr., Fraedrich & Aghayeva, vectored by the ambrosia beetle *Xyleborus glabratus* Eichhoff (Harrington *et al*. 2008), is responsible for Laurel wilt disease in the southeastern USA (Harrington *et al*. 2008) and in Japan, the ambrosia beetle *Platypus quercivorus* (Murayama) and its associate fungus *Raffaelea quercivora* Kubono et Shin. Ito was responsible for the catastrophic mass mortality and die-back of Japanese oak trees (Kubono & Ito 2002).

There are very few reports of members of the Ophiostomatales associated with subcortical beetles on native trees in southern Africa, despite the increased reports in the decline and death of native trees in the region (Chen *et al*. 2013; Roux *et al*. 2009; Malan 2006). Inadequate information on these insects and their fungal symbionts presents a serious hindrance to our understanding of the death of some of these native tree species. The recent discovery of some ambrosia beetles and their associated fungi causing the decline of the native *Euphorbia ingens* E. Meyer: Boissier in South Africa (van der Linde *et al.* 2012; Roux *et al.* 2009) highlighted the need for more research on these beetles and their effects on other native tree taxa in South Africa. During recent surveys of subcortical beetles associated with native trees in South Africa, many individuals of *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* were found to be infested by bark and ambrosia beetles. These beetles were most often associated with dying or recently dead trees. Given the gap in our knowledge, and the importance of beetle-associated Ophiostomatales to tree health, this study aimed to document the fungi associated with the bark and ambrosia beetles from these tree taxa, focussing specifically on Afromontane forests of the CFR of South Africa.

2.2. Materials and methods

2.2.1. Beetle sampling

Sampling was conducted in forests adjacent to the Harold Porter National Botanical Garden (S 34' 20.893 E 18' 55.519), Gouna Forest (S 33°57'2.584" E 23°2'9.80") and Gouldveld Forest (S 33°54'44.38" E 23°0'10.30") in the Western Cape Province of South Africa between

2011 and 2012. Bark and wood samples were collected from *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* trees that showed signs of subcortical beetle activity. The collected plant material was transferred to the laboratory and placed in insect emergence cages constructed from sealed cardboard boxes (ca. 49 x 49 x 32.6 cm) fitted with two transparent plastic bottles (5.7 cm diameter) on one side. Light penetrating through the plastic containers attracted beetles, causing them to emerge and accumulate in the bottles, from where they could be collected easily. Emergence cages were maintained at room temperature and inspected every 2-3 days for a period of fifty days. Individual beetles were aseptically placed in sterile vials, grouped into morpho-species, their numbers recorded and stored at 4ºC until further use (but not longer than 5 days). Reference collections of all beetle taxa collected in this study were stored in 70% ethanol. Reference material was sent for identification by expert taxonomists and is maintained in the Insect Collection of Stellenbosch University (USEC), Stellenbosch, South Africa.

2.2.2. Fungal isolation from beetles and beetle galleries

Wood from emergence cages was examined for the presence of ascocarps of members of the Ophiostomatales. When present, ascospores were removed from the apices of ascomatal necks with a dissecting needle and transferred to 2 % Malt Extract Agar (MEA; Biolab, Midrand, South Africa) amended with 0.05 g/L cycloheximide (Harrington 1981). Depending on availability, 3 to 50 beetle individuals per beetle morpho-species were placed in eppendorf tubes containing 0.2 ml ddH_2O and vigorously shaken for 1 min on a vortex mixer. A subset of individuals were also crushed and homogenized in 0.2 ml ddH_2O. After washing, the arthropods were removed (when not crushed) and stored in 70% ethanol for later identification and representatives of the crushed beetles were stored for later identification. Water suspensions were spread on Petri dishes containing 2% MEA, streptomycin sulphate (0.04g/L) and cycloheximide (0.05g/L). When present, a single colony of all the suspected ophiostomatoid morpho-types growing on these primary isolation plates were chosen at random and purified as representatives of the different fungal taxa. Once purified, all cultures were maintained on Petri dishes containing MEA at 4°C until further use. Representative cultures of all morpho-types collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

2.2.3. Morphological characterisation

Where possible/available, fruiting structures (ascocarps with ascospores) of the Ophiostomatales taxa found in beetle galleries were collected and mounted in clear lactophenol on microscope slides. These were studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan). For isolates chosen as the types of new species, twenty-five measurements of all characteristic morphologically and taxonomically useful structures were made and the means (± standard deviation) were calculated. A Leica digital camera mounted on the microscope was used to take photographs.

2.2.5. DNA extraction, amplification and sequencing

Three or more isolates from beetles and their galleries, representing each fungal morpho-type, were selected for DNA sequence comparisons (Table 1). Using a sterile scalpel, fungal mycelium was harvested from 2 week old, actively growing colonies on MEA. Following the manufacturer's instructions, genomic DNA was extracted using a Sigma-Aldrich™ plant extraction kit (USA). Primers ITS1-f (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) were used to amplify the nuclear ribosomal internal transcribed spacer region (ITS1, ITS2) and the 5.8S gene region of the rDNA. In cases where amplification was difficult, ITS1–f was replaced with ITS1 (White *et al*. 1990). For DNA amplification, the reaction mixture for the Polymerase Chain Reaction (PCR) was 25 µL, consisting of 11.3 µL ddH_2O, 2 µL DNA, 2.5 µL 10X PCR reaction buffer (with $MgCl_2$), 2.5 µL deoxynucleotide triphosphate mix (dNTP) (5mM), 5 µL GC rich solution (Roche Applied Science, Mannheim, Germany), 0.5 µL of each primer (10mM), 0.5 µL $MgCl_2$ (25mM) and 0.2 µL FastStart Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany). Preliminary placement of fungi (holotypes) was done using ITS, and according to this, amplification of other gene regions for specific clades were chosen (Table 1) based on previous studies (De Beer *et al*. 2013).

For the relevant fungal morph-types, primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used to amplify part of the Beta-tubulin gene region and where amplification did not work, Bt2a (Glass & Donaldson 1995) replaced T10. CL2F and CL2R (Duong *et al*. 2012) were used for PCR for part of the Calmodulin gene, and where amplification was difficult, either CL2R2 (Duong *et al*. 2012) was used in place of CL2R or a combination of the primers CL3F and CL3R was used. The same PCR volumes used for ITS

primers were used for DNA amplification of the other gene regions, except that no GC solution was added. PCR conditions comprised of an initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 60 seconds elongation at 72°C. A final elongation step at 72°C for 8 minutes was performed before termination of the PCR process. In some cases the annealing temperature was lowered to 50°C to enable amplification.

All PCR runs for DNA amplification were performed on a Gene Amp^{R}, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). Agarose gel electrophoresis stained with GelRed (Biotium Inc, California, USA) was used to separate all the amplified (PCR) products that were viewed under ultraviolet light. Following the manufacturer's instructions, amplified PCR products were purified using EXOSAP-IT (USB Corporation, Cleveland, Ohio, U.S.A.). The respective PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) were used for the sequencing reactions of the purified fragments and then analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). PCR sequencing reaction volumes were 12 µL consisting of 2.1 µL 5X reaction buffer, 0.5 µL Big Dye (3.1), 1 µL of primer (10 mM), 2 µL purified PCR product and 6.4 µL ddH_2O. Both DNA strands were sequenced using the same primers as those used for PCR amplifications. The CLC Genomics Workbench software package (CLC Bio, Cambridge, MA) was used to edit and combine sequences from both strands for each isolate to create consensus sequences.

Sequences generated in this study were compared to published sequences for the relevant related taxa available from the GenBank sequence database (http://www.ncbi.nlm.nih.gov) (Table 1). Alignment of sequences with those downloaded from the GenBank was done online using MAFFT 6 (Katoh and Toh 2008) for each dataset. All datasets created were subjected to Bayesian inference (BI) and maximum likelihood (ML) analyses. ML analyses were performed with an online version of PhyML 3.0 (Guindon and Gascuel 2003). jModelTest 0.1.1 (Posada 2008) was used to determine the best fit substitution models using Akaike information criteria (Akaike 1974) and confidence support values for nodes were estimated using 1000 replication bootstrap analyses. Bayesian inference analyses were performed based on a Markov chain Monte Carlo approach (MCMC) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Starting from a random tree, two Markov chains independent of each other were run simultaneously for 10 million generations. At every 2000th generation, trees were sampled and burn-in trees (first 25 000 generations) were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

Table 1. Culture collection details and GenBank accession numbers for strains of ophiostomatoid fungi isolated from bark beetles in this study.

Fungi species	**ITS**	ßT	**CAL**	**Host plant**	**Arthropod sp.**	**Collection Site**
Sporothrix aemuluphilus	38	38		*R. melanophloeos*	*X. aemulus*	Harold Porter National Botanical Garden
		T277	T277	*R. melanophloeos*		
		T278		*R. melanophloeos*		
Sporothrix pallida	T35	T35		*Olea capensis*	*Ctonoxylon.* sp. 1	Gouldveld Forest
	T57	T57		*Olea capensis*	*Lanurgus* sp. 1	Groenkop Forest
	T76	T76	T76	*Olea capensis*		Gouldveld Forest
Raffaelea rapaneae	T92	T92		*R. melanophloeos*	Platypodinae sp.	Gouldveld Forest
	T93	T93		*R. melanophloeos*		
	T110	T110		*R. melanophloeos*		
Raffaelea scabbardiae	T28	T28		*Olea capensis*	*Lanurgus* sp. 1	Goudeveld Forest
	T32	T32		*Olea capensis*		
	T43	T43		*Olea capensis*		
	T44	T44		*Olea capensis*		

2.2.4. Growth in culture

The optimal growth conditions of suspected new species were determined using the type cultures. A disk of agar (10 mm diam) that was covered with mycelium was removed from the edges of actively growing 1 week old cultures. These were placed mycelial side downward facing in the centres of 90 mm Petri dishes containing 20 mL MEA. These were incubated for 10 days in the dark at different temperatures ranging from 5 ºC to 35 ºC at intervals of 5 ºC. For each temperature interval, 5 replicates were used. Two colony diameters perpendicular to each other were taken after incubation and averaged for each isolate and means and standard deviations for each fungal species were calculated.

2.4. Results

2.4.1. Beetles

Four beetle species (three Scolytinae species and one Platypodinae species) were collected from *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* trees. All were associated with members of the Ophiostomatales. *Lanurgus* sp. 1 Eggers and *Ctonoxylon* sp. 1 Hagedorn (Scolytinae, Fig. 1) were both collected from *O. capensis* ssp. *macrocarpa*, while *Xyleborinus aemulus* Wollaston (Scolytinae) and the Platypodinae (Fig. 1) species were collected only from *R. melanophloeos*. Numbers of individuals of each species collected were: *ca*. 2000 individuals of *Lanurgus* sp. 1, *ca*. 300 individuals of *Ctonoxylon* sp. 1, six individuals of *X. aemulus* and only three individuals of the Platypodinae species. In addition, 27 individuals of another beetle species, *Hypothenemus* sp. Westwood, were collected from *R. melanophloeos*, but this species was not associated with any Ophiostomatales.

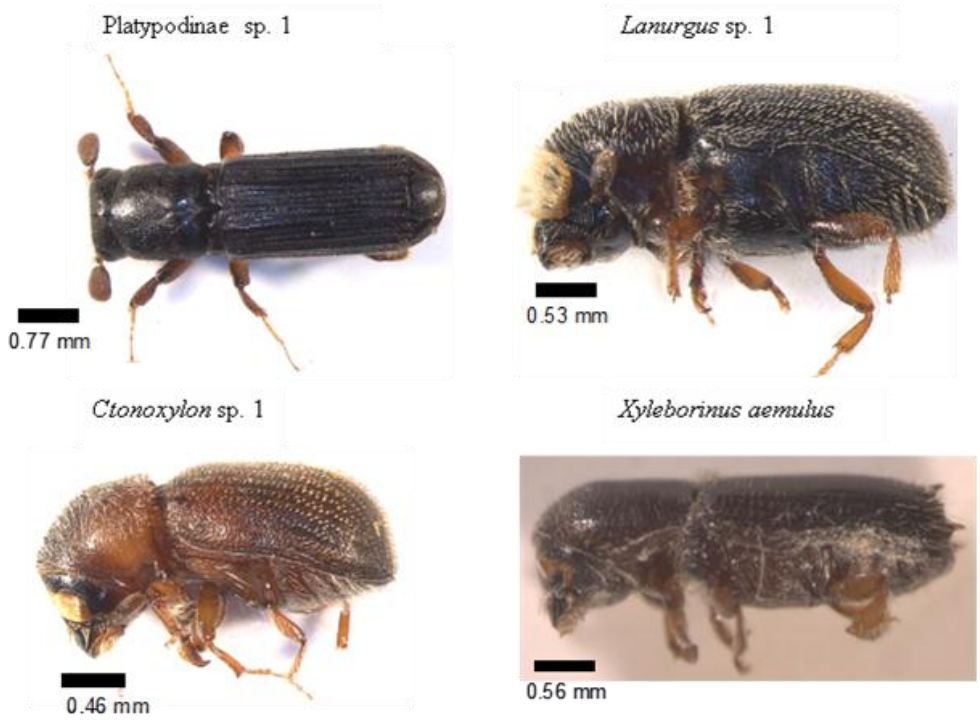

Figure 1: Bark and ambrosia beetles collected from *Olea capensis* ssp. *macrocarpa* and *Rapanea melanophloeos* in the Cape Floristic Region of South Africa.

2.4.2. Fungal isolates and morphological characterisation

A total of 38 fungal isolates that morphologically resembled members of the Ophiostomatales were obtained from the four beetle species. Of these, 15 were isolated from *Lanurgus* sp. 1, 10 from *Ctonoxylon* sp. 1, eight from *X. aemulus* and five from the Platypodinae species. Based on micro-morphology and colony characteristics, these isolates were grouped into four different operational taxonomic units (OTU's). One of the OTU's had an anamorph that resembled *Sporothrix* and was associated with both *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 collected from *O. capensis* ssp. *macrocarpa*. *Xyleborinus aemulus*, collected from *R. melanophloeos*, was associated with a second OTU with a *Sporothrix*-like anamorph. *Lanurgus* sp. 1 from *O. capensis* ssp. *macrocarpa* was associated with a third OTU that resembled species in the genus *Raffaelea*. The Platypodinae beetle from *R. melanophloeos* was associated with the fourth OTU that also resembled species in the genus *Raffaelea*.

2.4.4. Phylogenetic analyses

ITS data confirmed the placement of the collected fungal OTU's in the order Ophiostomatales. Based on initial placement using ITS (data not shown), the phylogenetic placements of the two OTU's with *Sporothrix*-like anamorphs were further investigated using ITS (*Ophiostoma* data set), βT and CAL data, as these grouped with taxa in the *S. schenckii*-*O. stenoceras* complex (De Beer & Wingfield 2013). The phylogenetic placement of the two OTU's with *Raffaelea*-like anamorphs was determined using only ITS data (*Grosmannia* data set). The aligned ITS (*Ophiostoma*) data set consisted of 54 taxa and 723 characters, the βT data set consisted of 45 taxa and 276 characters, while the CAL data set contained 38 taxa and 684 characters. The aligned ITS (*Grosmannia*) data set included 63 taxa and 734 characters. Statistical values obtained from the analyses of the different data sets and the substitution models chosen are presented in Table 2.

Analyses of ITS (*Ophiostoma*), βT and CAL data showed that the *Sporothrix*-like OTU associated with *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 on *O. capensis* ssp. *macrocarpa* grouped with *Sporothrix pallida* (Tubaki) Matsushima and its synonyms *S. albicans* S.B. Saksena and *S. nivea* Kreisel & F. Schuer) with strong support (Meyer *et al* 2008) (Figs 2 & 3; ITS (*Ophiostoma*) tree not presented). The other *Sporothrix*-like OTU from *Xyleborinus aemulus* on *R. melanophloeos* (hereafter referred to as: *Sporothrix aemuluphilus* sp. nov.) grouped

strongly as distinct, but sister to *Ophiostoma candidum* Kamgan-Nkuek., Jol. Roux & Z. W. de Beer using the three same markers (Figs 2 & 3).

Due to a lack of reference data for CAL and βT for the closest taxa to the two OTU's with *Raffaelea*-like anamorphs, phylogenetic analyses were only performed using ITS (Fig. 4). The taxon from *Lanurgus* sp. 1 on *O. capensis* ssp. *macrocarpa* (hereafter referred to as: *Raffaelea scabbardiae* sp. nov) grouped strongly as a distinct taxon sister to *O. deltoideosporum* (Olchowecki & J. Reid) Georg Hausner, J. Reid & Klassen and the taxon from the Platypodinae beetle (hereafter referred to as: *Raffaelea rapaneae* sp. nov.) grouped strongly as distinct, but sister to *R. canadensis* L.R. Batra (Fig. 4). Both *O. deltoideosporum* and *R. canadensis* are believed to be grouped in *Raffaelea s.str*., although the specific placement of *O. deltoideosporum* is still not fully resolved (De Beer & Wingfield 2013).

Table 2. Parameters used and statistical values obtained from Maximum Likelihood (ML) and Bayesian Inferece (BI) analyses of the four datasets.

	Dataset →	**ITS (*Ophiostoma*)**	**ITS (*Grosmannia*)**	ßT	**CAL**
Number of characters		723	734	276	684
ML	Substitution model	GTR+I+G	GTR+I+G	HKY+G	GTR + G
	Gamma shape	0.839	1.251	0.161	0.484
BI	Average Effective Sample Size	1282	1218	1103	2060
	Potential Scale Reduction Factor	1	1	1	1
	GTR Submodel Probability	0.23	0.15	0.072	0.2

2.4.3. Growth in culture

After 10 days of growth in the dark, *S. aemuluphilus* sp. nov. had a mean colony diameter of 24.2 mm (±1.4) at an optimal temperature of 30ºC. Cultures of *R. scabbardiae* sp. nov. grew

optimally at 30ºC, with a mean colony diameter of 23.8 mm (±1.3). *Raffaelea rapaneae* sp. nov. had a colony diameter of 21 mm (±0.95) when grown at its optimal temperature of 20ºC.

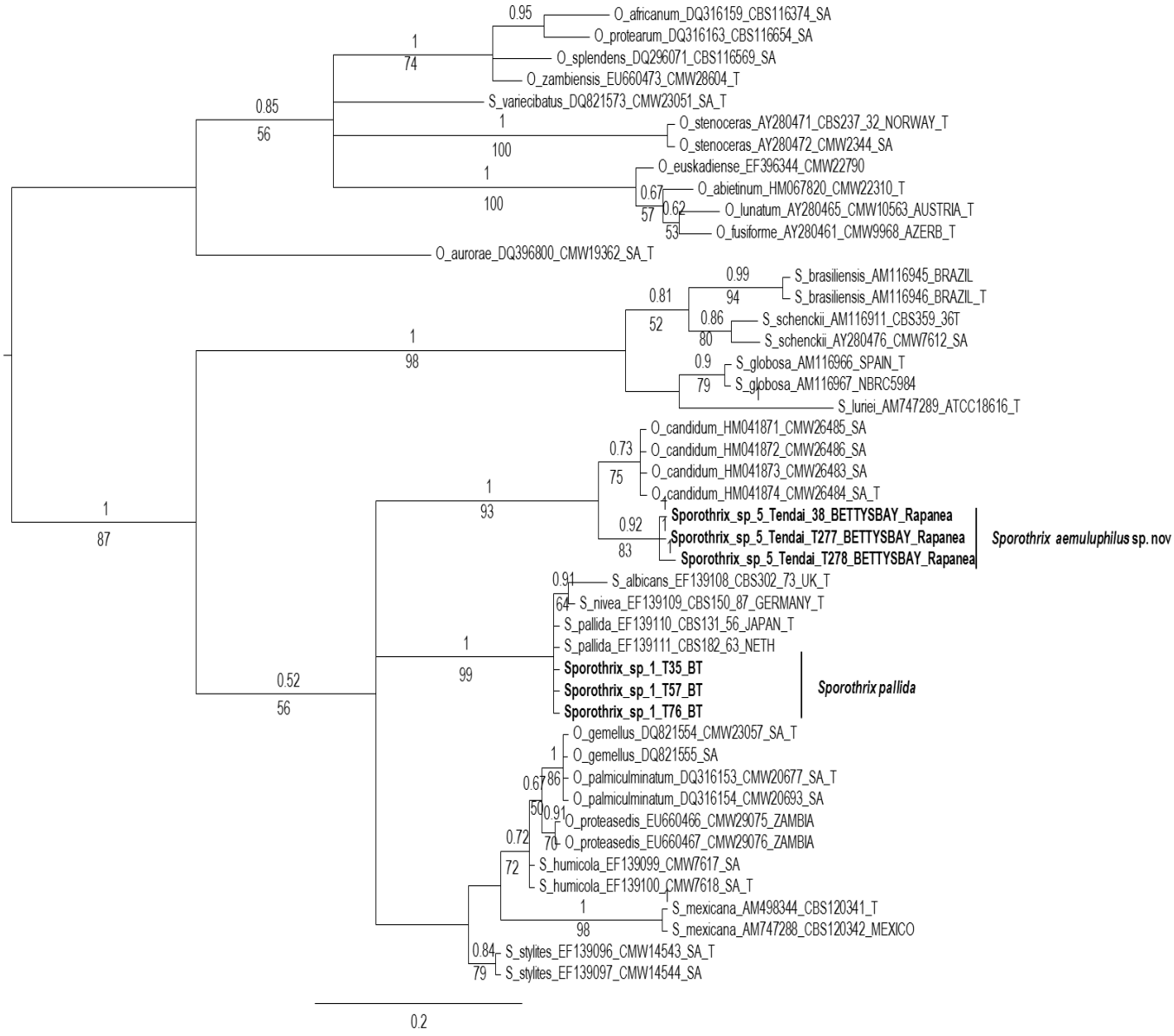

Figure 2: Bayesian Inference consensus tree (βt data) of members of the *S. schenckii-O. stenoceras* complex (De Beer *et al*. 2013). Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood Analysis. Isolates in bold were collected in

this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes.

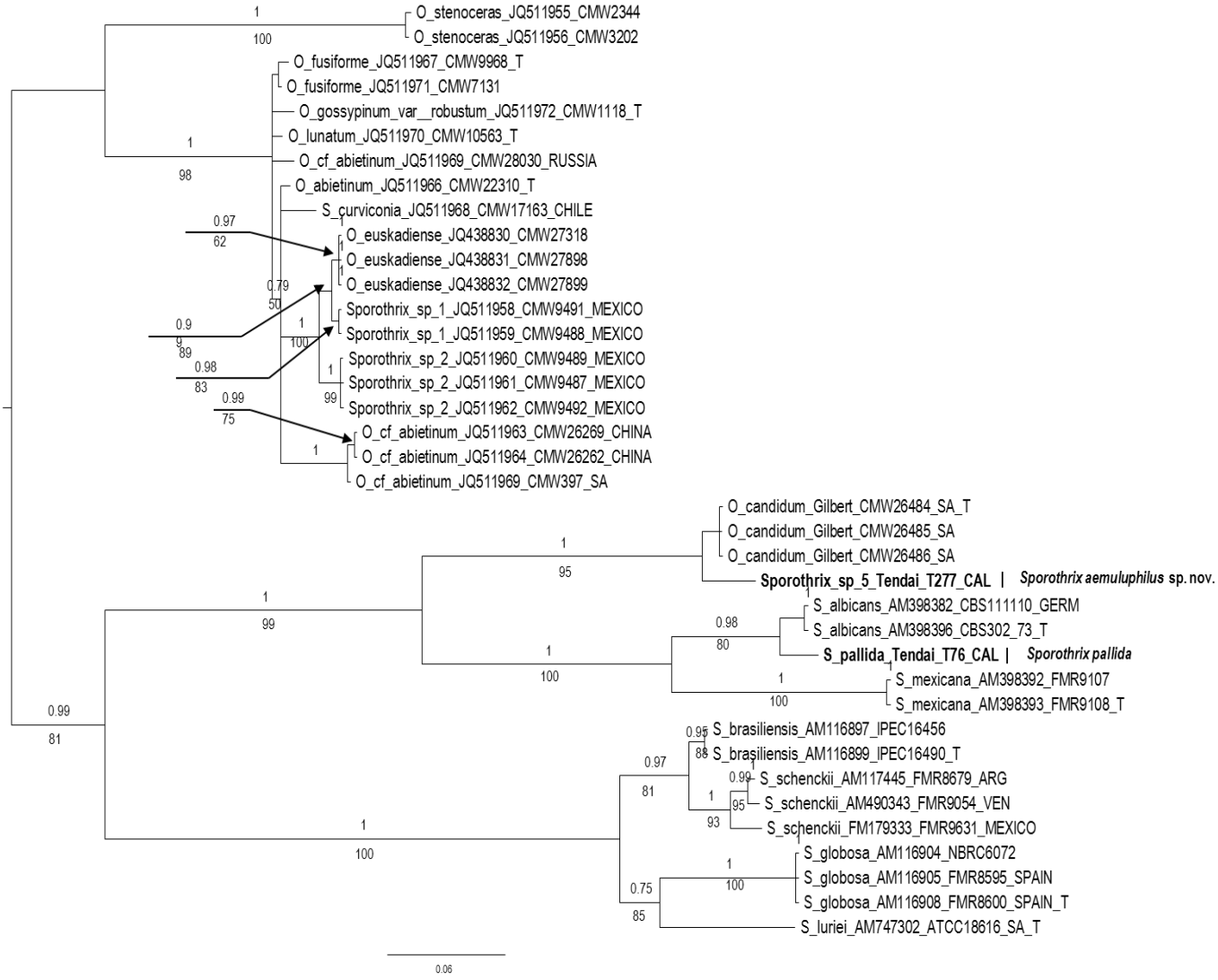

Figure 3: Bayesian Inference consensus tree (CAL data) of members of the *S. schenckii-O. stenoceras* complex (De Beer and Wingfield 2013). Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood Analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes.

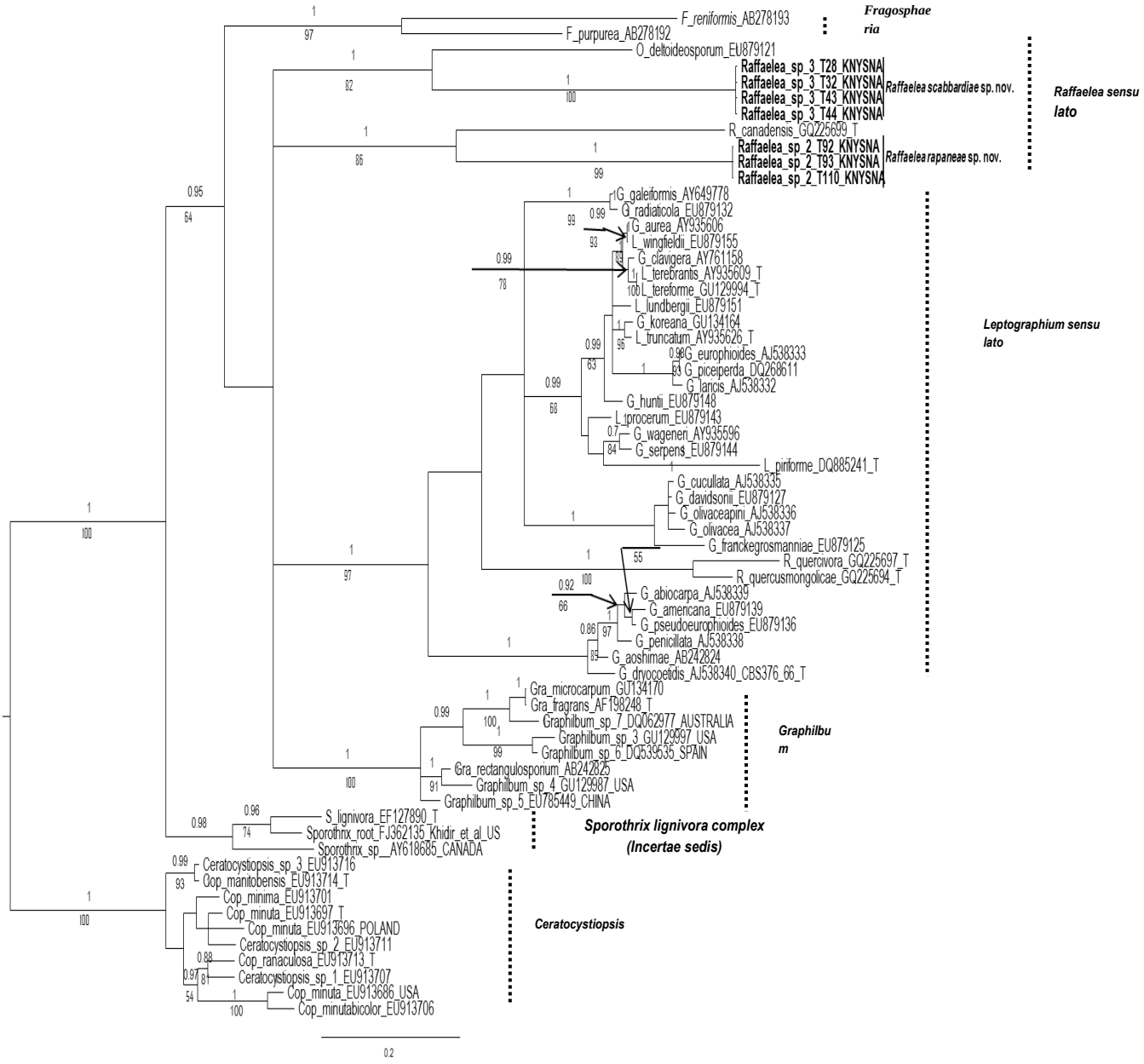

Figure 4: Bayesian Inference consensus tree (ITS *Grosmannia* data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood Analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. Dashed lines demarcate the different species complexes that the fungi belong to.

2.4.5. Taxonomy

Based on micro-morphological and phylogenetic analyses three of the four collected taxa are recognised as distinct and undescribed taxa (all except *O. pallida*). These are therefore here described as new species as follows:

Sporothrix aemuluphilus **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 6.

Etymology: The epithet *aemuluphilus* refers to the close association between this species and the beetle *Xyleborinus aemulus*.

Ascomata embedded in and superficial on the host substrate, bases black, globose, 81-162 (140 ± 25) µm diam, hyphal ornamentation absent. *Ascomatal* necks black, 278-743 (278 ± 743) µm long, 22-45 (36 ± 7) µm wide at the base, 7-11 (9 ± 1.1) µm wide at the tip (Fig 5A), osteolar hyphae present, slightly curved, hyaline, 13-20 (13 ± 3) µm long (Fig 5B). *Asci* evanescent. *Ascospores* allantoid, aseptate, hyaline, sheaths absent, 2.9-4.5 x 0.9-1.3 µm (Fig. 5C), accumulating in a sticky jelly-like droplet at the tip of the neck, becoming whitish when dry. *Colonies* of *Sporothrix*-like anamorph whitish in colour on MEA, odourless, circular with entire edge and fluffy on MEA (Fig. 5D). Colony diameter reaching 24.2 mm (±1.4) after 10 days of growth on MEA at an optimum growth temperature of 30ºC in the dark. No growth below 5ºC or above 35ºC. *Conidiophores* hyaline, tapering towards the tip, 4.5-8.2 x 0.8-1.2 µm (Fig. 5E), *Conidiogenous* cells forming directly from tips of conidiophores, hyaline, prominent denticles present 0.6-1.4 (0.9 ± 0.3) µm wide (Fig. 5E). *Conidia* hyaline, aseptate, oblong shaped, holoblastic, 3.8-7.2 x 0.9-1.5 µm (Fig. 5F).

Substrate: Isolated from *Xyleborinus aemulus* and its galleries on *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Gouldveld and Gourna forest. Isolated from *Xyleborinus aemulus* associated with *Rapanea melanophloeos* April, 2010, T. Musvuugwa, **holotype** PREM (pending), culture ex-holotype 38 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype

T277 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T278 CMW (pending) = CBS (pending).

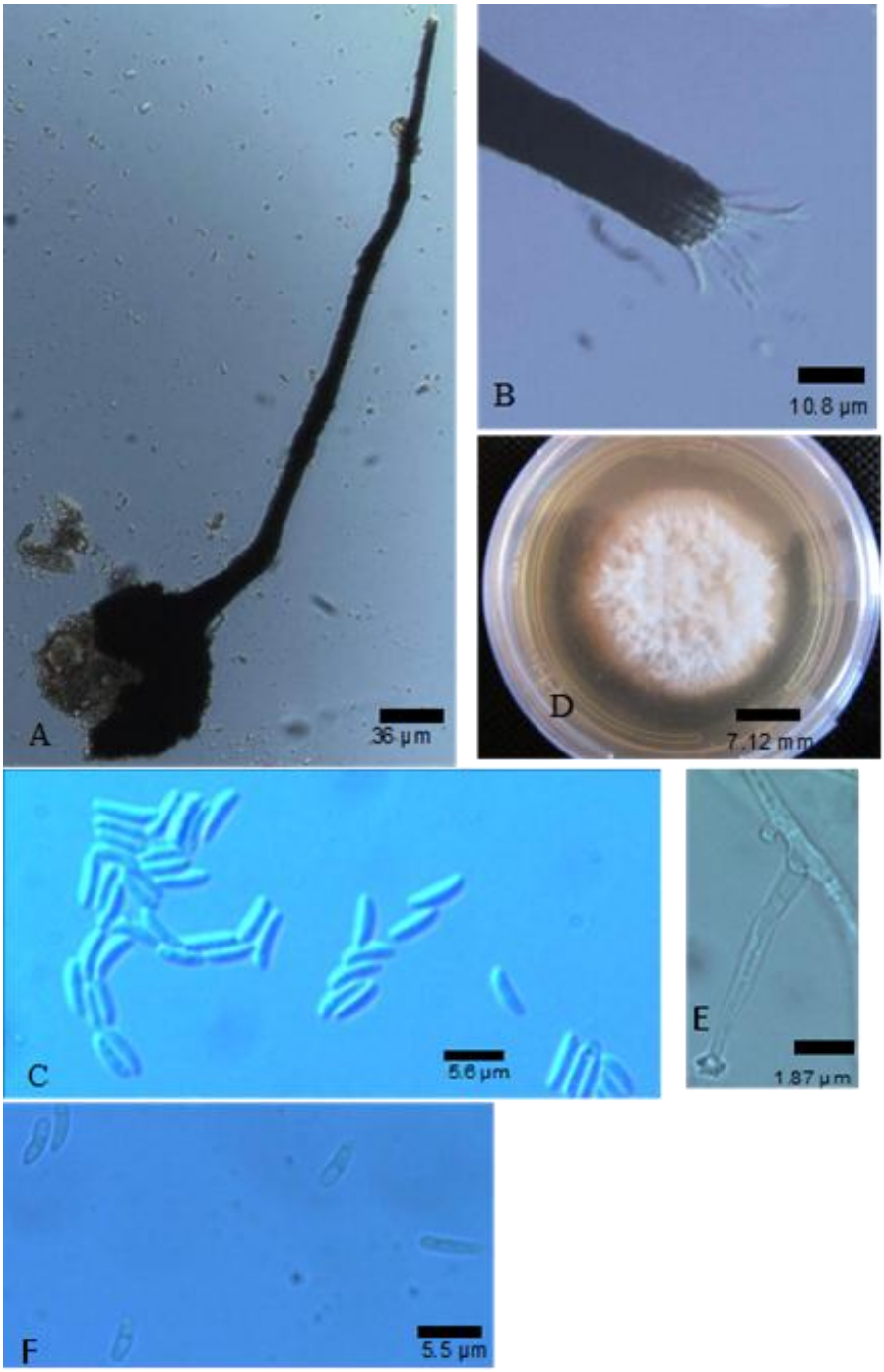

Figure 5: Micrographs of *Sporothrix aemuluphilus* sp. nov. A. Perithecium removed from bark of *Rapanea melanophloeos* colonised by *Xyleborinus aemulus*. B. Tip of perithecial neck showing ostiolar hyphae. C. Ascospores. D. Two-week-old colony on MEA. E. Conidiophore with conidiogenous cells showing denticles. F. Conidia.

Raffaelea scabbardiae **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 6.

Etymology: The epithet *scabbardiae* (*scabbard* = sheath) refers to the sheathed ascospores produced by this species.

Ascomata superficial on the host substrate, bases globose, black, with no hyphal ornamentation, 92-142 (112 ± 9) µm diam; necks black, 311-692 (392 ± 74) µm long, 24-40 (33 ± 4) µm wide at the base, 5-18 (12 ± 4) µm wide at the apex, osteolar hyphae absent (Fig. 6A & 6B). *Asci* evanescent. *Ascospores* cylindrical, aseptate, hyaline, sheaths present, 2.3-3.4 x 0.8-1.5 µm (Fig. 6C), accumulating in a gelatinous droplet at the tip of the neck, becoming white to cream colored when dry. *Colonies* white to cream coloured on MEA. Odourless, circular with entire edge and rough surface (Fig. 6D). Colony diameter reaching 23.8 mm (±1.3) after 10 days of growth on MEA at optimal growth temperature of 30ºC in the dark. No growth below 10ºC or above 35ºC. *Conidiophores* hyaline, 7.1-8.9 x 0.7-1.1 µm (Fig. 6E), *Conidiogenous* cells forming directly from apex of conidiophores, hyaline (Fig. 6E), *Conidia* aseptate, hyaline, thick walled, obovate shaped, 3.5-4.9 x 0.7-1.1 µm (Fig. 6F-G). Conidia produced in single, directly from hyphae (Fig. 6F).

Substrate: Isolated from *Lanurgus* sp. 1 (Scolytinae) and its host plant *Olea capensis* ssp. *macrocarpa.*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Gouldveld and Gourna forest. Isolated from *Lanurgus* sp. associated with *Olea capensis*, October 2011, T. Musvuugwa, **holotype** PREM (pending), culture ex-holotype T43 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T28 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T32 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T44 CMW (pending) = CBS (pending).

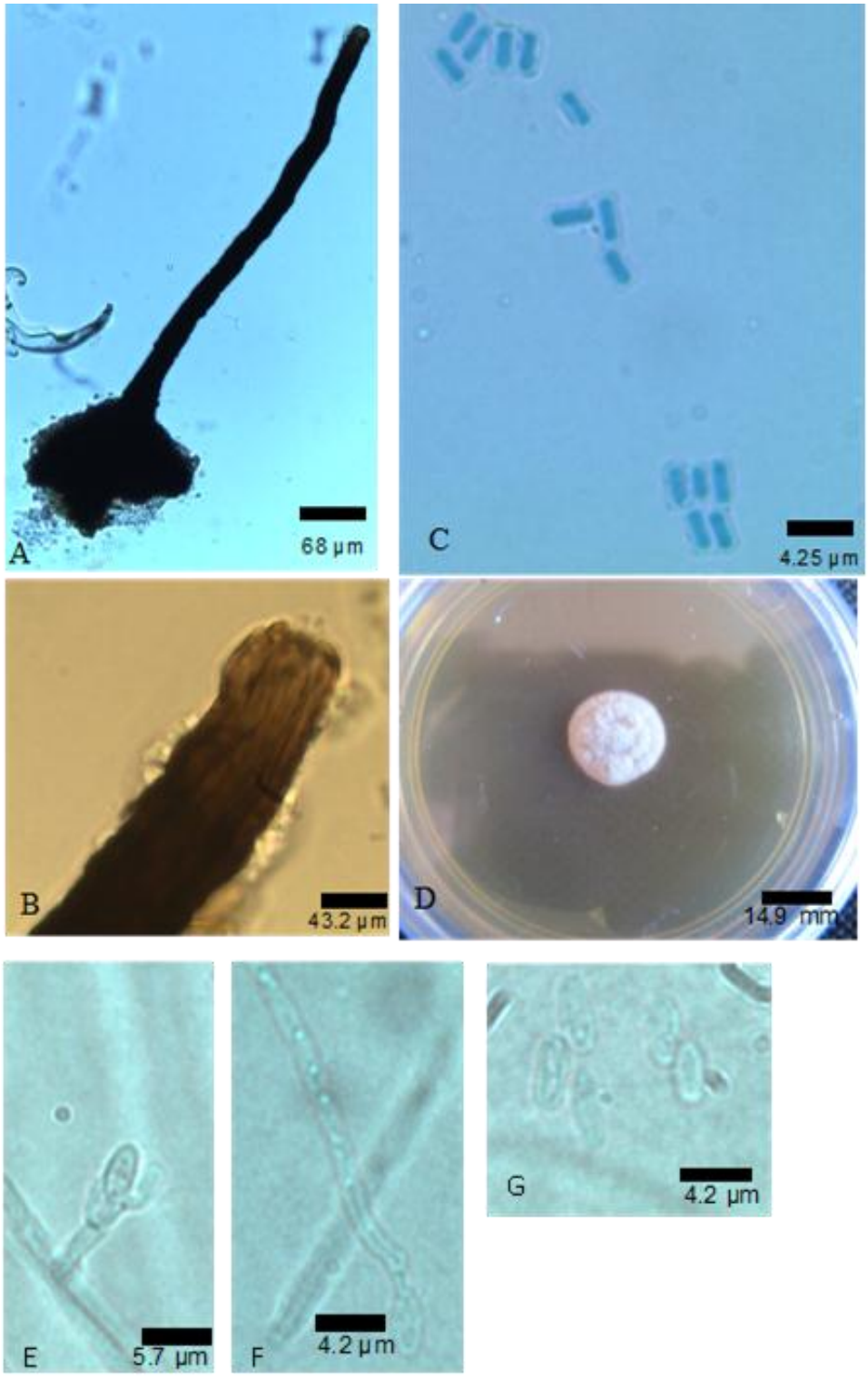

Figure 6: Micrographs of *Raffaelea scabbardiae* sp. nov. A. Perithecium removed from the gallery of *Lanurgus* sp. 1 in wood of *Olea capensis* ssp. *macrocarpa.* B. Tip of perithecial neck. C. Ascospores. D. Two-week-old colony on MEA. E. Conidiophores. F-G. Conidia.

Raffaelea rapaneae **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 7.

Etymology: The epithet *rapaneae* refers to the host plant genus (*Rapanea melanophloeos*) from which this species was collected.

Ascomata not observed. *Colonies* cream turning blackish on MEA, odourless, surface tough and leathery, which wrinkles and forms cracks under the wrinkled area (Fig 7A). Colony diameter reaching 21 mm after 10 days on MEA at 20ºC. Optimal growth at 20ºC. No growth below 10ºC or above 25 ºC, cream in the center and black along the edges, somewhat circular with rough surface and edge. *Conidiophores* hyaline, becoming tapering towards the tips, segmented appearance 10.6-15.5 x 1.5-1.7 µm (Fig. 7B-C), *Conidiogenous* cells arising from tips of conidiophores, hyaline (Fig. 7C), *Conidia* aseptate, hyaline, turning brown when mature, thick walled, spherical, form in masses 2.3-3.5 x 2.4-3.4 µm (Fig. 7C-D).

Substrate: Isolated from a Platypodinae beetle species collected from the wood of *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Gourna forest. Isolated from Platypodinae sp. associated with *Rapanea melanophloeos*, February 2012, T. Musvuugwa, **holotype** PREM (pending), culture ex-holotype T92 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T93 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T110 CMW (pending) = CBS (pending).

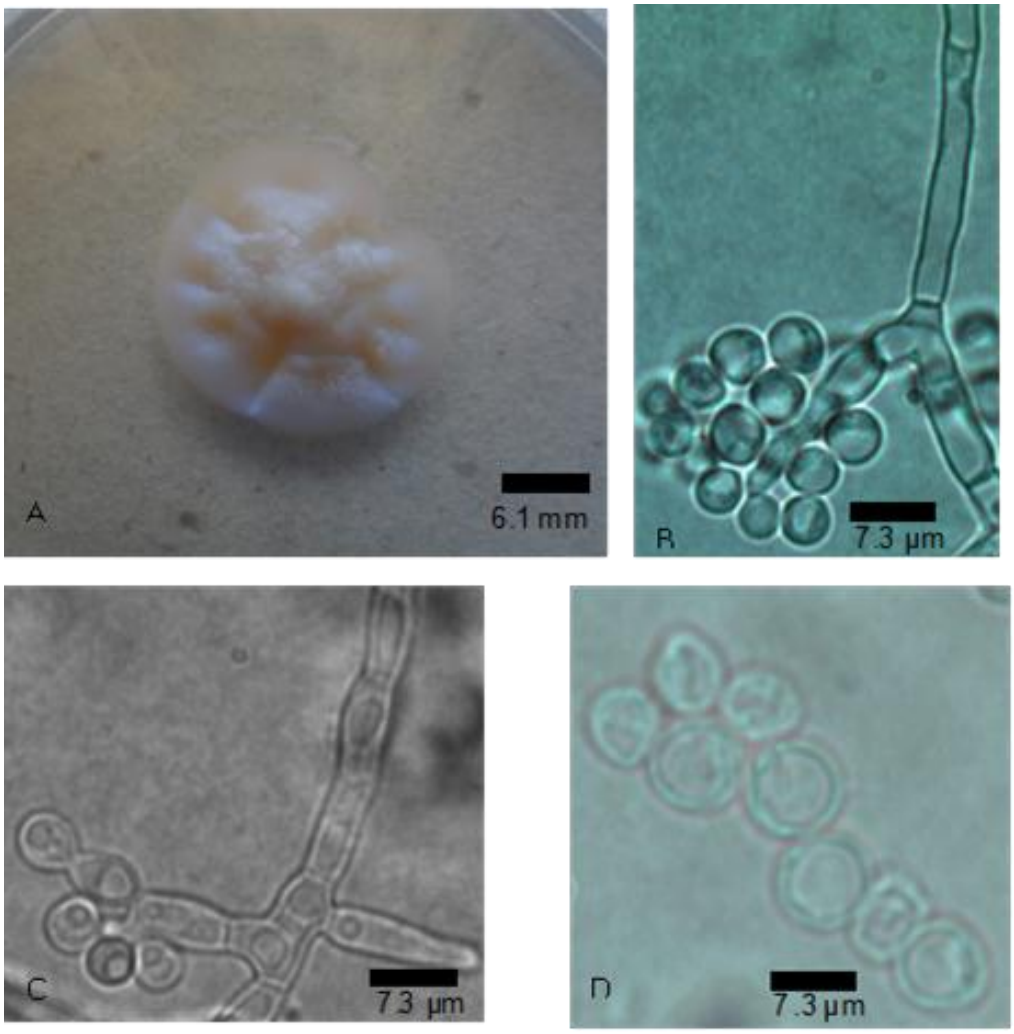

Figure 7: Micrograph of *Raffaelea rapaneae* sp. nov. A. Two-week-old colony on MEA. B-C. Conidiophores. D. Conidia.

2.5. Discussion

This represents the first study on the Ophiostomatales associated with sub-cortical beetles from trees native to the Afromontane forests of the CFR, South Africa. Previous studies focussed on either those taxa associated with wounds on native trees (e.g. Kamgan *et al*. 2008) or on the beetle associates of exotic tree taxa (e.g. Zhou *et al.* 2001). Three species from two genera were newly described here from only four beetles and from only two tree species. Considering the large diversity of native plant and sub-cortical beetles associated with these forests, it is reasonable to assume that many taxa still await discovery. This study therefore forms a platform for future studies on the diversity, evolution and ecology of these important fungi.

Both the *Sporothrix* species collected in this study belong to the *S. schenckii-O. stenoceras* complex, which is part of a larger contingent, *Ophiostoma sensu lato* (De Beer & Wingfield 2013). Besides the human pathogens such as *S. schenckii* and *S. luriei*, the *S. schenckii-O. stenoceras* complex mostly contains species that are associated with environmental samples such as soil and water sediments. However, some of the species in this complex are arthropod-associated. Examples include taxa collected from *Protea* infructescences, which are specifically associated with mites (Roets *et al*. 2009; 2007) and a single species that has infrequently been isolated from a bark beetle collected from a *Pinus* spp. (*O. aurorae* X.D. Zhou & M.J. Wingf) (de Meyer *et al*. 2008, Zhou *et al*. 2006). Given the number of southern African species in this complex, it has been suggested that southern Africa may be the centre of diversity for this group of fungi (Kamgan *et al*. 2012).

Our isolates of *S. pallida* were collected from both *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1. *Sporothrix pallida* is known from many environmental habitats (e.g. water sediments, soil and the sporophore of a slime-mould) and from various European countries (e.g. Germany, Spain, Italy, Netherlands, and England) and Japan (de Meyer *et al*. 2008). This fungus was also recently isolated from a human patient as the causative agent of a corneal ulcer (Morrison *et al*. 2013). To the best of our knowledge our findings represent the first example of this fungus forming associations with Scolytinae beetles. It is also the first example of a member of the Ophiostomatales associated with beetles belonging to the genera *Lanurgus* and *Ctonoxylon*.

Similar to *S. pallida*, the newly described *S. aemuluphilus* was found to be associated with a Scolytinae beetle, *Xyleborinus aemulus*. It is closely related to *O. candidum* that is known to be associated with a Cerambycidae beetle on exotic *Eucalyptus* spp. trees in South African plantations (Kamgan *et al*. 2012). *Sporothrix aemuluphilus* and *O. candidum* differ in many characteristics, including optimal growth temperature (30 ºC for *S. aemuluphilus* and 25 ºC for *O. candidum*), ornamentation at the base of ascoma (present in *O. candidum* and absent in *S. aemuluphilus*) and dimensions of morphological characters (Nkuekam *et al*. 2012). Despite quite extensive surveys from wounds on trees in the Afromontane forests where *S. aemuluphilus* occurs, including those on the same *R. melanophloeos* host, this species has never been found previously (Kamgan *et al*. 2008). Therefore, *S. aemuluphilus* seems to represent the only species in the *S. schenckii*-*O. stenoceras* complex that is known exclusively as a Scolytinae beetle associate. Whether this fungus is involved in beetle nutrition is unknown. However, this beetle seems to have an ambrosial-type of tunnelling system, which is usually seen in beetle taxa that depend on fungi for survival (De Fine Licht & Biedermann 2012; Jordal & Cognato 2012). *Xyleborinus saxesenii*, an ambrosia beetle in the same genus as *X. aemulus*, is mutualistically associated with fungi in the Ophiostomatales (Biedermann 2012). This suggests that the association between *X. aemulus* and *S. aemuluphilus* may be mutualistic, but this needs to be confirmed in future studies.

The two *Raffaelea* species collected in this study group with members of the *Raffaelea s. str*. clade (De Beer *et al*. 2013). Although the two species group in different clades within *Raffaelea s. str.*, they are closely related. Most of the species in *Raffaelea s. str.* are associated with ambrosia beetles (De Beer *et al*. 2013). This was also found to be the case with the two *Raffaelea* species collected here. Morphologically they generally exhibit *Hyalorhinocladiella*-like anamorphs and reduced conidiogenous structures, often producing pigmented conidia (De Beer *et al*. 2013). Only a few other *Raffaelea* species have been isolated in South Africa. These include *R. albimanens* D.B. Scott & J.W. du Toit, which was associated with the ambrosia beetle *P. externedentatus* Fairm. from *Ficus sycomorus* L. in the Dukuduku Forest in KZN province. *R. arxii* D.B. Scott & J.W. du Toit, represents another South African example. It is associated with *Xyleborus torquatus* Eichh. and was collected from *Cussonia umbellifera* Sond. (Scott & du Toit 1970).

Raffaelea scabbardiae was associated with the ambrosia beetle *Lanurgus* sp. 1 from *Olea capensis* ssp. *macrocarpa* and represents the first report of any Ophiostomatales from both the beetle and tree host. It is phylogenetically most closely related to *O. deltoideosporum* with which it shares some morphological characters. Their ascospores, for example, are similar in that they are cylindrical with an ossiform sheath and both form compact colonies on agar (De Beer *et al*. 2013). However, they differ in terms of their perithecia, which are small with an average neck length of 180 µm in *O. deltoideosporum*, while those of *R. scabbardiae* are 392 µm long. The perithecial necks of *O. deltoideosporum* have unique ostiolar hyphae at their apices and their perithecial bases are occasionally ornamented with septate spines (Olchowecki & Reid 1974), both of which are characters absent in *R. scabbardiae*. *Ophiostoma deltoideosporum* is known from Canada, where it was isolated from stained pine wood (Olchowecki & Reid 1974). This species, together with *R. scabbardiae* and *O. seticolle* (R.W. Davidson) de Hoog & R.J. Scheff. (which also exhibits similar ascospores and perithecia) (Davidison 1966), are the only members in the group with known teleomorphs. Although most of the species in the *Raffaelea s. str*. clade are associated with ambrosia beetles, *Raffaelea scabbardiae* is the first species in the group that is associated with a beetle in the genus *Lanurgus*.

Raffaelea rapaneae was isolated from a Platypodinae beetle associated with *R. melanophloeos*. Phylogenetically it is closely related to *R. canadensis*, which was first isolated in Canada from the ambrosia beetle *Platypus wilsoni* Swaine infesting *Pseudotsuga menziesii* (Mirb.) Franco (Batra 1963). More recently the species was also found to be causing laurel wilt disease symptoms on avocado trees in California (Eskalen & McDonald 2011). Morphologically *R. rapaneae* and *R. canadensis* share some similarities such as tough and leathery colonies, which wrinkle and form cracks under the wrinkled area (Batra 1963). They differ in characters such as their optimal growth temperatures, which is 25 ºC for *R. canadensis* and 20 ºC for *Raffaelea rapaneae* and the colour of colonies on agar (greenish black for *R. canadensis* and creamish black for *Raffaelea rapaneae*) (Batra 1967). The beetle associates of both of these fungi belong to the subfamily Platypodinae, a beetle subfamily associated with many *Raffaelea* species. *Raffaelea ambrosiae* Arx & Hennebert, for example, is associated with *Platypus cylindricus* Fab. and *P. compositus* Say (Batra 1967; Baker 1963), *R. santoroi* Guerrero is associated with *P. sulcatus* Chap (Guerrero 1966) and

the South African *R. albimanens* is associated with *P. externedentatus* (Scott & du Toit 1970).

The beetles species collected in this study were mostly associated with recently dead trees, although they were collected from weakened living trees in a few cases. These beetles can therefore be classified as secondary beetles, known to attack stressed, weakened and recently dead trees (Avtzis *et al*. 2012; Six & Wingfield 2011; Paine *et al*. 1997). Although secondary beetles have been responsible for the destruction of large populations of stressed trees (for example Breshears *et al*. 2005), we have seen no evidence of this at the sites included in this study.

2.6. Conclusion

Four members of the order Ophiostomatales were collected in this study from four subcortical beetles infesting *R. melanophloes* and *O. capensis* ssp. *macrocarpa*. This represents the first report of subcortical beetles associated with the Ophiostomatales in the CFR. Phylogenetic analyses confirmed that one new species belongs to the *S. schenckii-O. stenoceras* complex, while two other new species belong to *Raffaelea s. str*. Given that three out of the four fungal species collected are new species, it is highly likely that there are more Ophiostomatales beetle-associates waiting discovery in the CFR. More research should be conducted to include other native tree taxa and sub-cortical beetles. Although this study was an important first step towards understanding relationships between subcortical beetles and their fungal symbionts on native trees in the CFR, the exact nature of these relationships are still unclear and needs to be a focus in future studies.

References

Alamouti, S.M., Tsui, C.K.M. and Breuil, C. 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. *Mycological Research* 113: 822–835.

Atkinson, T.H. and Peck, S.B. 1994. Annotated checklist of the bark and ambrosia beetles (Coleoptera: Platypodidae and Scolytidae) of tropical southern florida. *The Florida Entomologist* 77: 313-329.

Atkinson, T.H. and Equihua, A. 1986. Source biology of the scolytidae and platypodidae (Coleoptera) in a tropical deciduous forest at Chamela, Jalisco, Mexico. *The Florida Entomologist* 69:303-310.

Avtzis, D.N., Bertheau, C. and Stauffer, C. 2012. What is next in bark beetle hylogeography? *Insects* 3: 453-472.

Baker, J.M. 1963. Ambrosia beetles and their fungi with particular reference to *Platypus cylindrus* Fab. Symbiotic associations, (Nutman, P.S. and Mosse, B. eds.), 13th Symposium, Society for General Microbiology., pp. 232-265. Cambridge: University Press.

Barras, S.J. and Perry, T.J. 1972. Fungal symbionts in the prothoracic mycangium of *Dendrocfonus frontalis*. *Zeitschrift fiir angwandefe Enfomologie* 71: 95-104.

Batra, L.R. 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. *Science* 153: 193-195.

Batra, L.R. 1966. Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science* 66: 213-236.

Batra, L.R. 1963. Ambrosia fungi: A taxonomic revision, and nutritional studies of some species. *Mycologia* 59: 976-1017.

Beaver, A.R. 2005. A remarkable new species of *Cyclorhipidion hagedorn*, and new records of bark and ambrosia beetles from Gabon (Coleoptera: Curculionidae, Scolytinae and Platypodinae). *Entomologist's monthly magazine* 141: 113-119.

Beaver, R.A. 1989. Insect-fungus relationships in the bark and ambrosia beetles. In: *Insect-fungus interactions*, (Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. eds.), Academic Press, London, United Kingdom, pp. 121–143.

Beaver, R.A. 1977. Bark and ambrosia beetles in tropical forests. *BIOTROP Special Publication* 2: 133–147.

Beaver, A.R. and Liu, L.-Y. 2013. A synopsis of the pin-hole borers of Thailand (Coleoptera: Curculionidae: Platypodinae). *Zootaxa* 3646: 447-486.

Berryman, A.A. 1972. Resistance of conifers to invasion by bark beetle fungus associations. *Bioscience* 22: 598–602.

Brasier, C.M. 2000. Intercontinental spread and continuing evolution of the Dutch elm disease pathogens. In: *The elms: breeding, conservation and disease management*, (Dunn, C.P. ed.), Boston, USA, Dordrecht, The Netherlands, London, UK: Kluwer Academic Publishers, pp. 61–72.

Brasier, C.M. & Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Breshears, D.D., Cobb, N.S., Rich, P.M., Price, K.P., Allen, C.D., Balice, R.G., Romme, W.H., Kastens, J.H., Floyd, M.L., Belnap, J., Anderson, J.J., Myers, O.B. and Meyer, C.W. 2005. Regional vegetation die-off in response to

global-change-type drought. *Proceedings of the National Academy of Sciences of the United States of America* 102: 15144-15148.

Bridges, J.R. and Moser, J.C. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* 8: 9–12.

Browne, F.G. 1968. Pests and diseases of forest plantation trees. Clarendon Press, Oxford, pp. 1330.

Chen, S.F., Wingfield, M.J., Roets, F. and Roux, J. 2013. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea* in South Africa. *Plant Pathology* 62: 667-678.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013. A nomenclature for ophiostomatoid genera and species in the Ophiostomatales and Microascales. In : *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.). CBS Biodiversity Series 12, pp 245-322.

De Fine Licht, H.H. and Biedermann, P.H.W. 2012. Patterns of functional enzyme activity in fungus farming ambrosia beetles. *Frontiers in Zoology* 9:13.

De Meyer, E.M., De Beer, Z.W., Summerbell, R.C., Moharram, A.M., De Hoog, G.S., Vismer, H.F. and Wingfield, M.J. 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* 100:647–661.

Duong, T. A., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2012. Phylogeny and taxonomy of species in the *Grosmannia serpens* complex." *Mycologia* 104: 715-732.

Farrell, B.D., Sequeira, A.S., O'Meara, B.C., Normark, B.B., Chung, J.H. and Jordal, B.H. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution* 55: 2011–2027.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113-118.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* 61: 1323-1330.

Guindon, S. and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696-704.

Haberkern, K.E., Illamn, B.L. and Raffa, K.F. 2002. Bark beetles and fungal associates colonizing white spruce in the Great Lakes region. *Canadian Journal of Forest Research* 32:1137–1150.

Harrington, T.C. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: *Insect-Fungal Associations: Ecology and Evolution*, (Vega, F.E. and Blackwell, M. eds.), Oxford University Press, New York, pp. 1–22.

Harrington, T.C. 1993. Biology and taxonomy of fungi associated with bark beetles. In: *Beetle-pathogen interactions in conifer forests*, (Schowalter, T.D. and Filip, G.M. eds.) Academic Press, NY, pp .37–58.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* 73: 1123-1129.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D.N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* 104:399-404.

Heybroek, H.M. 1993. Why bother about the elm? In: *Dutch Elm Disease Research* Cellular and Molecular Approaches, (Sticklen, M.B. and Sherald, J.L. eds.), Springer-Verlag, New York, pp. 1–8..

Holsten, E.H. 2001. The northern spruce engraver: *Ips perturbatus*. USDA For. Serv. Anchorage, AK (http://www.fs.fed.us/r10/spf/fhp/ipstext.htm).

Hulcr, J., Mogia, M., Isua, B. and Novotny, V. 2007. Host specificity of ambrosia and bark beetles (Coleoptera, Curculionidae: Scolytinae and Platypodinae) in a New Guinea rainforest. *Ecological Entomology* 32: 762–772.

Hudgins, J.W., Christiansen, E., Franceschi, V.R. 2004. Induction of anatomically based defence responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. *Tree Physiology* 24: 251-264.

Jacobs, K. and Wingfield, M.J. 2001. *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. APS press, St Paul, Minnesota, U.S.A.

Jordal, B.H. and Cognato, A.I. 2012. Molecular phylogeny of bark and ambrosia beetles reveals multiple origins of fungus farming during periods of global warming. *BMC Evolutionary Biology* 12:133.

Jordal, B.H., Beaver, R.A., Kirkendall, L.R. 2001. Breaking taboos in the tropics: inbreeding promotes colonization by wood-boring beetles. *Global Ecology and Biogeography* 10:345–357.

Kamgan Nkuekam, G., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2012. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Katoh, K. and Toh, H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* 9: 286-298.

Klepzig, K.D and Six, D.L. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis* 37: 189-205.

Klepzig, K.D., Moser, J.C., Lombardero, F.J, Hofstetter, R.W. and Ayres, M.P. 2001. Symbiosis and competition: Complex interactions among beetles, fungi and mites. *Symbiosis* 30: 83–96.

Knižek, M. and Beaver, R. 2004. Taxonomy and Systematics of Bark and Ambrosia Beetles. In: *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis*, (Lieutier, F., Day, K.R., Battisti, A., Grégoire, J.-C. and Evans, H.F. eds.), Kluwer Academic Publishers: Dordrecht, The Netherlands, pp. 41–54.

Kubono, T. and Ito, S. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Malan, R. 2006. Aspects of population biology and ecology of Euphorbia ingens on the Mokopane game breeding center in the Limpopo Province of South Africa. Assignment 3, Plant studies 4A, B. Pretoria, South Africa: Tech Nature Conservation, Department of Nature, Conservation, Tshwane University of Technology.

O'Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103-116.

Paine, T.D., Raffa, K.F. and Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* 42:179–206.

Payne, T.L. 1980. Life history and habits. In: *The southern Pine Beetle*, (Thatcher, R.C., Searcy, J.L., Coster, J.E. and Hertel, G.D. eds.), USDA Forest Service Technical Bulletins 1631: 7-23.

Pipe, N.D., Brasier, C.M. and Buck, K.W. 2000. Evolutionary relationships of the Dutch elm disease fungus *Ophiostoma novo-ulmi* to other *Ophiostoma* species investigated by restriction fragment length polymorphism analysis of the rDNA region. *Journal of Phytopathology* 148:533–539.

Price, T.S., Doggett, C., Pye, J.L., and Holmes, T.P. 1992. A history of southern pine beetle outbreaks in the southeastern United States. Sponsored by the Souhtern Forest Insect Workshop Conference. The Georgia Forestry Commission, Macon, GA, p. 65.

Roets, F., Dreyer, L.L., Crous, P.W. and Wingfield, M.J. 2009. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* 38: 143-152.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Ronquist, F.R. and Huelsenbeck, J.P. 2003. MrBayes: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

Roux, J., Malan, R., Howitt, M., Six, D.L. and Wingfield, M.J. 2009. Fungi associated with diseased *Euphorbia ingens* in South Africa. *46th Biannual Conference of the Southern African Society for Plant Pathology*, 25th–28th January 2009, Gordons Bay, South Africa.

Schedl, K.E. 1972. Monographie der Famile Platypodidae (Coleoptera). W. Junk, the Hague, p. 322.

Scott, D.B. and Du Toit, J.W. 1970. Three new *Raffaelea* species. *Transactions of the British Mycological Society* 55: 181–186.

Six, D.L. 2012. Ecological and evolutionary determinants of bark beetle - fungus symbioses. *Insects* 3: 339-366.

Six, D.L. 2003. Bark beetle-fungus symbioses. In: *Insect Symbioses*, (Bourtzis, K. and Miller, T. eds.), CRS press, Boca Raton, FL, USA, pp. 97-114.

Six, D.L. and Wingfield, M.J. 2011. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. *Annual Review of Entomology* 56:255-272.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, U.S.A.

Van Der Linde, J.A, Roux, J., Wingfield, M.J. and Six, D.L. 2012. Die-off of giant Euphorbia trees in South Africa: Symptoms and relationships to climate. *South African Journal of Botany* 83:172-185.

VanWyk, B. and VanWyk, P. 1997. Field Guide to Trees of Southern Africa. Cape Town, South Africa: Struik Publishers.

Webber, J.F. 1990. The relative effectiveness of *Scolytus scolytus*, *S. multistriatus* and *S. kirschii* as vectors of Dutch elm disease. *European Journal of Forest Pathology* 20: 184–192.

Werner, R.A. and Holsten, E.H. 1984. Scolytidae associated with felled white spruce in Alaska. *Canadian Entomologist* 116: 465–471.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A sequencing guide to methods and applications*, (Innis, M.S., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds.), Academic Press, San Diego, pp. 315-322.

Wingfield, M.J., Harrington, T.C. and Solheim, H. 1995. Do conifer bark beetles require fungi to kill trees? In: *Proceedings of bark beetles, blue-stain fungi, and conifer defense systems*, (Christiansen, E. ed.) Norwegian Forest Research Institute Symposium, Aas Norway, p. 6.

Whitney, H.S. 1982. Relationships in bark beetles and symbiotic organisms. In: *Bark beetles in North America conifers* (Mitton, J.B. & Sturgeon, K.B. eds.). University of Texas, Austin, pp. 183-211.

Wood, S.L. 1982. The bark and ambrosia beetles of north and central America (Coleoptera: Scolytidae). A taxonomic monograph. *Great Basin Naturalist Memoirs* 6: 1–1359.

Wood, S.L. and Bright, D.E. 1992. A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic index. *Great Basin Naturalist Memoirs* 13: 1–1553.

Zhou, X.D., De Beer, Z.W. and Wingfield, M.J. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* 53: 290-300.

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D. and Wingfield, M.J. 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* 55: 75-97.

Chapter 3: Wounds on *Rapanea melanophloeos* provide habitat for a large and unexplored diversity of ophiostomatoid fungi

Abstract

Rapanea melanophloeos, an important canopy tree in Afromontane forests, is commonly utilised for medicinal bark harvesting. Wounds created from these activities provide entrance for many fungi, including arthropod-associated pathogenic members of the Ophiostomatales and Microascales (ophiostomatoid fungi). In this study we assess the diversity of wound-associated ophiostomatoid fungi on storm-damaged *R. melanophloeos* trees in the Afromontane forests of South Africa. Five fungal species in the Ophiostomatales were identified based on micro-morphological and molecular phylogenetic analyses. These included *Ophiostoma stenoceras* and four newly described taxa *Sporothrix reniformis* sp. nov., *S. rapaneae* sp. nov., *S. lunateae* sp. nov. and *S. noisomeae* sp. nov. Four of these are members of the *S. schenckii-O. stenoceras* complex (*O. stenoceras*, *S. reniformis* sp. nov., *S. rapaneae* sp. nov., *S. lunateae*) and the other is a member of the *S. lignivora* complex (*S. noisomeae*). In addition to other taxa known from this host, the present study shows that there is a rich, yet still poorly explored, diversity of ophiostomatoid fungi associated with *R. melanophloeos* in Afromontane forests. More taxa are likely to be discovered with increased research effort. These must be assessed in terms of pathogenicity towards this ecologically and economically important tree.

Key words: storm damage wounds, Afromonatane forests, *Ophiostoma*

3.1. Introduction

Ophiostomatoid fungi represent genera that are morphologically similar, although not phylogenetically closely related (De Beer *et al*. 2013a, Viljoen *et al*. 1999,). The group includes genera such as *Ophiostoma* Syd., *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Raffaelea* Arx & Hennebert and *Leptographium* Lagerb. & Melin in the order Ophiostomatales, and *Ceratocystis* Ellis & Halst., *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas and *Graphium* Corda in the order Microascales (De Beer *et al*. 2013b). A common morphological similarity shared by these fungi is the globose ascomata with usually elongated necks that give rise to masses of sticky spores at their tips, an entomochoric adaptation to allow spore dispersal via arthropods (Malloch & Blackwell 1993; Wingfield *et al*. 1993; Upadhyay 1981).

Ophiostomatoid fungi contain some of the best known tree pathogens (Brasier & Buck 2001; Zhou *et al*. 2001; Heybroek 1993; Wingfield *et al*. 1993). Some of these are responsible for very high rates of tree mortality, which has led to significant losses to forests (Harrington *et al*. 2008; Brasier & Buck 2001; Zhou *et al*. 2001; Heybroek 1993). Well-documented examples include *Raffaelea quercivora* Kubono et Shin. Ito, responsible for oak die-back and mortality of Japanese oak trees (Kubono & Ito 2002) as well as the pathogens *Ophiostoma ulmi* (Buismann) Nannf and *O. novo-ulmi* Brasier, responsible for the Dutch Elm disease pandemics in Europe and the United States of America (Brasier 2008; Brasier 2000; Pipe *et al*. 2000; Wingfield *et al*. 1993). A number of pathogenic *Leptographium* species have also been reported. Examples include *L. procerum* (W.B. Kendr) M.J. Wingf. that is associated with pine root disease (Jacobs & Wingfield 2001), *L. calophylli* (Wiehe) JF. Webber, K. Jacobs & MJ. Wingfield that causes vascular wilt disease of takamaka trees (Webber *et al*. 1999; Wiehe 1949) and *L. wageneri* (Goheen & F.W. Cobb) T.C. Harr. that causes black stain root disease on conifers (Harrington 1993; Cobb *et al*. 1987). Some taxa are not pathogenic, or just weakly pathogenic, but may also cause significant problems in the forestry industry. For example, many species of *Ophiostoma* are responsible for sapstain of lumber and pulpwood, which degrades the quality of wood and results in substantial economic losses (Zhou *et al*. 2001; Seifert 1993; Lagerberg *et al*. 1927).

Most ophiostomatoid fungi infect their hosts via wounds (Wingfield *et al*. 1993). Such wounds are caused by various agents, including animals (e.g. arthropods), natural phenomena such as hail, frost or heavy rains as well as human practises such as bark stripping and siviculture (Vermeulen *et al*. 2012; Kamgan *et al*. 2008; Morris *et al* 1993; Moller & Devay 1968). *Ophiostoma minus*, for example, colonises wounds created through mechanical injury or partial debarking (Gibbs 1993). Fungi are transported to these wounds through agents such as arthropods (Harrington & Wingfield 1998; Hinds 1972; Moller & Devay 1968) or wind in the case of asexual states of *Sporothrix* with dry spores (Wingfield *et al*. 1993). Despite the importance of ophiostomatoid fungi as tree pathogens, reports of these fungi in Africa, including South Africa, are very limited. A few studies focused on taxa associated with native tree hosts (Kamgan *et al*. 2008; Roets *et al*. 2008; 2006; Marais & Wingfield 2001; 1997; De Beer *et al*. 1995), but most knowledge of the fungi in South Africa is based on species associated with non-native host tree species (Kamgan *et al*. 2012a; Zhou *et al*. 2006; 2001; De Beer *et al*. 1995).

The Afromontane forests of South Africa are found from Table mountain in the extreme southwest of the Cape Floristic Region, are most extensive in the Tsitsikamma areas of the southern Cape coastal regions, and extends northwards along the coastal mountains into the KwaZulu-Natal (Lubke & McKenzie 1996). They occur as small fragmented patches within river valleys, on mountains, foothills and on coastal platforms (Geldenhuys 2010), and represent the outliers of the tropical African Afromontane forests (Turpie *et al*. 2003). *Rapanea melanophloeos* Mez is one of the more abundant and very important canopy trees found in these forests. It is a fast growing, evergreen forest pioneer, but can also be found as large trees in mature forests (Van Wyk & Van Wyk 1997). The bark of this plant is often harvested for medicinal purposes (Vermeulen *et al*. 2012). This creates wounds through which fungi can infect (Vermeulen *et al*. 2012). In South Africa, wound-associated ophiostomatoid fungi have been collected from this species, some of which have proven to be very pathogenic (Kamgan *et al*. 2008). For example, *Ceratocystis tsitsikammensis* Kamg. Nkuek. & Jol. Roux was isolated from wounds on *R. melanophloeos* in the Tsitsikamma forests and could kill seedlings of this species within a relatively short period (Kamgan *et al*. 2008). Despite the fact that pathogens are of critical importance to ecosystem function (Castello *et al*.1995), there has been

little focus on them, especially on those associated with native trees in the Afromontane forests of South Africa (Taylor 2001). The recently described virulent pathogen, *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf. & Jol. Roux, that causes the death of *Rapanea* trees in the Harold Porter Botanical garden (Chen *et al*. 2013), illustrates the importance of documenting these fungal taxa.

In addition to *C. tsitsikammensis*, only a few other wound infecting ophiostomatoid fungi have been isolated from *R. melanophloeo*s. These include a *Pesotum fragrans* (Mathiesen-Käärik) G. Okada & Seifert-like fungus and *O. quercus* (Georgévitch) Nannf. (Kamgan *et al*. 2008). More recently, a few bark/ambrosia–beetle associated taxa have also been identified from *R. melanophloeos*, and were newly described as *Sporothrix aemuluphilus* Musvuugwa, LL. Dreyer & F. Roets and *Raffaelea rapaneae* Musvuugwa, LL. Dreyer & F. Roets (Musvuugwa *et al*. 2013, Chapter 2). Clearly this tree species is under considerable threat, and it has already been observed that *R. melanophloeos* trees in several sites of the Afromontane forests are declining. There is thus an urgent need to study other fungi, especially ophiostomatoid species, associated with *R. melanophloeos* trees in their natural habitats. The objective of this study was therefore to assess the diversity of wound-associated ophiostomatoid fungi on *Rapanea* trees at various sites across its distribution range in South Africa.

3.2. Materials and methods

3.2.1. Sampling of plant material and fungal isolation

Wounds on *Rapanea melanophloeos* trees were surveyed in the Harold Porter National Botanical Garden (S 34' 20.893 E 18' 55.519), Groenkop Forest Reserve (S 33°56'32.10" E 22°32'50.38") and Weza Forest (30°36’12.00” S 29°39’59.00” E) in the Afromontane forests of the Cape Floristic Region and KwaZulu-Natal Province. All wounds were presumably caused by weather damage during strong winter storms. Bark and wood samples were collected from these wounds during late winter (August) of 2010 - 2012, placed in separate sampling bags and transferred to the laboratory where they were examined for the presence of members of the Ophiostomatales using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan). When no fungi were present, collected material was placed in moisture

chambers (resealable plastic bags with ddH_2O-moistened paper towels) at room temperature (~23°C) in the dark for up to four weeks to stimulate fungal growth. When present, masses of ascospores and/or conidia were collected from the apices of sporulating structures using a sterile needle. They were transferred to 2% malt extract agar (MEA) emended with 0.05g/L cycloheximide (Harrington 1981) to select for members of the Ophiostomatales. Isolates were stored in the dark at room temperature and examined daily for fungal growth. Isolates resembling anamorphic states of ophiostomatoid fungi were purified by transferring single hyphal tips from the edges of actively growing fungal colonies to fresh MEA plates. All purified cultures were maintained on petri dishes containing MEA at 4°C until further use. Representative cultures of all morpho-types collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

3.2.2. Morphological characterisation

Where available, ascocarps and ascospores of ophiostomatoid fungi were collected from plant material, mounted in clear lactophenol on microscope slides and studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan). Twenty-five measurements of all morphologically and taxonomically useful structures were made of isolates suspected to represent new taxa. The maximum and minimum measurement for each taxonomically useful character was noted and means (± standard deviation) were calculated. Photographs of the structures were taken using a Leica digital camera mounted on the microscope.

3.2.3. Fungal identification

Based on morphological characteristics, all fungal cultures suspected to be ophiostomatoid fungi were grouped into Operational Taxonomic Units (OTU). Three or more pure isolates collected in this study, representing each OTU, were randomly chosen for DNA sequence comparisons (Table 1). For DNA extraction, fungal mycelium was harvested from edges of 2 week old actively growing colonies of pure cultures on MEA using a sterile scalpel. A Sigma-Aldrich™ plant extraction kit

(USA) was used for the extraction of DNA based on the manufacturer's instructions. To amplify the nuclear ribosomal internal transcribed spacer region (ITS1, ITS2), including the 5.8S gene region of the rDNA, the primers ITS1-f (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) were used and where amplification was difficult, ITS1-f was replaced with ITS1 (White *et al*. 1990). Preliminary placement of fungi (holotypes) using ITS was used as baseline to decide on additional markers to amplify for each fungal morpho-type (Table 1). For amplification of part of the Beta-tubulin gene, the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used and in cases where amplification was difficult, Bt2a (Glass & Donaldson 1995) was used in combination with T10. PCR for the calmodulin marker was done using the primers CL2F (5′-GACAAGGAYGGYGATGGT-3′) and CL2R (5′-TTCTGCATCATGAGYTGSAC-3′) (Duong *et al*. 2012) and in cases where amplification was difficult, CL2R2 (Duong *et al*. 2012) was used in stead of CL2R (or a combination of CL3F and CL3R was used). For each marker, PCR reaction components, volumes and conditions followed those described by Musvuugwa *et al*. (2013, Chapter 2).

All products were amplified using a Gene Amp^{R}, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). Amplified PCR products were separated using agarose gel electrophoresis stained with GelRed (Biotium, Inc., California, USA) and visualized under ultraviolet light. All amplified PCR products were cleaned using EXOSAP-IT (USB Corporation, Cleveland, Ohio, U.S.A.) following the manufacturer's instructions. Purified fragments were sequenced using the respective PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) and analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). The PCR sequencing reaction volumes were the same as those used in Musvuugwa *et al*. (2013, Chapter 2) and the same primers used for PCR amplifications were used to sequence both DNA strands. Resultant sequences were edited and used to construct consensus sequences using the CLC Genomics Workbench software package (CLC Bio, Cambridge, MA).

BLAST algorithm (Altschul *et al*. 1990) searches were performed using ITS sequences to preliminary identify the fungal isolates and to compare and find similarities with sequences published on the GenBank sequence database (http://www.ncbi.nlm.nih.gov). Sequences of closely related taxa to isolates collected in this study (Table 1) were downloaded from the GenBank database and they were aligned with sequences generated in this study using MAFFT 6 (Katoh & Toh 2008) for each data set. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on all the datasets created. An online version of PhyML 3.0 (Guindon & Gascuel 2003) was used to perform ML analyses. To determine the best fit substitution models jModelTest 0.1.1 (Posada 2008) was used using Akaike information criteria (Akaike 1974). Statistical confidence support values for nodes were determined using 1000 replication bootstrap analyses. MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) was used to perform BI analyses employing a Markov chain Monte Carlo approach (MCMC). Two Markov chains independent of each other were run simultaneously for 10 million generations starting from a random tree. A sample frequency of 2000 was implemented and the burn-in trees (first 25 000 generations) were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

3.2.4. Growth in culture

To determine the optimal growth conditions of potential new species a disk of agar (10 mm diam) covered with mycelium was removed from edges of actively growing, one week old cultures. They were placed upside down in the centres of 90 mm fresh Petri dishes containing 20 mL MEA. The Petri dishes were incubated in the dark at different temperatures ranging from 5 ºC to 35 ºC at intervals of 5 ºC for a period of 10 days. Five replicates were used for each temperature interval. After 10 days, two colony diameters perpendicular to each other were measured for each colony and results were averaged (± standard deviation) for each species.

Table 1. Culture collection and GenBank accession numbers for strains of ophiostomatoid fungi isolated from *Rapanea melanophloeos* that were sequenced in this study.

Fungi species	**ITS**	**βT**	**CAL**	**Collection site**
Sporothrix lunateae	T124	T124	T124	Weza Forest
	T125	T125	T125	
	T6	T6	T6	
Sporothrix noisomeae	T3	T3		Weza Forest
	T11	T11	T11	
	T12		T12	
	T13	T13	T13	
Sporothrix rapaneae		T148	T148	Groenkop Forest
	T149	T149		
	T60	T60	T60	
Sporothrix reniformis	39	39		Harold Porter National Botanical Garden
	T197	T197		
	NO	T198		
Ophiostoma stenoceras	T1	T1	T1	Weza Forest
	T10	T10	T10	
		T187	T187	

3.3. Results

3.3.1. Fungal isolates and morphological characterisation

In total, 39 isolates that morphologically resembled fungi in the Ophiostomatales were obtained from wounds on several *R. melanonophloeos* trees sampled at the different sites (Table 1). Based on colony characteristics and micro-morphology, the isolates collected in this study were grouped into five different operational taxonomic units (OTU's) that all resembled *Sporothrix* species. Of the 39 isolates obtained, 8 were from an OTU collected from Harold Porter National Botanical Garden (Table 1). They were characterised by perithicia (collected from bark only) with brownish ascomatal bases that had hyphal ornamentation present and produced whitish to cream-coloured colonies that were fluffy at the centre. Seven isolates collected from Groenkop Forest were assigned to another OTU. They were characterised by black ascomata collected from bark and the production of white fluffy colonies on MEA (Table 1). The third OTU identified included nine isolates collected from Weza Forest. They were characterised by producing white colonies that grew slowly compared to other OTU's (Table 1). A second OTU comprising of 10 isolates was also collected at Weza Forest (Table 1). They were characterised by black ascomata collected from bark and also produced white colonies that turned blackish with age. Five isolates of a fifth OTU also collected from Weza Forest (Table 1) were characterised by black ascomata that only started to grow in culture after three weeks and slow-growing colonies, which either became dark brown or remained white with age.

3.3.2. Phylogenetic analyses

All the fungal OTU's collected belong to the Ophiostomatales based on their initial placement from ITS data. This phylogenetic placement led to further investigation using ITS, βT and CAL data. Alignment of data sets resulted in 84 taxa and 785 characters for ITS, 73 taxa and 280 characters for βT and 58 taxa and 720 for CAL. Statistical values obtained from the analyses of the different data sets are summarized in Table 2, along with the substitution models chosen. GenBank accession numbers

for other taxa used in the phylogenetic analyses are presentenced on the respective trees for each data set (Figs. 1-3).

For the OTU collected from the Harold Porter National Botanical Garden, phylogenetic analyses were only performed using ITS and βT (Figs. 1 & 2) and could not be performed for the CAL gene region due to amplification failure. Analysis of this particular OTU (hereafter referred to as: *Sporothrix reniformis* sp. nov.) using ITS data resulted in its isolates grouping together as a strongly supported distinct taxon sister to a clade comprising of *S. aemuluphilus* Musvuugwa, LL. Dreyer and F. Roets, *O. candidum* Kamgan-Nkuek., Jol. Roux & Z. W. de Beer and the OTU from Groenkop forest collected in this study. Analyses using βT data resulted in a strongly supported distinct taxon sister to *S. aemuluphilus*.

Analyses of ITS, βT and CAL data for the OTU collected from Groenkop forest (hereafter referred to as: *Sporothrix rapaneae* sp. nov.) resulted in trees with similar placement of isolates. The ITS data did not distinguish between *S. rapaneae*, *S. aemuluphilus* and *O. candidum*, which formed a single group (Fig. 1). Analyses of the βT data set showed that *S. rapaneae* grouped as a distinct taxon sister to *O. candidum* (Fig. 2), while analyses of the CAL dataset resulted in the isolates forming a strongly supported distinct taxon sister to *S. aemuluphilus* (Fig. 3). When comparing the βT and CAL data, the distinct taxon formed as sister to *S. aemuluphilus* (CAL data) is strongly supported, while the distinct taxon formed as sister to *O. candidum* (βT data) is not very strongly supported.

Analyses of ITS data for the first OTU collected from Weza Forest (hereafter referred to as: *Sporothrix lunateae* sp. nov.) showed its isolates grouping into a distinct clade with strong support as sister to *O. aurorae* X.D. Zhou & M.J. Wingf and also closely related to a bigger clade comprising of the species *O. fusiforme* D.N. Aghayeva & M. J. Wingfield, *O. lunatum* D.N. Aghayeva & M. J. Wingfield and *O. abietinum* Marmolejo & Butin (Fig. 1). Analyses of ßT and CAL data showed similar placement of *Sporothrix lunateae* (Figs. 2 & 3), where it grouped as a strongly supported distinct taxon sister to a big clade that forms smaller clades of species such as *O. fusiforme*, *O. lunatum* and *O. abietinum*.

The second taxon from Weza Forest (hereafter referred to as: *Sporothrix noisomeae* sp. nov.) grouped strongly as distinct taxon in analyses of all three data sets. Analyses

of ITS data showed its isolates grouping into a strongly supported clade sister to the genus *Graphilbum* (Fig. 1). Based on ßT data, the taxon formed a distinct clade sister to the *Sporothrix lignivora* complex (De Beer *et al*. 2013) with strong support (Fig. 2), while with CAL data a strongly supported distinct clade was also formed (Fig. 3). Analyses of ITS, ßT and CAL data showed that the fifth OTU grouped with isolates of *Ophiostoma stenoceras* (Robak) Nannf. with strong support (Figs. 1-3).

Table 2. Parameters used and statistical values obtained from Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of the three datasets

	Dataset →	**ITS**	**BT**	**CAL**
Number of characters		785	280	720
ML	**Substitution model**	GTR+I+G	K80+G	HKY+I+G
	Gamma shape	1.303	0.184	1.878
BI	**Average Effective Sample Size**	366	1368	1007
	Potential Scale Reduction Factor	1	1	1
	GTR Submodel Probability	0.24	0.124	0.299

3.3.3. Growth in culture

After a period of 10 days of growth in the dark, cultures of *S. reniformis* grew optimally at 25ºC, with a mean colony diameter of 35.7 mm (±1.3), while *S. rapaneae* grew at an optimal temperature of 25ºC and the mean colony diameter was 34.9 mm (±0.7). The mean colony diameter of *S. lunateae* was 38 mm (±1.2) at an optimal

temperature of 25ºC and *S. noisomeae* grew optimally at 25ºC with a mean colony diameter of 28.7 mm (±1.1) after 10 days of growth in the dark.

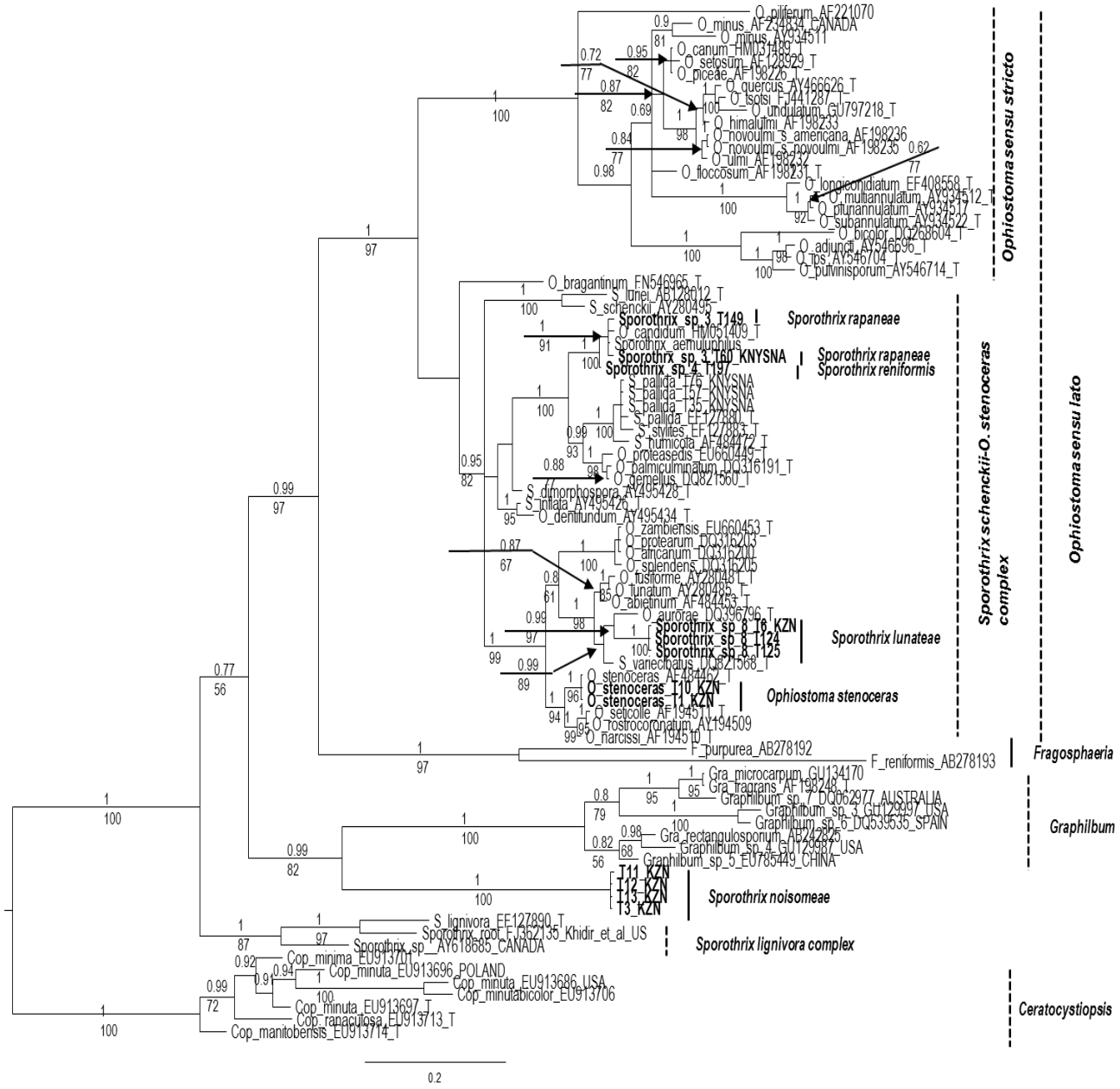

Figure 1: Bayesian Inference consensus tree (ITS data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. Dashed lines demarcate the different genera or species complexes that the fungi belong to.

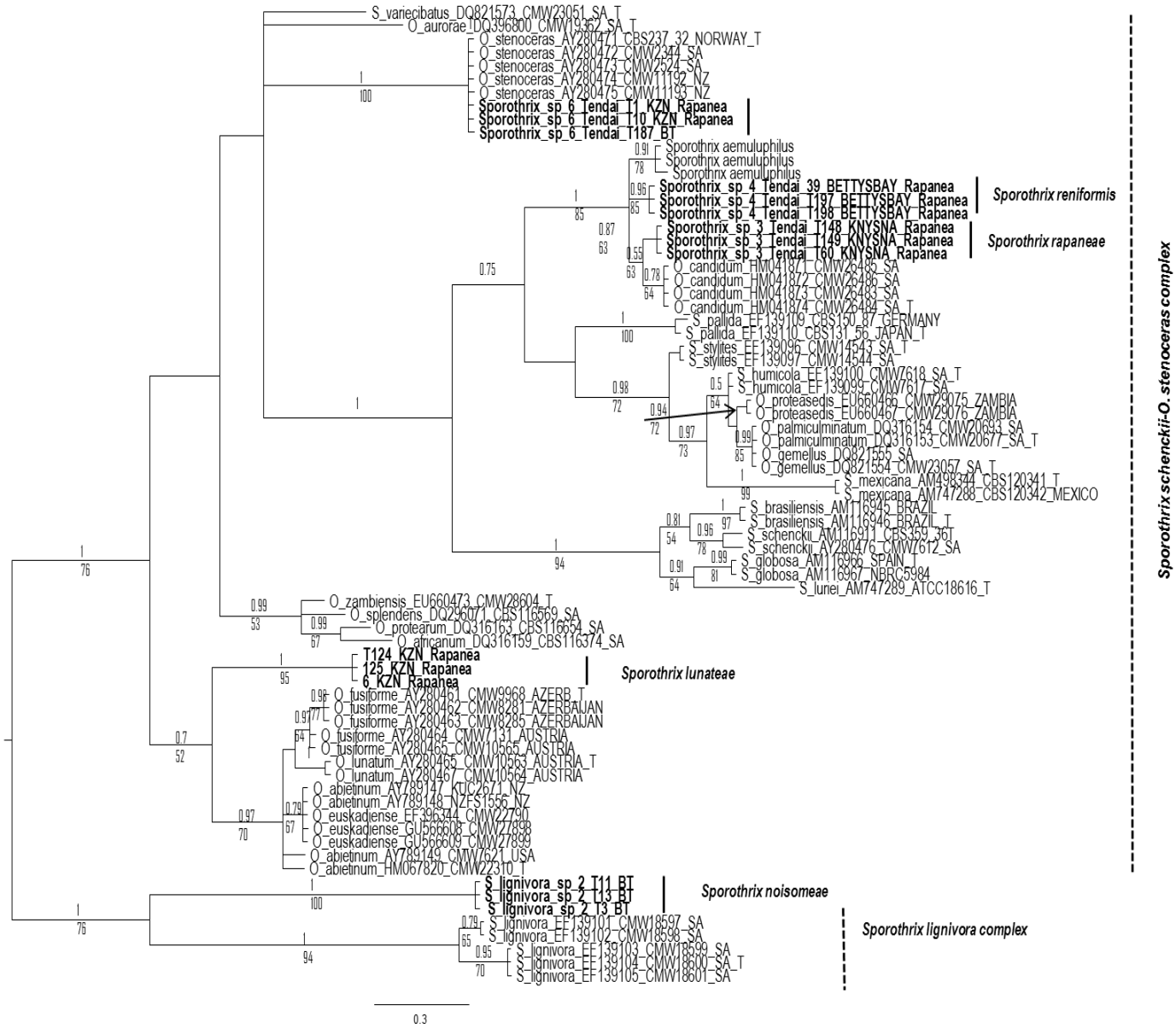

Figure 2: Bayesian Inference consensus tree (βt data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. Dashed lines demarcate the different species complexes that the fungi belong to.

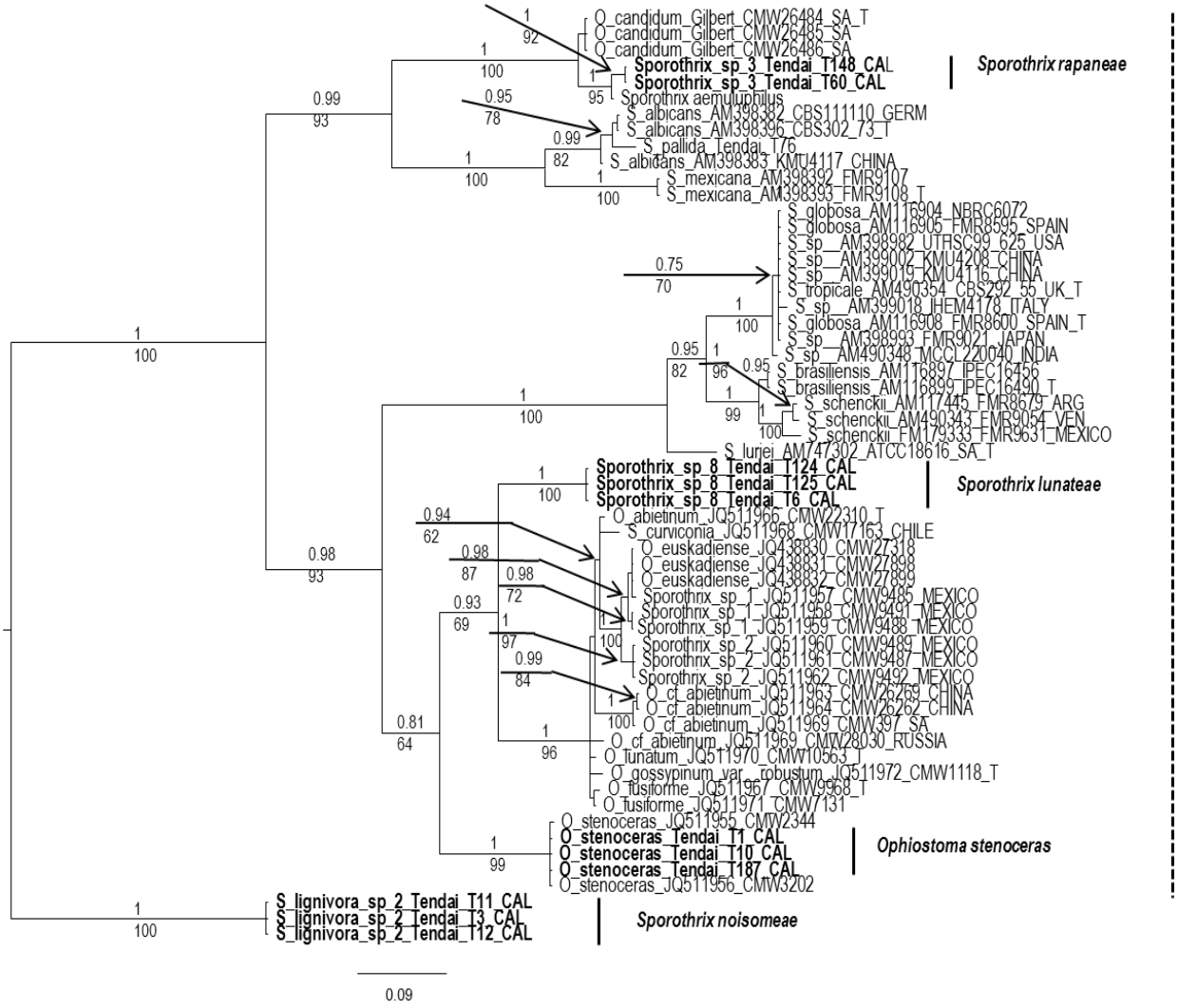

Figure 3: Bayesian Inference consensus tree (CAL data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes

3.3.4. Taxonomy

Based on phylogenetic analyses and micro-morphological characteristics four out of the five collected taxa (all except *Ophiostoma stenoceras*) were recognised as distinct new taxa. They are therefore described here as new species as follows:

Sporothrix reniformis **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 4.

Etymology: The epithet *reniformis* (reniformis = kidney shaped) refers to the kidney shaped ascospores produced by this species.

Ascomata superficial or embedded on the host substrate, bases globose, dark brownish to black, ornamental hyphae present, 95-178 (134 ± 28) µm diam; necks black, 384-777 (530 ± 121) µm long, 15-30 (24 ± 5) µm wide at the base, 5-12 (8 ± 2) µm wide at the apex, osteolar hyphae hyaline, 13-17 (15 ± 1.2) long (Fig. 4A, B & C). *Asci* evanescent. *Ascospores* kidney shaped, aseptate, hyaline, sheaths absent, 1.3-2.4 x 0.3-0.5 µm (Fig. 4D), accumulating in a transparent gelatinous droplet at the apex of the neck, becoming dry when old. *Colonies* whitish to cream-coloured, on MEA fluffy in the centre only. Odourless, circular with entire edge (Fig. 4E). Colony diameter reaching 35.7 mm (±1.3) after 10 days on MEA at an optimal growth temperature of 25ºC. No growth below 5ºC or above 35ºC. *Conidiophores* cylindrical, hyaline, tapering towards the tip, 5.1-7.9 x 0.5-1.1 µm (Fig. 4F), *Conidiogenous* cells arising directly from tips of conidiophores, hyaline, form denticles 0.5-1.3 (0.9 ± 0.3) µm wide (Fig. 4G). *Conidia* hyaline, aseptate, obovate, tapering at one end, thick walled, 3.4-4.4 x 0.6-0.9 µm (Fig. 4 H).

Substrate: Isolated from damaged wood and bark of *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Harold Porter National Botanical Garden, Betty's Bay. Isolated from *Rapanea melanophloeos*, August 2010, T. Musvuugwa, holotype PREM (pending), culture ex-holotype 39 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T197 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T198 CMW (pending) = CBS (pending).

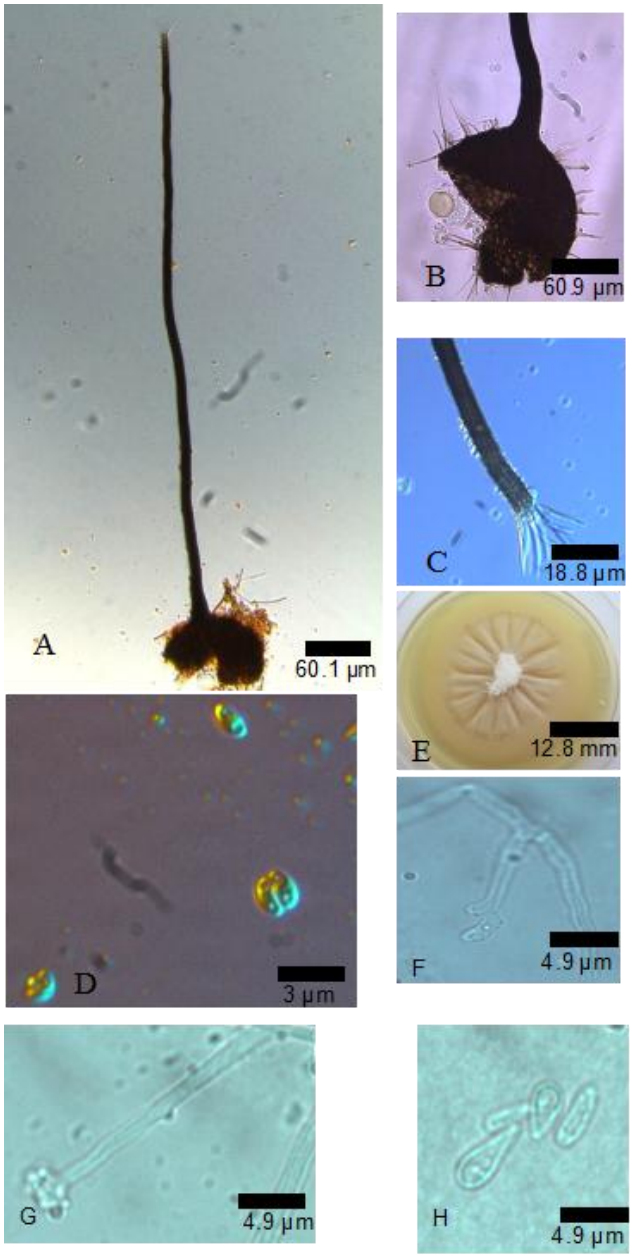

Figure 4. Micrographs of *Sporothrix reniformis*. A. Perithecium removed from the bark of *Rapanea melanophloeos*. B. Globose base showing ornamental hyphae. C. Tip of perithecial neck. D. Ascospores. E. Two-week-old colony on MEA at 25ºC. F. Conidiophores. G. Conidiogenous cells showing denticles. H. Conidia.

Sporothrix rapaneae **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 5.

Etymology: The epithet *rapaneae* refers to the host plant genus (*Rapanea melanophloeos*) from which this species was collected.

Ascomata superficial on the host substrate, bases globose, black, with no ornamental hyphae, 125-165 (147 ± 11) µm diam; necks black, 473-905 (670 ± 129) µm long, 11-30 (24 ± 5) µm wide at the base, 3-6 (4 ± 1) µm wide at the apex, ostiolar hyphae present, hyaline, 22-35 µm (26 ± 3) long (Fig. 5A & B). *Asci* evanescent. *Ascospores* allantoid, slightly curved, aseptate, hyaline, 2.8-4.7 x 0.3-0.6 µm (Fig. 5C), accumulating in a sticky gelatinous droplet at the tip of the neck, becoming white to cream colored when dry. *Colonies* white, fluffy, on MEA, circular, edges entire (Fig. 5D). Colony diameter reaching 34.9 mm (±0.7) after 10 days on MEA at an optimal growth temperature of 25ºC. No growth below 5ºC or above 30ºC. *Conidiophores* hyaline, tapering at the tip, cylindrical, 4.1-7.9 x 0.7-1.2 µm (Fig. 5E), *Conidiogenous* cells forming directly from tips of conidiophores, hyaline, prominent denticles present 0.4-1.3 (0.86 ± 0.2) µm wide (Fig. 5E). *Conidia* hyaline, aseptate, thin-walled, smooth, oblong shaped, 3.9-5.5 x 0.5-1.1 µm (Fig. 5F).

Substrate: Isolated from bark of *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Groenkop. Isolated from *Rapanea melanophloeos*, October 2011, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T60 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T148 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T149 CMW (pending) = CBS (pending).

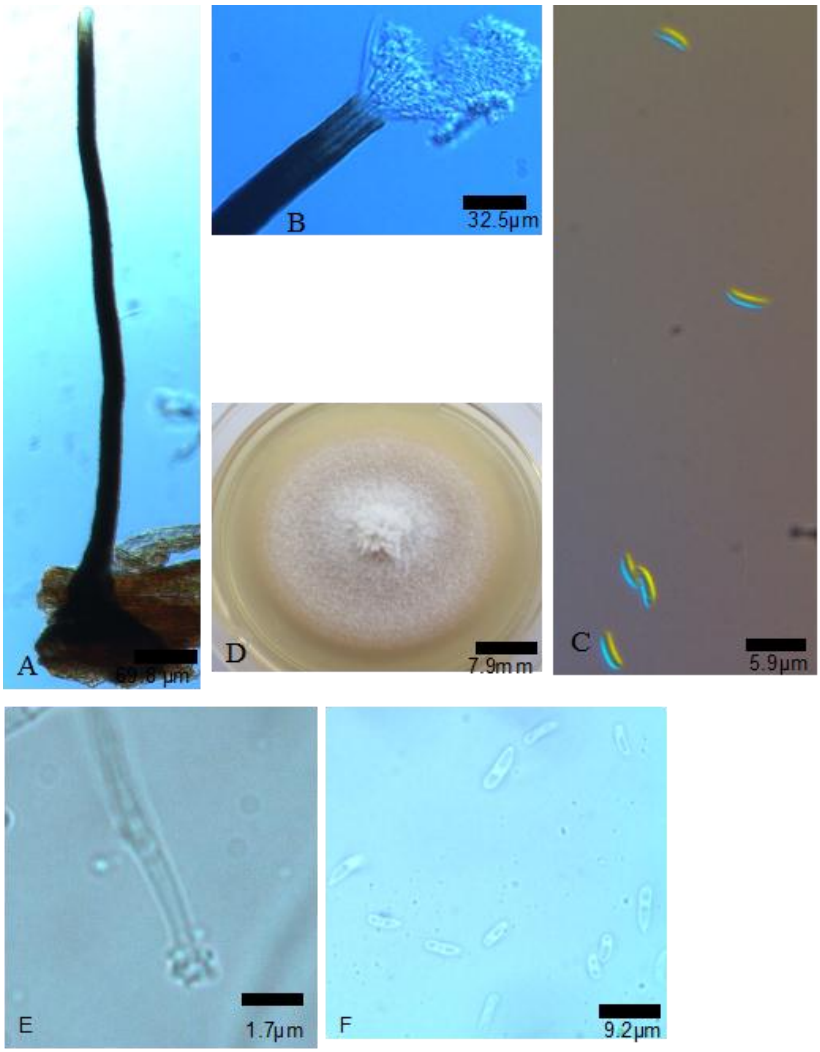

Figure 5. Micrographs of *Sporothrix rapaneae*. A. Perithecium removed from bark of *Rapanea melanophloeos*. B. Tip of perithecial neck. C. Ascospores. D. Two-week-old colony on MEA. E. Conidiogenous cells showing prominent denticles. F. Conidia.

Sporothrix lunateae **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig 6.

Etymology: The epithet *lunateae* (*lunate* = crescent shaped) refers to the crescent shaped ascospores produced by this species.

Ascomata embedded and superficial on the host substrate, bases globose, brownish to black, with no hyphal ornamentation, 87-101 (94 ± 5) µm diam; necks dark brown, 1212-1501 (1319 ± 103) µm long, 19-30 (23 ± 4) µm wide at the base, 6-9 (7 ± 1) µm wide at the tip, ostiolar hyphae hyaline, 12-17 (15 ± 2) long (Fig. 6A & B). *Asci* evanescent. *Ascospores* crescent-shaped, hyaline, aseptate, 1.9-2.6 x 0.5-0.8 µm (Fig. 3C), accumulating transparent sticky masses at the apex of the neck, becoming white with age. *Colonies* white, fluffy appearance on MEA. Odourless, circular with entire edges (Fig. 6D). Colony diameter reaching 38 mm (±1.2) after 10 days on MEA at optimal of 25ºC. No growth below 5ºC or above 35ºC. *Conidiophores* hyaline, tapering towards the apex, cylindrical shaped 3.9-8 x 0.9-1.4 µm (Fig. 6E-F), *Conidiogenous* cells formed from tips of conidiophores, hyaline, becoming denticulate, 0.9-2.8 (1.6 ± 0.6) µm wide (Fig. 6E-F). *Conidia* hyaline, aseptate, oblong shaped, holoblastic, forming singly 4.2-4.8 x 0.7-1.2 µm (Fig. 6G).

Substrate: Isolated from wood and bark of *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, KwaZulu-Natal, Weza Forest Reserve. Isolated from *Rapanea melanophloeos*, September 2010, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T6 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T124 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T125 CMW (pending) = CBS (pending).

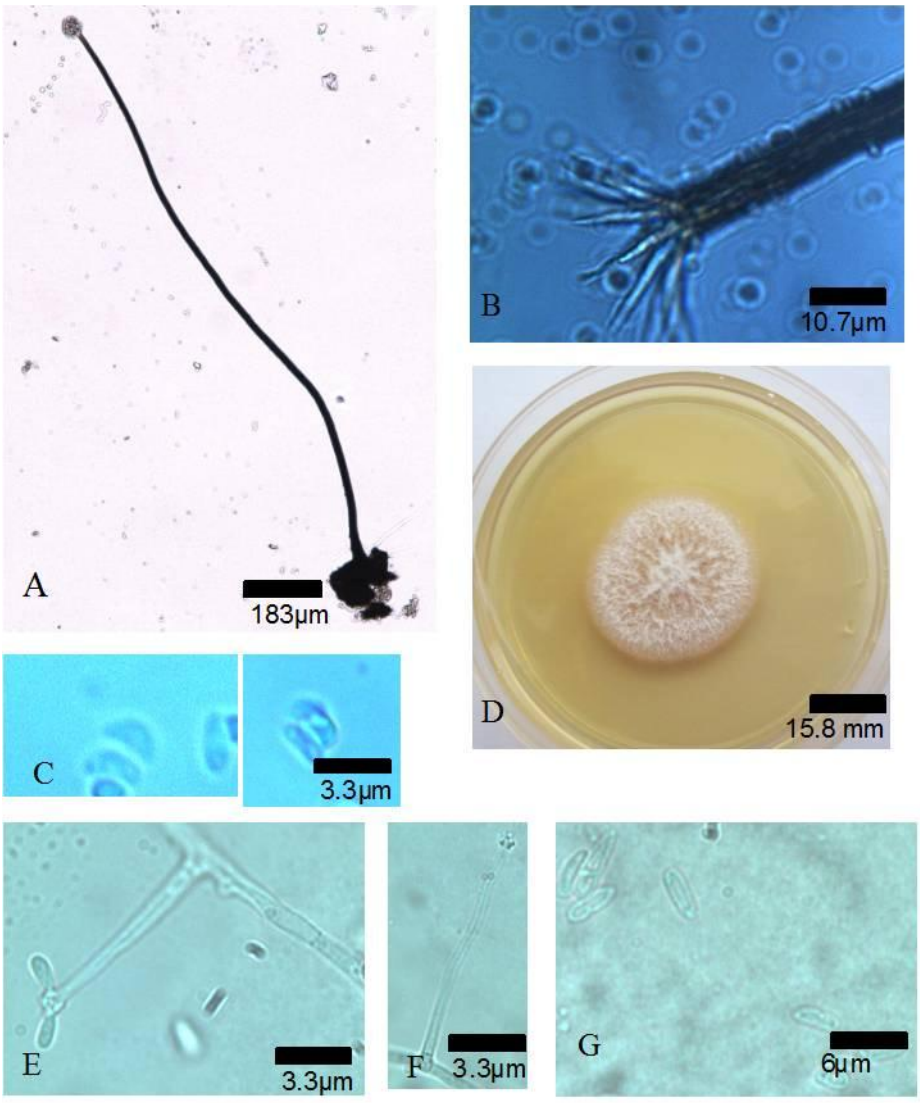

Figure 6. Micrographs of *Sporothrix lunateae*. A. Perithecium removed from bark of *Rapanea melanophloeos*. B. Tip of perithecial neck. C. Ascospores. D. Two-week-old colony on MEA. E-F. Conidiogenous cells showing prominent denticles. G. Conidia.

Sporothrix noisomeae **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending.

Etymology: The epithet *noisomeae* (*noisome* = foal smell) refers to the foal smell produced by the colonies of this species.

Ascomata superficial on the host substrate, bases globose, black, with no hyphae , 104-146 (126 ± 15) µm diam; necks black, 658-1270 (1034 ± 265) µm long, 12-31 (23 ± 5) µm wide at the base, 4-9 (7 ± 1) µm wide at the apex, ostiolar hyphae hyaline, 24-33 (28 ± 3) long. *Asci* evanescent. *Ascospores* allantoid, aseptate, hyaline, 2.0-2.6 x 0.5-0.8 µm, accumulating a gelatinous droplet at the tip of the neck, becoming whitish with age. *Colonies* white, turning blackish with age on MEA. Foal smelling when older, circular, edges entire. Optimal growth is at 25ºC with a colony diameter of 28.7 mm (±1.1) after 10 days on MEA. No growth below 5ºC or above 30ºC. *Conidiophores* 18.3-21.4 x 0.9-1.8 µm, hyaline, cylindrical (Fig. 7E-F), *Conidiogenous* cells arising from apex of conidiophores, hyaline (Fig. 7F), *Conidia* aseptate, hyaline, thin walled, smooth, spherical, form in masses, sometime brown in color, 2.2-3.8 x 2.2-3.4 µm (Fig. 7G).

Substrate: *Rapanea melanophloeos*.

Distribution: South Africa, KwaZulu-Natal

Specimens examined: South Africa, KwaZulu-Natal, Weza Forest Reserve. Isolated from *Rapanea melanophloeos*, September 2010, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T11 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T3 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T12 CMW (pending) = CBS (pending), culture ex-paratype T13 CMW (pending) = CBS (pending).

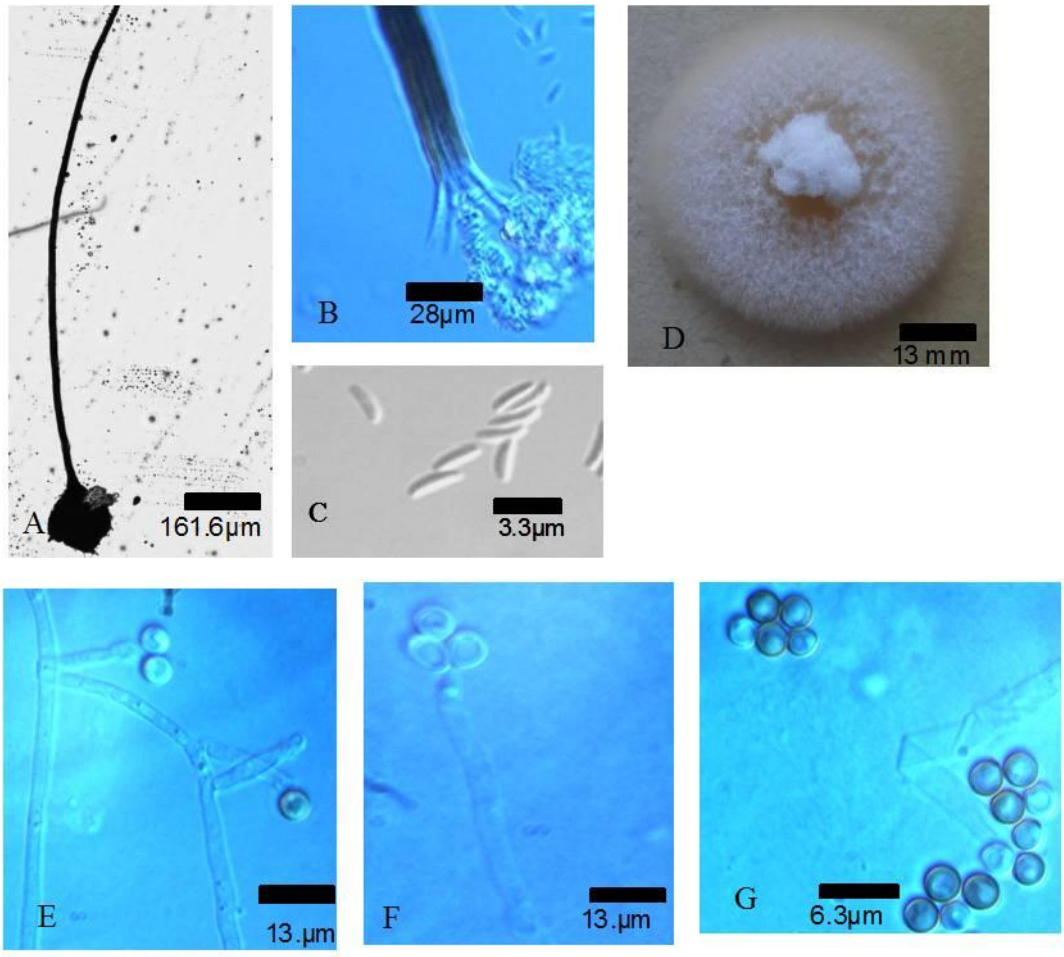

Figure 7. Micrographs of *Sporothrix noisomeae*. A. Perithecium removed from bark of *Rapanea melanophloeos*. B. Tip of perithecial neck. C. Ascospores. D. Two-week-old colony on MEA. E-F. Conidiophores. G. Conidia.

3.4. Discussion

This study reports five members of the Ophiostomatales associated with wounds from *R. melanophloeos* trees found in the Afromontane forests of the CFR of South Africa. These species add to the several other species in the Ophiostomatales that have already been isolated from wounds of the same host in previous studies in these forests (Kamgan *et al*. 2008) and recently from subcortical beetles associated with *Rapanea* (Musvuugwa *et al*. 2013, Chapter 2). The total number of Ophiostomatales species now known from this host is nine. Four species from two different species complexes were newly described here. Given the relatively high number of members of Ophiostomatales collected from *R. melanophloeos* from only a few studies done in

the Afromontane forests (present study and previous studies e.g. Musvuugwa *et al*. 2013, Chapter 2; Kamgan *et al*. 2008), there is a possibility of even more Ophiostomatales fungi associated with this tree species. An interesting next step would be to assess the type of relationship these fungi share with this host tree.

Four of the species collected in this study belong to the *S. schenckii-O. stenoceras* complex (De Beer *et al*. 2013b), including three of the newly described species. This adds to an already high diversity of species from southern Africa found in this complex (Musvuugwa *et al*. 2013, Chapter 2; Kamgan *et al*. 2012; Roets *et al*. 2009; 2007; de Meyer *et al*. 2008; Zhou *et al*. 2006). This complex includes species collected from different host types, including the mite-associated species from *Protea* infructescences (Roets *et al*. 2009; 2007), human pathogens (Marimon *et al*. 2007; Hektoen & Perkins 1900) and subcortical beetle-associated species (Musvuugwa *et al*. 2013, Chapter 2). Many taxa are also known from "environmental" samples such as soil, air and decaying wood (De Beer & Wingfield 2013b). The ecology of species in this complex thus varies greatly and it would be interesting to determine the ecology of the taxa associated with wounds on *Rapanea*.

The newly described *Sporothrix reniformis* was collected from *Rapanea* hosts in indigenous forests surrounding the Harold Porter National Botanical Garden. The species is closely related to *S. aemuluphilus*, a recently described species from the same *R. melanophloeos* host (Musvuugwa *et al*. 2013, Chapter 2). *Sporothrix aemuluphilus* is an associate of the ambrosia beetle *Xyleborinus aemulus* Wollaston (Scolytinae) that infest *R. melanophloeos* trees at the same site (Harold Porter National Botanical Garden) from which *S. reniformis* was collected (Musvuugwa *et al*. 2013, Chapter 2). Besides being isolated from the same host tree and from the same area, these two taxa are also similar in characters such as the presence of osteolar hyphae and similar colony growth forms on MEA (Musvuugwa *et al*. 2013, Chapter 2). The two species differ in other characters such as the presence of ornamental hyphae on perithecial bases in *S. reniformis*, which are absent in *S. aemuluphilus* and the kidney-shaped ascospores in *S. reniformis*, while *S. aemuluphilus* has allantoid shaped spores (Musvuugwa *et al*. 2013, Chapter 2).

Sporothrix rapaneae was collected from Groenkop forest and analyses of βT and CAL data resolved it as sister to *O. candidum* and *S. aemuluphilus*, respectively. The

relationship with *S. aemuluphilus* was, however, more strongly supported than that with *O. candidum*. Similarities shared with *S. aemuluphilus* include the presence of osteolar hyphae and the production of allantoid-shaped ascospores. *Sporothrix rapaneae* differs from *S. aemuluphilus* in its optimal growth temperatures (30 ºC for *S. aemuluphilus* and 25 ºC *S. rapaneae*) and average sizes of morphological structures (Musvuugwa *et al*. 2013, Chapter 2). *Sporothrix rapaneae* and *S. aemuluphilus* differ from *O. candidum* in basal ornamentation of perithecia, which is present in *O. candidum*, but absent in *S. rapaneae*, the production of ascomata on colonies of *O. candidum*, but not in *S. rapaneae* as well as in average sizes of morphological structures (Nkuekam *et al*. 2012). They are similar in having the same optimal growth temperatures and having osteolar hyphae (Nkuekam *et al*. 2012). *Ophiostoma candidum* was isolated from a wound on the exotic *Eucalyptus cloeziana* F. Muell in the Limpopo Province of South Africa where it was found to be associated with a Cerambycidae beetle (Kamgan Nkuekam *et al*. 2012). There is no arthropod associate currently known for *Sporothrix rapaneae*.

Phylogenetic analyses of the three gene regions suggest that *Sporothrix lunateae* is closely related to several species (e.g. *O. fusiforme*, *O. lunatum* and *O. abietinum*), although ITS data showed it to be the sister species to *O. aurorae*. *Ophiostoma aurorae* was isolated from the root-feeding *Hylastes angustatus* (Herbst) that infest *Pinus patula* Schiede & Deppe in South Africa (Zhou *et al*. 2006). It is characterised by ornamented bases with light grey hyphae and allantoid ascospores (Zhou *et al*. 2006). These characters were not observed in *S. lunateae.* Several of the species in a clade closely related to the one *S. lunateae* belongs to, are wood-inhabiting species (De Beer *et al*. 2013b). *Ophiostoma fusiforme*, for example, was isolated from *Populus nigra* L. and *Quercus petraea* (Matt.) Liebl. in Azerbaijan and Austria, respectively. *Ophiostoma lunatum* is known from Austria, where it was isolated from *Carpinus betulus* L. and *Larix decidua* Mill., while *O. abietinum* was isolated from pine wood (Aghayeva *et al*. 2004). Although they are all wood inhabiting species with *Sporothrix*-like anamorphs and share other similarities such as presence of osteolar hyphae, they still differ in several characters such as their basal hyphal ornamentation (present in *O. fusiforme* and *O. lunatum*), the shape of their ascospores and the average measurements of morphological structures (Aghayeva *et al*. 2004).

The isolates of *Ophiostoma stenoceras* collected in this study were from Weza Forest. It is a well-known sapwood colonising fungus that was first described in Norway from ground wood pulp (Robak 1932). It has also been isolated from several hardwood trees and many coniferous hosts, mostly in the Northern Hemisphere (Griffin 1968). It has rarely been collected in the Southern Hemisphere, although it is known from pine trees in New Zealand (Schirp *et al* 1999) and from *Eucalyptus* spp. in South Africa (De Beer *et al.* 2003). It has several synonyms, including *O. ponderasae* (T.E. Hinds & R.W. Davidson) Georg Hausner, J. Reid & Klassen, *O. albidum* Math.-Käärik (De Beer *et al*. 2003) and *Ceratocystis eucastaneae* R.W. Davidson, (Updhyay 1981). Although this species causes slight gray stain on pines (Kaarik 1980), it is not considered economically important (Griffith 1968).

Sporothrix noisomeae, another newly described species from Weza Forest, belongs to the *Sporothrix lignivora* complex (De Beer *et al*. 2013b) based on βT data. Species found in this complex differ from other *Sporothrix* species in the *S. schenckii-O. stenoceras* complex in that their ßT introns (-/4/5) differ (De Beer *et al*. 2013b). Species found in this complex include *S. lignivora* isolated from wooden utility poles in South Africa? (De Meyer *et al*. 2008), and two undescribed taxa isolated from Thuja in Canada (Lim *et al*. 2005) and from Yucca roots in the USA (Khidir *et al*. 2010). Our phylogenetic analyses of *S. noisomeae* supports results of De Beer *et al* (2013b) and Linnakoski *et al* (2010) that the species in this complex form a distinct lineage with significant distance from other genera and complexes in the Ophiostomatales. De Beer *et al* (2013b) suggested that due to the distance of which this complex groups from other complexes and genera in the Ophiostomatales, the group may represent a separate genus. The complex is, however, still treated as of uncertain status until more data and material can be included (De Beer *et al*. 2013b). This uncertainty mostly results from previous studies on species in this complex, in which phylogenetic placement differed depending on the specific marker used (De Beer *et al*. 2013b). Similar conflicting placements were also detected in this study, with ITS data showing a distinct clade sister to *Graphilbum*, while ßT data proposed a distinct clade sister to *S. lignivora*. Based on CAL data, it formed a distinct clade, but it was not clear what the sister of this group would be as CAL data of *Sporothrix lignivora* and *Graphilbum* species are still lacking.

3.5. Conclusion

In this study we collected five members of the Ophiostomatales, including four new species, associated with wounds on *R. melanophloeos*. Four of the species belong to the *S. schenckii-O. stenoceras* complex, while the fifth one has provisionally been placed in the *Sporothrix lignivora* complex. Based on these results and those from previous studies, there is evidence of a relatively high diversity of ophiostomatoid fungi associated with this host tree in the Afromontane forests of South Africa. It is recommended that future studies look at the total number of taxa associated with this important tree, and that the pathogenicity of all the associated fungi is tested. If many prove to be pathogenic, it may prompt conservation managers to restrict bark harvesting activities on this species.

References

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W. and Kirisits, T. 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* 96: 866–878.

Brasier, C.M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57: 792–808.

Brasier, C.M. 2000. Intercontinental spread and continuing evolution of the Dutch elm disease pathogens. In: *The elms: Breeding, Conservation and Disease Management*, (Dunn, C.P. ed.), Boston, USA, Dordrecht, The Netherlands, London, UK: Kluwer Academic Publishers, pp. 61–72.

Brasier, C.M. and Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Castello, J.D., Leopold, D.J. and Smallidge, P.J. 1995. Pathogens, patterns and processes in forest ecosystems. *BioScience* 45: 16-24.

Chen, S.F., Wingfield, M.J., Roets, F. and Roux, J. 2013. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native Rapanea in South Africa. *Plant Pathology* 62: 667-678.

Cobb, F.W. (Jr), Lawson, T.T. and Popenuck, T.L. 1987. Interactions among the tree variants of *Verticicladiella wageneri* and the three host types. *Phytophathology* 77: 1640-1646.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013a. The ophiostomatoid fungi: their dual position in the *Sordariomycetes* In: *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W., Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 1-19.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013b. A nomenclature for ophiostomatoid genera and species in the *Ophiostomatales* and *Microascales*. In: *Ophiostomatoid Fungi: Expanding Frontiers*, (Seifert, K.A., De Beer, Z.W., Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 245-322.

De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2003. The *Ophiostoma piceae* complex in the southern hemisphere: a phylogenetic study. *Mycological Research* 107: 469-476.

De Beer, Z.W., Wingfield, M.J. and Kemp, G.H.J. 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* 91: 6.

De Meyer, E.M., De Beer, Z.W., Summerbell, R.C., Moharram, A.M., De Hoog, G.S., Vismer, H.F. and Wingfield, M.J. 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* 100:647–661.

Duong, T. A., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2012. Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. *Mycologia* 104: 715-732.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113-118.

Geldenhuys, C.J. 2010. Distribution, size and ownership of forests in the Southern Cape. *South African Forestry Journal* 158: 51-66.

Gibbs, J.N. 1993. The biology of Ophiostomatoid fungi causing sapstain in trees and freshly cut long. In: *Ceratocystis* and *Ophiostoma : Taxonomy, Ecology, and Pathogenicity*, (Wingfield, M.J., Seifert, K.A. and Webber, J.F. eds.), American Phytopathological Society Press, St. Paul, Minnesota, pp. 157-160.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* 61: 1323-1330.

Griffin HD. 1968. The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* 46: 689–718.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* 73: 1123-1129.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D.N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* 104: 399-404.

Harrington, T.C. and Wingfield, M.J. 1998. The *Ceratocystis* species on conifers. *Canadian Journal of Botany of Botany* 76: 1446-1457.

Hektoen, L. and Perkins, C.F. 1900. Refractory subcutaneous abscesses caused by *Sporothrix schenckii*, a new pathogenic fungus. *Journal of Experimental Medicine* 5: 77–89.

Heybroek, H.M. 1993. Why bother about the elm? In: *Dutch Elm Disease Research. Cellular and Molecular Approaches*, (Sticklen, M.B. and Sherald, J.L. eds.), Springer-Verlag, New York , pp. 1–8.

Hinds, T.E. 1972. Insect transmission of *Ceratocystis* species associated with Aspen cankers. *Phytopathology* 62: 221-225.

Jacobs, K. and Wingfield, M.J. 2001 *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. APS press, St Paul, Minnesota, U.S.A.

Kamgan Nkuekam, G., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2012. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Kaarik A. 1960. Growth and sporulation of *Ophiostoma* and some other blueing fungi on synthetic media. *Symbolae Botanicae Upsalienses* 16:1–168.

Kaarik A. 1980. Fungi causing sapstain in wood. Swedish University of Agricultural Science, Department of Forest Products. *Report Number* R114: 1-112.

Katoh, K. and Toh, H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* 9: 286-298.

Khidir, H.H., Eudy, D.M., Porras-Alfaro, A., Herrera, J., Natvig, D.O. and Sinsabaugh, R.L. 2010. A general suite of fungal endophytes dominate the roots of two dominant grasses in a semiarid grassland. *Journal of Arid Environment* 74: 35-42.

Kubono, T. and Ito, S. 2002. *Raffaelea quercivora* sp. nov. associated with massmortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Lagerberg, T., Lundberg, G and Melin, E. 1927. Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvårdsföreningens Tidskrift* 25: 145-272.

Linnakoski, R., De Beer, Z.W., Athiainen, J, Sidorov, E., Niemela, P., Pappinen, A. and Wingfield, M.J. 2010. Ophiostoma spp. associated with pine and spruce-infesting bark beetles in Finland and Russia. Persoonia 25: 72-93.

Malloch, D. and Blackwell, M. 1993. Dispersal biology of the ophiostomatoid fungi. In: *Ceratocystis and Ophiostoma: taxonomy, ecology and pathogenicity*, (Wingfield, M.J., Seifert, K.A and Webber, J.F. eds.), APS Press, St. Paul, U.S.A, pp. 195–206.

Marais, G.J. and Wingfield, M.J. 2001. *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. *Mycological Research* 105: 240-246.

Marais, G.J. and Wingfield, M.J. 1997. *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. *Canadian Journal of Botany* 75: 362-367.

Marimon, R., Cano, J., Gene, J., Sutton, D.A., Kawasaki, M. and Guarro, J. 2007. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. *Journal of Clinical Microbiology* 45:3198–3206.

Moller, W.J. and Devay, J.E. 1968. Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* 58: 1499-1507.

Morris, M.J., Wingfield, M.J. and De Beer, C. 1993. Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* 42: 814-817.

Kamgan Nkuekam, G., De Beer Z.W., Wingfield, M.J. and Roux, J. 2012a. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533.

O'Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103-116.

Pipe, N.D., Brasier, C.M. and Buck, K.W. 2000. Evolutionary relationships of the Dutch elm disease fungus *Ophiostoma novo-ulmi* to other *Ophiostoma* species investigated by restriction fragment length polymorphism analysis of the rDNA region. *Journal of. Phytopathology* 148:533–539.

Robak, H. 1932. Investigations regarding fungi on Norwegian ground wood pulp and fungal infection at wood pulp mills. *Nyt Mag Naturvid* 71: 185-330.

Roets, F., Dreyer, L.L., Crous, P.W. and Wingfield, M.J. 2009. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* 38: 143-152.

Roets, F., De Beer, Z.W., Wingfield, M.J., Crous, P.W and Dreyer, L.L. 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* 100: 496-510.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Roets, F., de Beer, W., Dreyer, L.L., Zipfel, R., Crous, P.W. and Wingfield, M.J. 2006. Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. *Studies in Mycology* 55: 199-212.

Ronquist, F.R. and Huelsenbeck, J.P. 2003. MrBayes: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

Schirp, A., Farrell, R.L. and Kreber, B. 1999. Effect of New Zealand staining fungi on structural wood integrity of radiata pine. In: *The 2nd New Zealand Sapstain Symposium. Proceedings of Symposium, Rotorua, New Zealand, 18-19 November, FRI Bull* 215: 99-104.

Seifert, S.A., Wingfield, M.J. and Kendrick, W.B. 1993. A nomenclature for described species of *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis*, *Ceratostomella* and *Sphaeromella*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A., Webber, J., eds.), American Phytopathological Society, St. Paul, Minnesota, U.S.A, pp. 269-287.

Taylor, J.E., Lee, S. and Crous, P.W. 2001. Biodiversity in the Cape Floral Kingdom: fungi occurring on Proteaceae. *Mycological Research* 105: 1480-1484.

Turpie, J.K., Heydenrych, B.J. and Lamberth, S.J. 2003. Economic value of terrestrial and marine biodiversity in the Cape Gloristic Region: implications for defining effective and socially optimal conservation strategies. *Biological Conservation* 112: 233-251.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, U.S.A.

VanWyk, B. and VanWyk, P. 1997. Field Guide to Trees of Southern Africa. Cape Town, South Africa: Struik Publishers.

Vermeulen, W.J., Geldenhuys, C.J. and Esler, K.J. 2012**.** Response of *Ocotea bullata*, *Curtisia dentata* and *Rapanea melanophloeos* to medicinal bark stripping in the southern Cape, South Africa: implications for sustainable use. *Southern Forests* 74: 183–193.

Viljoen, C.D., Wingfield, B.D. and Wingfield, M.J. 1999. Relatedness of*Custingophora olivaceae* to *Gondwanamyces* spp. from *Protea*. *Mycological Research* 103: 497-500.

Webber, J.F., Jacobs, K. and Wingfield, M.J. 1999. A re-examination of the vascular wilt pathogen of takamaka (*Colophyllum inophyllum*). *Mycological Research* 103: 1588-1592.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A sequencing guide to methods and applications*, (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J.), Academic Press, San Diego, pp. 315-322.

Wingfield, M.J.; Seifert, K.A. and Webber, J.A. 1993. *Ceratocystis and Ophiostoma*: Taxonomy, Ecology and Pathogenicity. St Paul, MN, USA: APS Press.

Zhou, X.D., De Beer, Z.W. and Wingfield, M.J. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* 53: 290-300.

Chapter 4: Three new ophiostomatoid fungi from wounds on storm-damaged trees in Afromontane forests of the Cape Floristic Region.

Abstract

Taxa such as plants, birds and mammals are well-studied in the Cape Floristic Region (CFR) of South Africa. However, little research has been done on micro-organisms such as fungi, even though these organisms play vital roles in the ecology and evolution of other organisms in this region. Ophiostomatoid fungi, a well-studied, tree-associated fungal group includes some of the globally most important pathogenic fungi, but very little is known about their presence and associations in the Afromontane forests of the CFR. To date, most research on them have been focussed on *Rapanea melanophloeos*, but numerous other important tree species have not yet been surveyed for their presence. Therefore, the aim of this study was to document the diversity of ophiostomatoid fungi associated with wounds on other Afromontane forest tree taxa in the CFR. Storm damaged trees in various Afromontane forest patches were surveyed and fungi were isolated from bark and wood samples. Three undescribed ophiostomatoid species, belonging to three different genera and two orders, were identified based on micro-morphological and phylogenetic analyses. These are newly described here as *Graphilbum roseus* sp. nov., *Graphium ilexiense* so. nov. and *Sporothrix capensis* sp. nov. *Sporothrix capensis*, a species that groups within the *S. schenckii* - *O.stenoceras* species complex, was associated with *Olea capensis*. *Graphilbum roseus* was isolated from several host tree species including *Curtisia dentata*, *Halleria lucida* and *Pterocelastrus tricuspidatus* while *Graphium ilexiense* was the first ophiostomatoid fungus to be isolated from *Ilex mitis*. Results show the diversity of ophiostomatoid fungi in the region to be large, but still greatly understudied. Along with previous studies on ophiostomatoid fungi from this region, this study forms a platform for future studies on the ecological significance of this important group of fungi within the CFR.

Key words: Ophiostomatales, Microascales, wound-associated, phylogeny

4.1. Introduction

The Cape Floristic Region (CFR), an internationally recognised biodiversity hotspot at the southern tip of South Africa (Myers *et al*. 2000), is characterised by high levels of plant gamma-diversity and endemism (Goldblatt & Manning 2000). Even though taxa such as plants, birds and mammals have been well-documented in the CFR (Kerley *et al*. 2003; Brooks *et al*. 2001; Stattersfield *et al*. 1998; Cowling & Hilton-Taylor 1994), generally few studies have been conducted on the diversity of less conspicuous taxa such as fungi. This is despite the fact that fungal diversity in the CFR is also thought to be high, given that in South Africa there is an estimated 200 000 fungal species associated with native plants (Crous *et al*. 2006). Based on previous research, for example Kamgan *et al*. (2008), a large proportion of these fungal taxa may be associated with trees in Afromontane forests, an important component of the CFR vegetation (Mucina & Rutherford 2006; Goldblatt & Manning 2002). Currently, however, very little is known about fungi associated with native trees in these forests (Taylor *et al*. 2001; Castello *et al*. 1995).

The Afromontane forests are evergreen, with trees reaching 10-30 meters in height (Turpie *et al*. 2003). Some important canopy trees include Assegaaihout (*Curtisia dentata* C.A. Sm.), various Yellowwoods (*Podocarpus* spp. Persoon), Ironwood (*Olea capensis* L. ssp. *macrocarpa* (C. H. Wright) I. Verd.) and Cape Beech (*Rapanea melanophloeos* Mez) (Van Wyk & Van Wyk 1997). In the CFR, most of these forests are found in the Tsitsikamma area. Further westward they occur in small, fragmented patches on mountains, foothills, coastal platforms, river valleys and dunes along the coastal regions of the Western Cape Province, South Africa (Geldenhuys 2010; Lubke & McKenzie 1996). These forests are of ecological, economic and cultural importance. Tree species such as *Ocotea bullata* (Burch.) Baill, *Olinia ventosa* (L.) Cufod. and *Podocarpus* spp. are economically important for timber used in carpentry (Turpie *et al*. 2003). Others are used culturally for medicinal purposes. The bark of *R. melanophloeos*, *C. dentata* and *O. bullata*, for example, is used by local people for the treatment of various ailments (Vermeulen *et al*. 2012).

The ophiostomatoid fungi represent one of the best-studied tree-associated fungal groups. This polyphyletic taxon (De Beer *et al*. 2013a) includes some of the globally best-known pathogenic fungi (Kamgan Nkuekam *et al*. 2012; Heath *et al*. 2009).

Many of its members can cause rot diseases, cankers and wilting that in some cases lead to death of the infected trees (Barnes *et al*. 2005; Roux *et al*. 2005; Wingfield *et al*. 1993). These fungi are grouped into two orders, the Microascales that contains the genera *Ceratocystis* Ellis & Halst., *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas and *Graphium* Corda and the Ophiostomatales that contains genera such as *Raffaelea* Arx & Hennebert, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Ophiostoma* Syd. and *Leptographium* Lagerb. & Melin (De Beer *et al*. 2013b). Some well-documented examples of pathogenic ophiostomatoid fungi include *Ceratocystis fimbriata* Ellis & Halst. *s.l*. and *C. manginecans* M. van Wyk, responsible for causing mango blight disease in Brazil, Oman and Pakistan (Van Wyk *et al*. 2005; Ribeiro 1980; Viégas 1960), *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, responsible for Dutch elm disease in Europe and America (Brasier 2001; Pipe *et al*. 2000), *Ceratocystis fagacearum* (Bretz) Hunt, responsible for oak wilt disease (Wingfield *et al*. 1993; Sinclair *et al*. 1987; Bretz 1952), *Raffaelela lauricola* T.C. Harr., Fraedrich & Aghayeva, responsible for the Laurel wilt disease in south-eastern USA (Harrington *et al*. 2008) and *R. quercivora* Kubono et Shin. Ito, responsible for oak die-back and mortality of Japanese oak trees (Kubono & Ito 2002).

Very little is known about the ophiostomatoid fungi in Africa, including South Africa (Taylor *et al*. 2001). Most research published on these fungi in South Africa focussed on the members associated with exotic plantation trees (Kamgan *et al*. 2012a; Zhou *et al*. 2006; Zhou *et al*. 2001; De Beer *et al*. 1995) and a few of these have proven to be pathogenic on their host trees. For example, *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris, which is responsible for wattle wilt disease in *Acacia mearnsii* De Wild., has led to significant economic losses in South African plantations (Barnes *et al*. 2005; Roux *et al*. 2005; Roux *et al*. 1999; Wingfield *et al*. 1996). *Ceratocystis fimbriata* has been reported on *Eucalyptus* species, where it caused serious wilting and dieback (Roux *et al.* 2004). A few studies focussed on ophiostomatoid fungi associated with native trees, some of which occur in the Afromontane forests of South Africa. Many were collected from tree wounds caused by weather, animals and/or human practices. Documented examples in the Microascales include *C. albifundus* and *C. savannae* Kamgan Nkuekam & Jol. Roux that are associated with several native hosts (Roux *et al*. 2007) and *C. tsitsikammensis* Kamgan & Jol. Roux from

Ocotea bullata and *Rapanea melanophloeos* (Kamgan *et al*. 2008). Taxa in the Ophiostomatales known from wounds on trees in this region include *Ophiostoma quercus* (Georgev.) Nannf. from *R. melanophloeos* (Kamgan *et al*. 2008; De Beer *et al*. 1995), *Sporothrix stenoceras* (Robak) Nannf. and the recently described *Sporothrix reniformis* Musvuugwa, LL. Dreyer & F. Roets, *S. noisomeae* Musvuugwa, LL. Dreyer & F. Roets, *S. lunateae* Musvuugwa, LL. Dreyer & F. Roets and *S. rapaneae* Musvuugwa, LL. Dreyer & F. Roets from *R. melanophloeos* trees (Musvuugwa *et al*. 2013, Chapter 3). In addition, a few taxa are known from subcortical beetles infesting native trees species in the Afromontane forests. These include *Sporothrix aemuluphilus* Musvuugwa, LL. Dreyer & F. Roets and *Raffaelea rapaneae* Musvuugwa, LL. Dreyer & F. Roets from *R. melanophloeos* as well as *S. pallida* (Tubaki) Matsush and *R. scabbardiae* from *O. capensis* ssp. *macrocarpa* (Musvuugwa *et al*. 2013, Chapter 2).

It is evident that a large diversity of ophiostomatoid fungi is associated with Afromontane forest trees in the CFR. However, very little is known about taxa associated tree taxa other than *R. melanophloeos*. During recent surveys of fungi associated with wounds on these trees, three possibly new and undescribed ophiostomatoid taxa were collected. The present study therefore sets out to evaluate the identity of these taxa based on morphological and molecular phylogenetic comparisons.

4.2. Materials and methods

4.2.1. Sampling of plant material and fungal isolation

Sampling was conducted in various native forests of the Cape Floristic Region, including Groenkop Forest Reserve (S 33°56'32.10" E 22°32'50.38"), Gouldveld (S 33°54'44.38" E 23°0'10.30"), Gouna (S 33°57'2.584" E 23°2'9.80") and Assegaibos (S 33°58'23.10" E 18°56‘11.38") during 2010 to 2012. Bark and wood samples were collected from wounds on various storm-damaged native trees from which ophiostomatoid fungi were recovered following the methods of Musvuugwa *et al*. 2013 (Chapter 3). Purified cultures were maintained on Petri dishes containing 2%

malt extract agar MEA (MEA; Biolab, Midrand, South Africa) at 4°C until further use. Representatives of pure cultures of collected isolates are preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

4.2.2. Morphological characterisation

Where available, perithicia and ascospores of the ophiostomatoid were collected from plant material and mounted in clear lactophenol on microscope slides. Conidia and conidiophores formed in culture were treated similarly and all structures were studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan). Photographs of structures were taken using a Leica digital camera mounted on the microscope. Based on micro-morphology and culture characteristics on MEA, isolates were grouped according to morpho-type after two weeks of growth at 25°C in the dark. Twenty-five measurements of all morphologically and taxonomically characteristic structures were made for isolates chosen as types of the suspected undescribed species. The maximum and minimum measurement for each taxonomically informative structure was noted and the means (± standard deviation) were calculated.

4.2.3. DNA extraction, amplification and sequencing

At least three isolates representing each morph-type were randomly chosen for DNA sequencing (Table 1). Following the manufacturer's instructions, a Sigma-Aldrich™ plant extraction kit (USA) was used for the extraction of genomic DNA. To amplify the ITS and 5.8S gene regions, the primers ITS1-f (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) were used. Preliminary phylogenetic placement of fungi based on ITS sequence data (Table 1) was used to determine which other gene regions to amplify for accurate identification, following De Beer *et al*. (2013a & b).

For the relevant isolates, amplification of Beta-tubulin gene fragments was performed using the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995). Primers LROR and LR5 (Vilgalys & Hester 1990) were used to amplify the

gene region comprising the nuclear large sub-unit rDNA. Part of the transcription elongation factor-1α gene was amplified using primers EF1-F and EF2-R (Jacobs *et al.* 2004). PCR for Calmodulin was achieved using primers CL2F (5′-GACAAGGAYGGYGATGGT-3′) and CL2R (5′-TTCTGCATCATGAGYTGSAC-3′) (Duong *et al*. 2012) and in cases where amplification was problematic, CL2R2 (Duong *et al*. 2012) was used in the place of CL2R or a combination of CL3F and CL3R was used. For each gene region, PCR reaction mixtures, volumes and conditions for amplification followed methods described in Musvuugwa *et al*. (2013) (Chapter 2).

Amplified PCR products were separated using agarose gel electrophoresis stained with GelRed (Biotium, Inc., California, USA) and visualized under ultraviolet light. Following the manufacturer's instructions, all amplified PCR products were cleaned using the EXOSAP-IT kit (USB Corporation, Cleveland, Ohio, U.S.A.). Purified fragments were sequenced using the respective PCR primers and a Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) and analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). Both DNA strands were sequenced and the consensus sequences were constructed using CLC Genomics Workbench software package (CLC Bio, Cambridge, MA).

4.2.4. Phylogenetic analyses

Datasets were analysed using Bayesian inference (BI) and maximum likelihood (ML) procedures. A Markov chain Monte Carlo approach (MCMC) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) was used to perform Bayesian inference analyses. Two Markov chains were run simultaneously independent of each other for 10 million generations starting from a random tree. A sample frequency of 2000 was implemented, in which burn-in trees (first 25 000 generations) were discarded. The remaining trees were pooled into a 50% majority rule consensus tree. An online version of PhyML 3.0 (Guindon & Gascuel 2003) was used to perform ML analyses. Using jModelTest 0.1.1 (Posada 2008), the best fit substitution models were determined using Akaike information criteria (Akaike 1974) and confidence support values for nodes were estimated using 1000 replication bootstrap analyses.

Table 1. Culture collection and GenBank accession numbers for strains of ophiostomatoid fungi isolated from different native CFR trees that were sequenced in this study.

Species	**ITS**	**Bt**	**EF**	**LSU**	**CAL**	**Host plant**	**Site**
Graphilbum roseus sp. nov.	T46		NA	T46	NA	*Curtisia dentata*	Gouna Forest
	T72		NA	T72	NA	*Curtisia dentata*	Gouna Forest
	T79		NA	T79	NA	*Pterocelastrus tricuspidatus*	Groenkop Forest
	T81		NA		NA		Gouna Forest
	T82		NA	T82	NA	*Curtisia dentata*	Gouna Forest
						Halleria lucida	
Graphium ilexiense sp. nov.	33	NA		NA	NA	*Ilex mitis*	Assegaaibosch
	T202	NA	T202	NA	NA	*Ilex mitis*	Assegaaibosch
	T203	NA		NA	NA	*Ilex mitis*	Assegaaibosch
Sporothrix capensis sp. nov.	T206	T206	NA	NA		*Olea capensis*	Gouldveld Forest
		T207	NA	NA		*Olea capensis*	Goudeveld Forest
	T39	T39	NA	NA		*Olea capensis*	Gouldveld Forest

4.2.5. Growth in culture

The temperature for optimal growth of selected type cultures of new taxa was determined by transferring mycelium covered disks of agar (10 mm diam) from edges of actively growing 1 week old cultures to the centres of 90 mm fresh Petri dishes containing 20 mL MEA. These plates were incubated in the dark at a range of different temperatures (from 5 ºC to 35 ºC at intervals of 5 ºC) for 10 days in the dark. The experiment was replicated five times. After the 10 day incubation period, colony diameters were determined by calculating the average of 2 perpendicular measurements per colony and then calculating the mean (± standard deviation) for each species at each temperature.

4.3. Results

4.3.1. Fungal isolates and morphological characterisation

In total, 21 isolates morphologically resembling ophiostomatoid fungi were isolated from wounds found on different native trees in the different native forests sampled. Based on micro-morphological and colony characteristics, these isolates were grouped into three different Operations Taxonomic Units (OTU's) (Table 1). These comprised of seven isolates resembling species in the genus *Sporothrix*, eight isolates resembling species in the genus *Graphilbum* and six isolates resembling species in the genus *Graphium*. Isolates in the OTU resembling species in *Sporothrix* were characterised by white to cream coloured colonies all collected from *O. capensis* ssp. *macrocarpa* in Gouldveld. Isolates belonging to the OTU resembling *Graphilbum* were collected from *Curtisia dentata*, *Halleria lucida* L., *Pterocelastrus tricuspidatus* Loes, *Trichocladus crinitus* (Thunb.) Pers. and *O. capensis* ssp. *macrocarpa* from the Groenkop and Gouna forests. These were characterised by producing synnemata that form conidia in sticky, pink droplets. Isolates of the OTU resembling *Graphium* were isolated from *Ilex mitis* (L.) Radlk. from Assegaibosch and produced white to greyish colonies.

4.3.2. Phylogenetic analyses

ITS data placed two of the collected OTU's into the order Ophiostomatales and the third OTU into the order Macroascales. The phylogenetic placement of the OTU with *Sporothrix*-like anamorphs was further investigated using ITS (*Ophiostoma* & *Graphilbum* data set) and ßT data. Placement of the OTU with *Graphilbum*-like anamorphs was assessed using ITS (*Ophiostoma* & *Graphilbum* data set) data only. The phylogenetic placement of the OTU belonging to the genus *Graphium* was assessed using both ITS (*Graphium* data set) and EF data. After alignment of the data sets the ITS (*Ophiostoma* & *Graphilbum* data set) data consisted of 75 taxa and 723 characters, the ßT data set consisted of 46 taxa and 269 characters, ITS (*Graphium* data set) consisted of 53 taxa and 560 characters and EF data set consisted of 19 taxa and 610 characters. GenBank accession numbers for all other taxa used in analyses are presented on the respective phylogenetic trees (Figs. 1-4). Table 2 presents

statistical values obtained from analyses of the different data sets and the substitution models chosen.

Analyses of ITS (*Ophiostoma* & *Graphilbum* data set) and ßT data resulted in similar phylogenetic placement (Fig. 1 & 2) of the *Sporothrix*-like OTU (hereafter referred to as *Sporothrix capensis* sp. nov.). Analyses of ITS data resulted in this species forming a weakly supported clade sister to *Sporothix rapaneae* Musvuugwa, LL. Dreyer & F. Roets and very closely related to *S. aemuluphilus* Musvuugwa, LL. Dreyer & F. Roets and *Ophiostoma candidum* Kamgan-Nkuek., Jol. Roux & Z. W. de Beer (Fig. 1). Analyses of ßT resulted in a strongly supported clade sister to *O. candidum* (Fig. 2). Phylogenetic analyses of the ITS data (*Ophiostoma* & *Graphilbum* data set) resulted in the OTU resembling *Graphilbum* (hereafter referred to as: *Graphilbum roseus* sp. nov.) grouping strongly as a distinct taxon sister to an unnamed *Graphilbum* species previously collected from *Pinus radiata* D. Don in Australia (Thwaites *et al*. 2005) (Fig. 1). The taxon resembling *Graphium* (hereafter referred to as: *Graphium ilexiense* sp. nov.) grouped together with *G. basitrancatum* (Matsush) Seifert & G. Okada as sister to *G. carbonarium* Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf based on analyses of ITS (*Graphium* data set) data (Fig. 3), while based on EF data it formed a distinct clade sister to *G. carbonarium* (Fig. 4).

Table 2. Parameters used and statistical values obtained from Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of the four datasets

	Dataset →	ITS (*Ophiostoma*)	ITS (*Graphium*)	BT	EF
Number of characters		723	560	269	610
ML	**Substitution model**	GTR+I+G	GTR+G	HKY+G	HKY+I+G
	Gamma shape	1.0030	0.5370	4.6660	0.7700
BI	**Average Effective Sample Size**	373	570	1053	1570
	Potential Scale Reduction Factor	1	1	1	1
	GTR Submodel Probability	0.366	0.158	0.114	0.191

4.3.3. Growth in culture

Cultures of the new fungal species belonging to *Sporothrix capensis* had a colony diameter of 42 mm (±1) at an optimal temperature of 25ºC after 10 days of growth in the dark. *Graphilbum roseus* grew optimally at 25ºC and reached a colony diameter of 29.5 mm (±1.4), while the *Graphium ilexiense* grew at an optimal temperature of 35ºC, with a mean colony diameter of 29.1 mm (±0.88) after growing for 10 days in the dark.

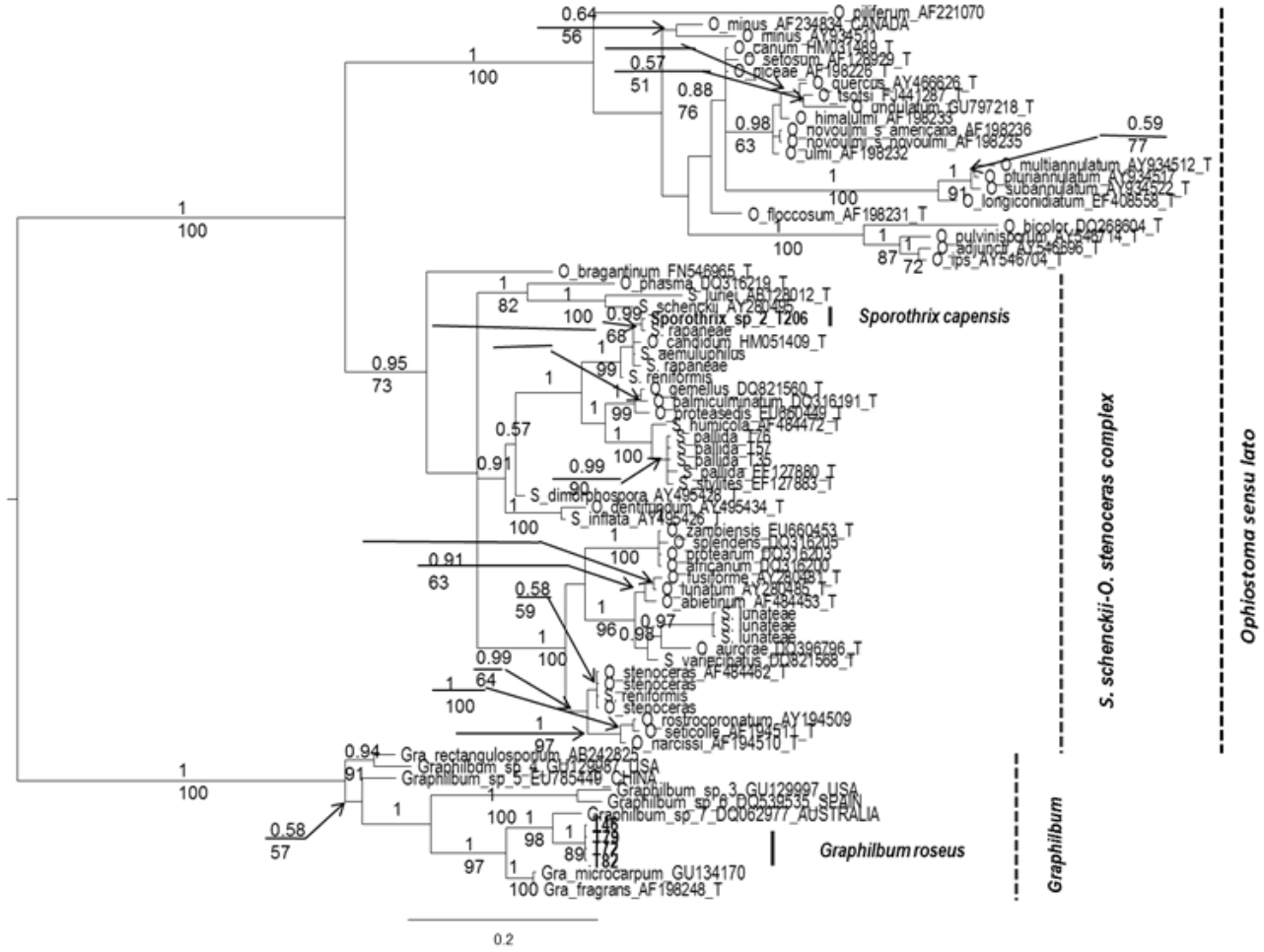

Figure 1: Bayesian Inference consensus tree (ITS *Ophiostoma* & *Graphilbum* data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. Dashed lines demarcate the different genera or species complexes that the fungi belong to.

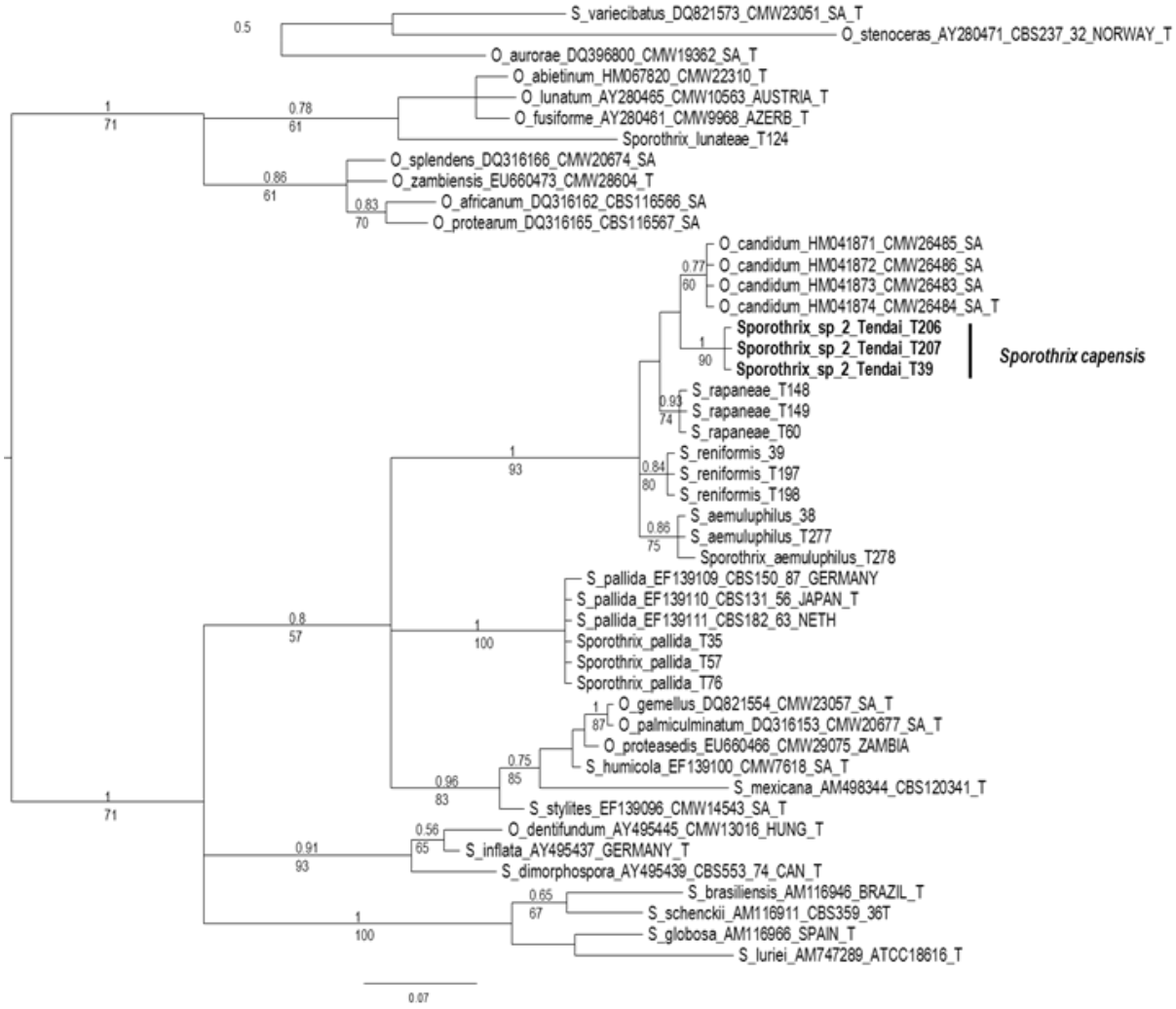

Figure 2: Bayesian Inference consensus tree (βt data) of taxa related to species isolated in the present study Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes.

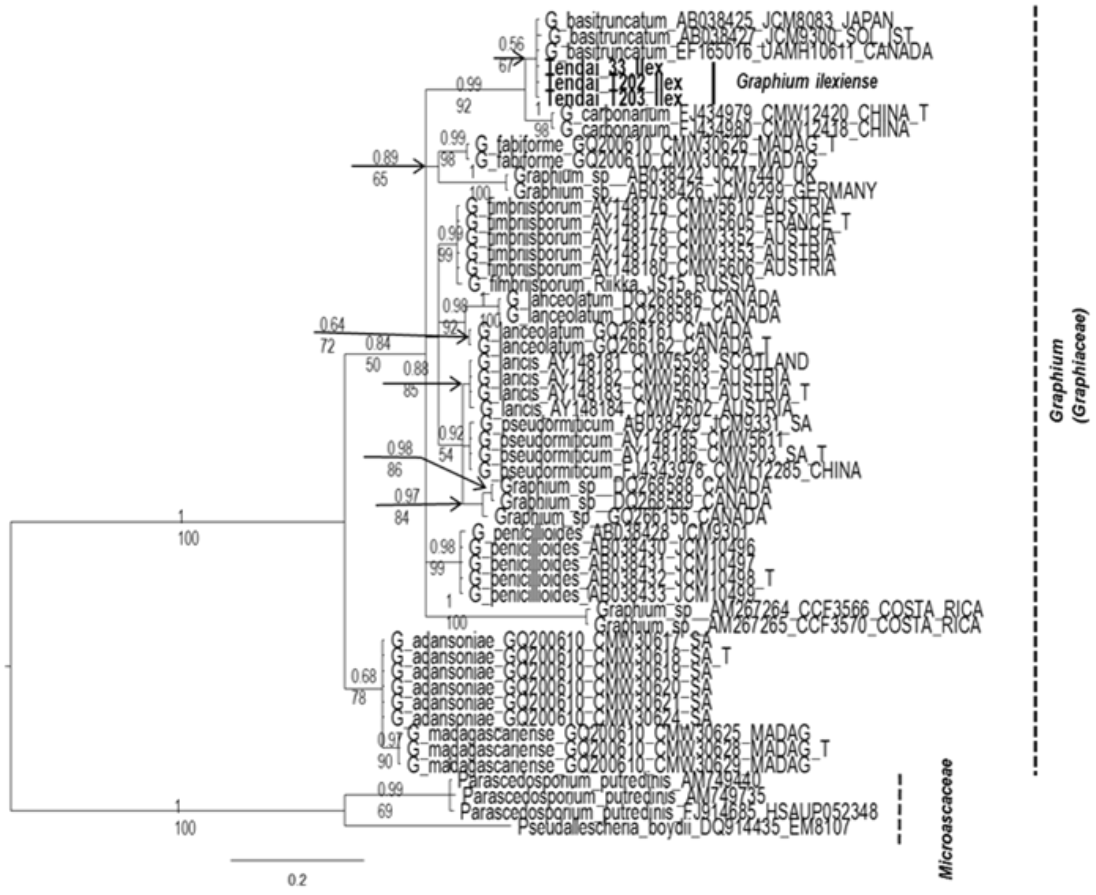

Figure 3: Bayesian Inference consensus tree (ITS *Graphium* data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. Dashed lines demarcate

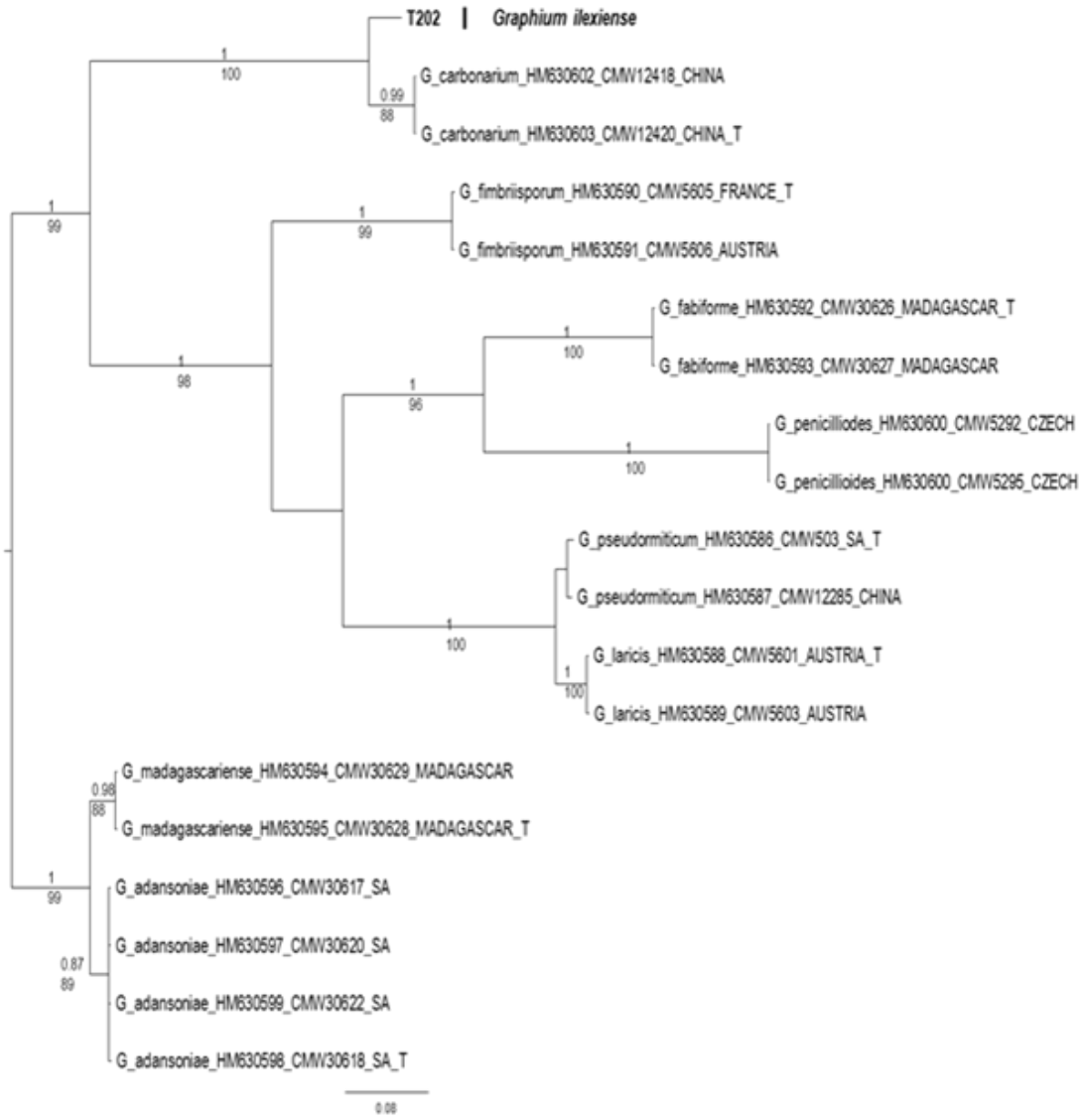

Figure 4: Bayesian Inference consensus tree (EF data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes.

3.3.4. Taxonomy

All three of the taxa collected from wounds on native Afromontane forest trees were recognised as new species based on phylogenetic analyses and micro-morphological characteristics. They are therefore described here as new species as follows:

Sporothrix capensis **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 5.

Etymology: The epithet *capensis* (capensis = of the Cape) refers to the Cape Floristic Region in which this fungus was collected.

Ascomata embedded in and superficial on the host substrate, bases 92-150 (118 ± 19) µm diam, globose, black, basal hyphae absent; necks black, 329-579 (421 ± 89) µm long, 17-32 (26 ± 5) µm wide at the base, 4-8 (5 ± 1) µm wide at the apex, osteolar hyphae, hyaline, 4.3-5.8 (5.3 ± 0.8) long (Fig. 5A & 5B). *Asci* evanescent. *Ascospores* allantoid, aseptate, hyaline, sheaths absent, 2.7-3.7 x 0.2-0.3 µm (Fig. 5C), accumulating in a transparent sticky droplet at the tip of the neck, translucent when dry. *Colonies* whitish to cream-coloured, fluffy towards the centre on MEA. Circular with entire edge (Fig. 5D), odourless. Colony diameter reaching 42 mm (± 1) after 10 days on MEA at an optimal growth temperature of 25ºC. No growth below 10ºC or above 35ºC. *Conidiophores* 4.2-8.3 x 0.6-1.0 µm, hyaline, tapering towards the apex, (Fig. 5E), *Conidiogenous* cells forming at the tips of conidiophores, hyaline, denticles present 0.5-1.5 (1 ± 0.3) µm long (Fig. 5E). *Conidia* hyaline, aseptate, oblong shaped, thin walled, holoblastic, 4.0-7.5 x 0.7-1.1 µm (Fig. 5F).

Substrate: Isolated from wound and bark of *Olea capensis* ssp. *macrocarpa*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Gouldveld. Isolated from an *Olea capensis* ssp. *macrocarpa* wound, October 2011, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T39 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T206 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T207 CMW (pending) = CBS (pending).

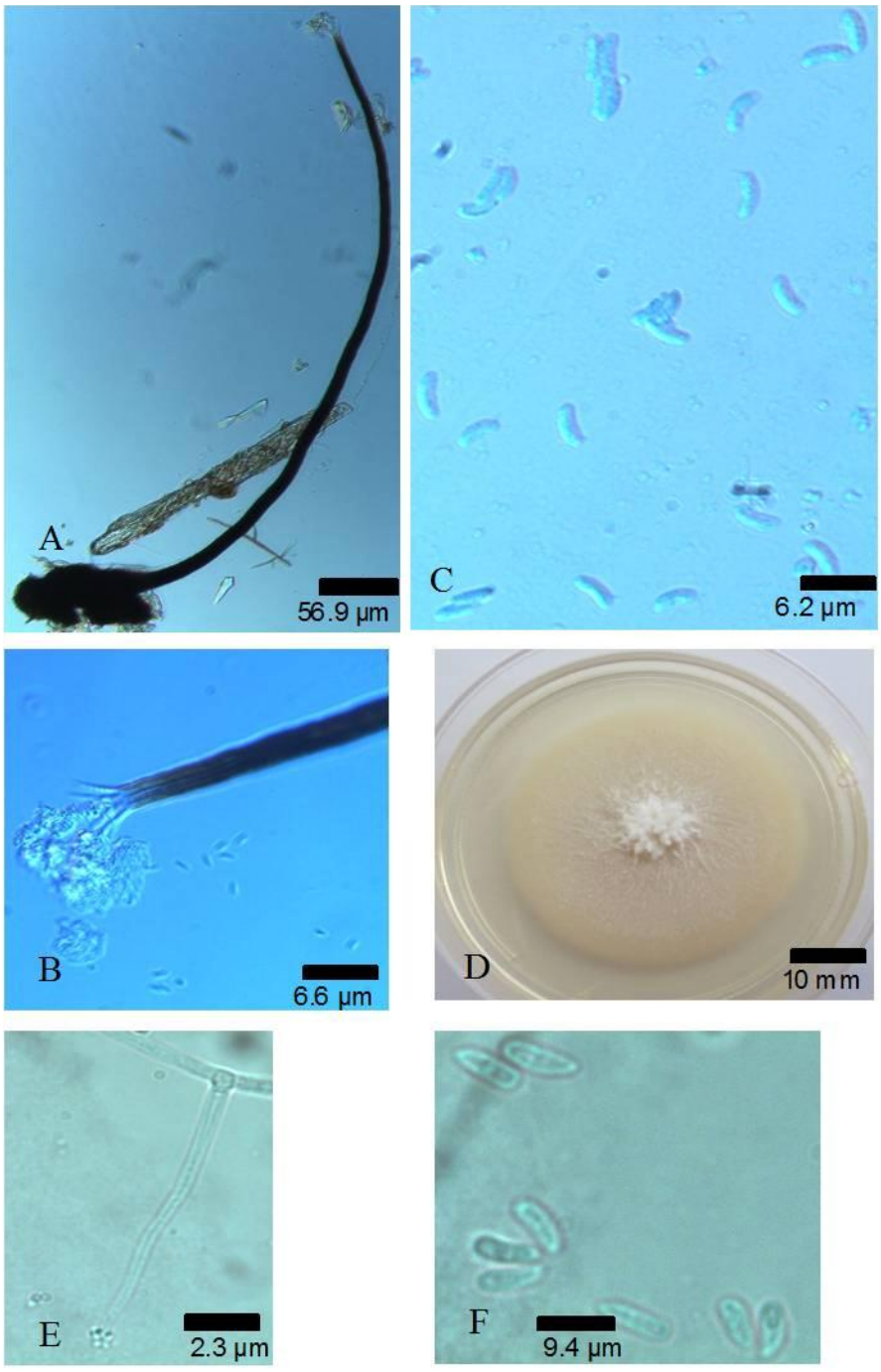

Figure 5. Micrographs of *Sporothrix capensis*. A. Perithecium removed from wounded bark of *Olea capensis*. B. Tip of perithecial neck. C. Ascospores. D. Two-week-old colony on MEA. E. Conidiophore showing conidiogenous cells with prominent denticles. F. Conidia.

Graphilbum roseus **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 6

Etymology: The epithet *roseus* (roseus = Latin for pink or rosy) refers to the pink colour of conidia produced by the synnemata.

Synnemata formed on the surface of the host substrate, also produced on agar after 4 weeks of growth in the dark at 25ºC, black at the base, becoming dark brown towards the apex, 210-833 (377 ± 188) µm long, 10-24 (19 ± 5) µm wide at the base, 7-13 (10 ± 2) µm wide at the apex. *Colonies* cream at the edges, becoming pinkish to brown at the centre, hard in texture at the edges on MEA, edges entire. Synnemata arising directly on media (Fig. 6). Colony diameter reaching 29.5 mm (±1.4) after 10 days on MEA at the optimal growth temperature of 25ºC. No growth below 10ºC or above 30ºC. *Conidiogenous* heads 60-91 µm over the widest part, brownish in color, becomes hyaline at the top (Fig. 6C), *Conidia*, hyaline, aseptate, smooth, thin walled and oblong shaped, forms in sticky slimy masses at tip of synnemata, pink, turning red when dry, 1.6 – 2.2 x 0.3 – 0.9 µm (Fig. 6D).

Substrate: Isolated from wound and bark of *Curtisia dentata*, *Halleria lucida*, *Pterocelastrus tricuspidatus*, *Trichocladus crinitus* and *O. capensis* ssp. *macrocarpa*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Gouna. Isolated from *Rapanea melanophloeos, Curtisia dentata*, *Halleria lucida* wounds, October 2011, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T46 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T72 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T82 CMW (pending) = CBS (pending). Western Cape Province, Groenkop. Isolated *Rapanea melanophloeos*, October 2011. T. Musvuugwa, **paratype** PREM (pending), culture ex-paratype T79 CMW (pending) = CBS (pending

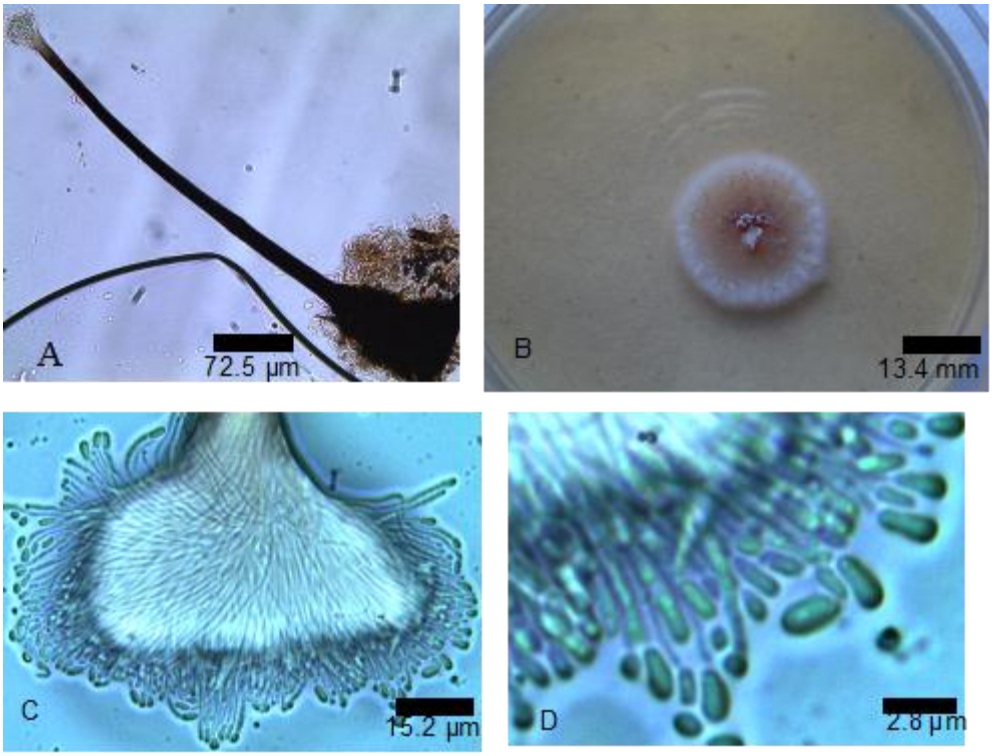

Figure 6. Micrograph of *Graphilbum roseus*. A. Synnemata. B. Two-week-old colony on MEA. C. Head of synnema. D. Conidia at the tips of conidiogenous cells.

Graphium ilexiense **Musvuugwa, LL. Dreyer and F. Roets sp. nov.** Mycobank: pending. Fig. 7

Etymology: The epithet *ilexiense* refers to the host plant genus (*Ilex mitis*) from which this species was collected.

Ascomata not observed. *Colonies* white, greyish in the centre, fluffy appearance on MEA. Irregular shaped and edges scalloped (Fig. 7). Grows at an optimal temperature of 35ºC on MEA with a colony diameter reaching 29.1 mm (±0.88) after 10 days. No growth below 10ºC. Causes black staining on the wood on its host. *Conidiophores* 16.1-18.5 x 0.4-1.2 µm, hyaline, oblong in shape (Fig. 7B-C), *Conidiogenous* cells hyaline, forming at the tips of conidiophores, (Fig. 7C). *Conidia* hyaline, aseptate, oblong to obovate shaped, thin walled, 2.1-3.5 x 0.4-0.8 µm (Fig. 7D).

Substrate: Isolated from wound on *Ilex mitis*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Assegaibosch, Stellenbosch. Isolated from *Ilex mitis* wound, April 2010, T. Musvuugwa, holotype PREM (pending), culture ex-holotype T202 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype 33 CMW (pending) = CBS (pending), **paratype** PREM (pending), culture ex-paratype T203 CMW (pending) = CBS (pending).

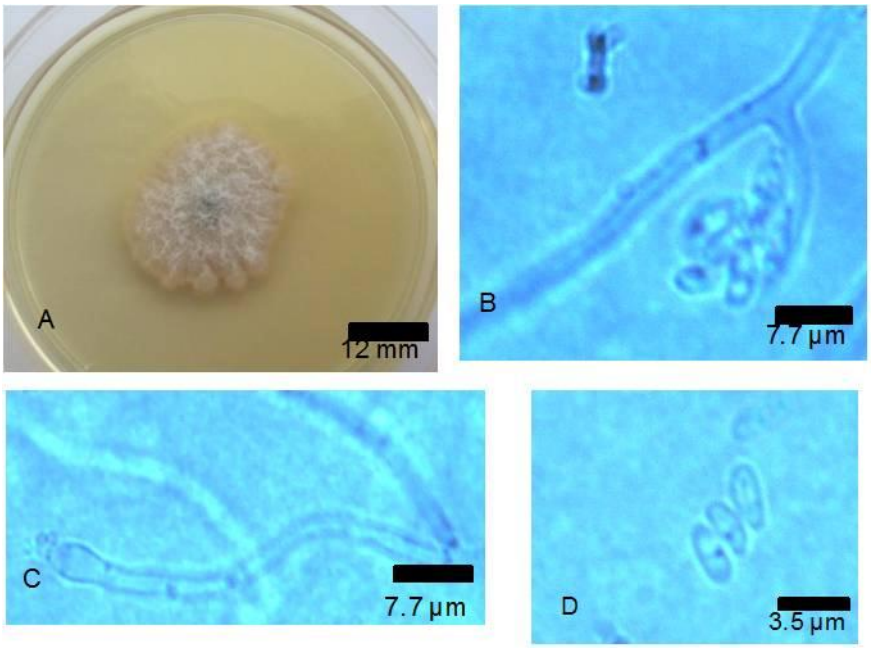

Figure 7. Micrographs of *Graphium ilexiense*. A. Two-week-old colony on MEA. B-C. Conidiophores. D. Conidia.

4.5. Discussion

Two species belonging to the Ophiostomatales and one species belonging to the Macroascales were isolated from wounds on native trees growing in the Afromontane forests of the CFR in this study. All proved to be new to science, and were described as members of three different genera. In previous CFR-based assessments of native host trees, a diverse array of ophiostomatoid fungal species were found associated with wounds (Musvuugwa *et al*. 2013, Chapter 3; Kamgan Nkuekam *et al*. 2008) and subcortical beetles (Musvuugwa *et al*. 2013, Chapter 3). Given that the three new species collected here belong to three different genera of ophiostomatoid fungi, it suggests that both the general diversity and the number of genera of ophiostomatoid fungi in the CFR may be much higher than had previously been anticipated. These findings suggest that additional research should be focussed on documenting the diversity of ophiostomatoid fungi associated with CFR tree hosts.

Sporothrix capensis belongs to the genus *Ophiostoma* and resolves within the *S. schenckii-O. stenoceras* complex (De Beer & Wingfield 2013) in which several species from these forests were recently placed (Musvuugwa *et al*. 2013, Chapter 2 &

3). It was isolated from wounds on *O. capensis* ssp. *macrocarpa*. Until the recent isolation of the beetle-associated *Sporothrix pallida* (Tubaki) Matsushima and *Raffaelea scabbardiae* Musvuugwa, LL. Dreyer & F. Roets (Musvuugwa *et al*. 2013, Chapter 2), no other ophiostomatoid fungus had ever been recorded from this tree species. Phylogenetically *Sporothrix capensis* formed a weakly supported clade sister to *S. rapanea*. It also resolved very close to *O. candidum* and *S. aemuluphilus* based on ITS data, but formed a strongly supported, distinct clade sister to *O. candidum* based on ßT data. *Ophiostoma candidum* is a known associate of Cerambycidae beetles that were isolated from wounds on *Eucalyptus cloeziana* F. Muell in South Africa (Kamgan Nkuekam *et al*. 2012). *Ophiostoma candidum* and *S. capensis* share some characters, such as the presence of osteolar hyphae and the same optimal growth temperatures (Kamgan Nkuekam *et al*. 2012). They differ in characters such as the production of ascomata on colonies in culture in *O. candidum*, but not in *S. capensis*. *Ophiostoma candidum* also displays hyphal ornamentation on bases of perithicia, but this was absent in *S. capensis*. Finally, the two species also differ in the average sizes of many morphological structures measured (Kamgan Nkuekam *et al*. 2012).

Graphilbum roseus belongs to *Graphilbum*, a genus that is the most distinct of all the well-supported Ophiostomatales genera (De Beer & Wingfield 2013). The genus comprises of only six known species and seven undescribed species (De Beer & Wingfield 2013) collected from various parts of the world, including Europe, America and Australia (Kim *et al*. 2005; Geldenhuis *et al.* 2004). *Graphilbum roseus* grouped strongly with *Graphilbum* sp. 7., one of the undescribed taxa, which is only known as an anamorph (De Beer & Wingfield 2013). It was initially reported as a *Pesotum* J.L. Crane & Schkn. species collected in Australia by Thwaites *et al*. (2005). It was isolated from *Pinus radiata* D. Don causing sapstain on the host tree (Thwaites *et al*. 2005). *Graphilbum roseus* share characters with other species in the genus, such as the presence of synnematous anamorphs that are *Pesotum*-like and the production of conidia in slimy masses (De Beer *et al*. 2013a). Although *G. roseus* was isolated from wounds, some of the other species in the genus are associated with conifer-infesting bark beetles (De Beer *et al*. 2013a). *Graphilbum fragrans* (Mathiesen-Käärik) Z.W. de Beer, Seifert & M.J. Wingf., for example, has been isolated from *Ips sexdentatus* Borner infesting *Pinus sylvestris* L. in Sweden (Mathiesen-Käärik 1953). In South Africa, the same species was associated with *Hylastes angustatus* Herbst infesting

Pinus patula Schiede ex Schltdl Cham. in Mpumalanga (Zhou *et al*. 2006). Unlike the other two species collected in this study, *G. roseus* was isolated from several host species, including *Curtisia dentata*, *Halleria lucida*, *Pterocelastrus tricuspidatus*, *Trichocladus crinitus* and *O. capensis* ssp. *macrocarpa*.

Graphium ilexiense belongs to the order Microascales. Based on ITS sequence data, it grouped with *G. basitrancatum* as sister to *G. carbonarium*. EF data resolved it as sister to *G. carbonarium* with very strong support. *Graphium carbonarium* was first described from China, where it was associated with a bark beetle (*Pissodes* sp. Germar) infesting *Salix babylonica* L. (Paciura *et al*. 2010). It is similar to *G. ilexiense* in that they both produce greyish colonies and they both produce black staining on wood (Paciura *et al*. 2010). The other closely related species, *G. basitrancatum*, was first isolated in the Solomon Islands from forest soil (Matsushima 1971). In Canada it has also been isolated from a leukaemia patient, confirming that this species can act as an opportunistic human pathogen (Deepali *et al*. 2007). A major difference between *G. ilexiense* and most other species in the genus is that it was wound-associated. Most other species, such as *G. carbonarium*, are bark beetle associated (De Beer *et al*. 2013b; Paciura *et al*. 2010; Geldenhuis *et al*. 2004; Okada *et al*. 2000). *Graphium* species isolated in South Africa include *G. adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux, which was isolated from *Adansonia digitata* L. (Cruywagen *et. al* 2010) and *G. pseudormiticum*, isolated from a bark beetle on exotic pine trees (Mouton *et al*. 1994). There are no sexual stages known for species in this genus (De Beer *et al*. 2013b). To the best of our knowledge, no other ophiostomatoid fungus has to date been isolated from *Ilex mitis*.

4.6. Conclusion

Three new ophiostomatoid species, belonging to three different genera, were collected from wounds on native Afromontane tree hosts in this study. This brings the current total of ophiostomatoid species known from these forests to 18. Other known species include *Ceratocystis tsitsikammensis* Kamg. Nkuek. & Jol. Roux and *Pesotum fragrans* (Mathiesen-Käärik) G. Okada & Seifert-like fungus and *O. quercus* (Georgévitch) Nannf. Future research in this niche is therefore encouraged. One of the species, *Graphilbum roseus*, was associated from several native hosts, while the other

two were only collected from one host each. Future research should also explore the reasons for this observed host non-specificity in one species, and apparent specificity in the other two species. Our results represent the first discovery of an ophiostomatoid species isolated from *Ilex mitis*. This is significant, as several other CFR plants species have thus far been viewed as not associated with ophiostomatoid fungi. Future studies should focus on such tree species in order to ultimately understand the true diversity of ophiostomatoid fungi associated with the Afromontane forests of the CFR.

References

Barnes, I., Nakabonge, G., Roux, J., Wingfield, B.D., Wingfield, M.J. 2005. Blackwell Publishing, Ltd. Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* 54: 189-195.

Brasier, C.M. & Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Bretz, T.W. 1952. The ascigerous stage of the oak wilt fungus. *Phytopathology* 42: 435-437.

Brooks, T., Balmford, A., Burgess, N., Fjeldsa, J., Hansen, L.A., Moore, J., Rahbek, C. and Williams, P. 2001. Towards a blueprint for conservation. Africa. *BioScience* 51: 613–624.

Castello, J.D., Leopold, D.J. and Smallidge, P.J. 1995. Pathogens, patterns and processes in Forest Ecosystems. *BioScience* 45: 16-24.

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., de Beer, Z.W., Wingfield, M.J., Hawksworth, D.L., 2006. How many species of fungi are there at the tip of Africa? *Studies in Mycology* 55: 13–33.

Cruywagen, E.M., de Beer Z.W., Roux, J. and Wingfield M.J. 2010. Three new *Graphium* species from baobab trees in South Africa and Madagascar. *Persoonia* 25: 61–71.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013a. The ophiostomatoid fungi: their dual position in the *Sordarimycetes*. In: *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 1-19.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013b. A nomenclature for ophiostomatoid genera and species in the *Ophiostomatales* and *Microascales*.

In : *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.). CBS Biodiversity Series 12, pp. 245-322.

De Beer, Z.W. and Wingfield, M.J. 2013. Emerging lineages in the Ophiostomatales. In: *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 21-46.

De Beer, Z.W., Wingfield, M.J. and Kemp, G.H.J. 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* 91: 6.

Deepali, K., Sigler, L., Gibas, C.F.C., Subhash, M., Schuh, A., Medeiros, B.C., Peckham, K. and Natul, H. 2007. *Graphium basitruncatum fungemia* in a patient with acute leukemia. *Journal of Clinical Microbiology* 45:1644–1647.

Duong, T. A., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2012. Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. *Mycologia* 104: 715-732.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113-118.

Geldenhuys, C.J. 2010. Distribution, size and ownership of forests in the Southern Cape. *South African Forestry Journal* 158: 51-66.

Geldenhuis, M.M., Roux, J., Montenegro, F., De Beer, Z.W., Wingfield, M.J. and Wingfield, B.D. 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Divers* 15: 137–151.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* 61: 1323-1330.

Goldblatt, P. and Manning, J. 2000. Cape plants: A conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria, South Africa; Missouri Botanical Garden Press, St. Louis, Missouri, USA.

Guindon, S. and Gascuel, O. 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology* 52: 696-704.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D.N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* 104: 399-404.

Heath, R.N., Wingfield, M.J., Wingfield, B.D., Meke, G., Mbaga, A. and Roux, J. 2009. *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* 34: 41-67.

Kamgan Nkuekam, G., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2012. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Kerley, G.I.H., Pressey, R.L., Cowling, R.M., Boshohof, A.F. and SimsCastley, R. 2003. Options for the conservation of large and medium-sized mammals in the Cape Floristic Region. *Biological Conservation* 112: 169–190.

Kim, J.J., Allen, E.A., Humble, L.M. and Breuli, C. 2005. Ophiostomatoid and basidiomycetous fungi associated with green, red and grey lodgepole pines after mountain pine beetle (*Dendroctonus ponderosae*) infestation. *Canadian Journal of Forestry Research* 35: 274-284.

Kubono, T. and Ito, S. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Mathiesen-Käärik, A. 1953. Eine Übersicht über die gewöhnlichsten mit Bokenkäfern assoziierten Bläuepilze in Schweden und eihige für Schwedenn neue Bläuepilze. *Meddelanden från Statens Skogsforskningsinstitut* 43: 1-74.

Matsushima, T. 1971. Microfungi of the Solomon Islands and Papua New Guinea. Published by the author, Kobe.

Mouton, M., Wingfield, M.J., Wyk, P.S. van and Wyk, W.J. van. 1994. *Graphium pseudormiticum* sp. nov.: a new hyphomycete with unusual conidiogenesis. *Mycological Research* 98: 1272–1276.

Mucina, L. and Rutherford, M. 2006. The vegetation of South Africa, Lesotho and Swaziland. Streletzia 19, Pretoria: *South African National Biodiversity Institute* 77: 93-94.

Myers, N., Mittelmeier, R.A., Mittelmeier, C.G., da Fonseca, G.A.B. and Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Okada, G., Jacobs, K., Kirisits, T., Louis-Seize, G.W., Seifert, K.A., Sugita, T., Takematsu, A. and Wingfield, M.J. 2000. Epitypification of *Graphium penicillioides* Corda, with comments on the phylogeny and taxonomy of *Graphium*-like synnematous fungi. *Studies in Mycology* 45:169–188.

O'Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103-116.

Paciura, D., Zhou, X.D., De Beer, Z.W., Jacobs, K., Ye, H. and Wingfield, M.J. 2010. Characterisation of synnematous bark beetle-associated fungi from China, including *Graphium carbonarium* sp. nov.. *Fungal Diversity* 40:75–88.

Pipe, N.D., Brasier, C.M. and Buck, K.W. 2000. Evolutionary relationships of the Dutch elm disease fungus *Ophiostoma novo-ulmi* to other *Ophiostoma* species investigated by restriction fragment length polymorphism analysis of the rDNA region. *Journal of Phytopathology* 148: 533–539.

Ribeiro, I.J.A. 1980. Seca da mangueira. Agentes causais e estudo da molestia. In: Anais do I Simposio Brasiliero Sobre a Cultura de Mangueira. Sociedade Brasileira de Fruticultura, Jaboticabal, pp. 123–130.

Ronquist, F.R. and Huelsenbeck, J.P. 2003. MrBayes: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

Roux, J., Heath, R.N., Labuschagne, L., Kamgan Nkuekam, G.and Wingfield, M.J. 2007. Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* 37: 292-302.

Roux, J., Meke, G., Kanyi, B., Mwangi, L., Mbaga, A., Hunter, G.C., Nakabonge, G., Heath, R.N. and Wingfield, M.J. 2005. Diseases of plantation forestry trees species in Eastern and Southern Africa. *South African Journal of Science* 101: 409-413.

Roux, J., VanWyk, M., Hatting, H. and Wingfield, M. J. 2004: *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* 53: 414–421.

Roux, J., Dunlop, R. and Wingfield, M.J. 1999. Susceptibility of elite *Acacia mearnsii* families to *Ceratocystis* wilt in South Africa. *Journal of Forestry Research* 4: 187–190.

Sinclair, W.A., Johnson, W.T. and Lyon, H. 1987. Diseases of trees and shrubs. Cornell University Press, Ithaca, Nwe York, USA, pp. 574.

Stattersfield, A.J., Crosby, M.J., Long, A.J. and Wedge, D.C. 1998. Endemic Bird Areas of the World. BirdLife Conservation Series 7. BirdLife International, Cambridge.

Taylor, J.E., Lee, S. and Crous, P.W. 2001. Biodiversity in the Cape Floral Kingdom: fungi occurring on Proteaceae. *Mycological Research* 105: 1480-1484.

Thwaites, J.M., Farell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E, Hadar, Y., Harrington, T.C. and McNew, D. 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* 43: 653-663.

Turpie, J.K., Heydenrych, B.J. and Lamberth, S.J. 2003. Economic value of terrestrial and marine biodiversity in the Cape Floristic Region: implications for defining

effective and socially optimal conservation strategies. *Biological Conservation* 112: 233-251.

Van Wyk, M., Al Adawi, A.O., Wingfield, B.D., Al Subhi, A.M., Deadman, M.L. and Wingfield, M.J. 2005. DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* 34: 587–590.

VanWyk, B. and VanWyk, P. 1997. Field Guide to Trees of Southern Africa. Cape Town, South Africa: Struik Publishers.

Vermeulen, W.J., Geldenhuys, C.J. and Esler, K.J. 2012**.** Response of *Ocotea bullata*, *Curtisia dentata* and *Rapanea melanophloeos* to medicinal bark stripping in the southern Cape, South Africa: implications for sustainable use. *Southern Forests* 74: 183–193.

Viegas, A.P. 1960. Mango blight. *Bragantia* 19: 163–182.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Sequencing Guide to Methods and Applications*, (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds.), Academic Press, San Diego, pp. 315-322.

Wingfield, M.J., De Beer, C., Visser, C. and Wingfield, B.D. 1996. A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* 19: 191-202.

Wingfield, M.J., Seifer, K.A., Webber, J.F. 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Society Press, St. Paul Minnestota, USA.

Zhou, X.D., De Beer, Z.W. and Wingfield, M.J. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* 53: 290-300.

Chapter 5: Association between sub-cortical beetles (Coleoptera: Curculionidae), mites (Acari) and ophiostomatoid fungi (Ascomycota: Ophiostomatales) on trees in the Cape floristic Region of South Africa

Abstract

Afromontane forests form a vital component of the Cape Floristic Region (CFR), but very little research has been conducted on the diversity of organisms associated with trees in these forests. Ophiostomatoid fungi are well-known associates of arthropods that infest forest trees and many can cause disease. They are usually associated with bark- and ambrosia-beetles, but some are also associated with other arthropods such as mites. In this study we document the diversity of ophiostomatoid fungi and their associated arthropods on CFR trees, focussing on those associated with wounds, sub-cortical beetles and mites. Sampling was conducted in various indigenous forest patches throughout the CFR. Bark and wood samples were collected from trees showing signs of subcortical beetle activity or from wounds on storm damaged trees. Ophiostomatoid fungi were isolated from sub-cortical beetles emerging from samples, their galleries, their phoretic mites and from wounds and wound-associated mites. Fungal isolates representing different Operational Taxonomic Units were identified using molecular markers (ITS, BT, CAL, EF, LSU). Fourteen species of fungi that belong to the Ophiostomatales and a single representative from the Microascales were isolated from only nine native tree hosts. Three species, including two apparently undescribed species, were isolated exclusively from *Pinus* species. Many species were found to be fairly specific towards their tree hosts. However, a few species were isolated from both native and non-native tree hosts. Similarly, one of the mite taxa was associated with both native and non-native arthropods and hosts. Interestingly, wound-associated fungi seemed to be less host specific than sub-cortical beetle associated taxa. Results of this study highlighted the very rich diversity of ophiostomatoid fungi, sub-cortical beetles and mites associated with trees in the CFR. It provides the foundation for future studies on the ecology of these important organisms.

Key words: Bark-beetle interaction, mite-fungus interaction, phoresy, fungal diversity

5.1. Introduction

The Cape Floristic Region (CFR) at the southern tip of South Africa is an internationally recognised biodiversity hotspot (Myers *et al*. 2000). The region displays exceptionally high levels of plant diversity and endemism (Goldblatt & Manning 2000), but information on other taxa such as micro-organisms is generally lacking. The paucity in knowledge regarding microbial diversity is not unique to the CFR, as in South Africa as a whole, only about 800 of the estimated 200 000 plant-associated fungal species have been described (Crous *et al*. 2006). This lack in knowledge on CFR fungal biodiversity has resulted in an overwhelming lack of research on the ecology of native fungi.

Afromontane forests form a relatively small, but vital, component of CFR vegetation and are found as small fragmented patches within river valleys, on mountains, foothills and on coastal platforms along the southern and southwestern coastal areas (Geldenhuys 2010; Morgenthal & Cilliers 2000). They are evergreen forests with closed canopies and are confined to fertile, well-watered areas (Geldenhuys 1997). The largest Afromontane forests in the CFR are located in the Tsitsikamma and Knysna regions. This vegetation type also extends through the northeastern parts of South Africa and northwards into Swaziland and Mozambique. The most common canopy trees in these forests include ironwood (*Olea capensis* L. ssp. *macrocarpa*), assegaaihout (*Curtisia dentata* C.A. Sm.), yellowwoods (*Podocarpus* Persoon spp.), Cape beech (*Rapanea melanophloeos* Mez) and stinkwood (*Ocotea bullata* (Burch.) Baill).

Although the CFR forests are extremely important for ecosystem functioning, very limited research has been conducted on the diversity of potential pathogens present in these forests (Castello *et al*. 1995). These studies are extremely important, as fungi have the potential to kill trees and disrupt normal ecosystem processes, especially under altered environmental conditions (Brasier 2008; Harrington *et al*. 2008; Pipe *et al*. 2000; Heiniger & Rigling1994;). For example, in the CFR a recent study identified a serious stem canker disease on *R. melanophloeos* that is caused by a new pathogen in a semi-natural botanical garden setting (Chen *et al*. 2013). The influence of exotic fungi on trees within these forests has also become apparent with species such as *Phytophthora cinnamomi* Rands and *Armillaria mellea* (Vahl. Fr.) Kummer causing

disease and death of many plant taxa in these forests (Wingfield *et al*. 2010; Doidge & Bottomley 1931).

Studies on CFR tree-associated pathogens have been extended recently to include the ophiostomatoid fungi (Ascomycota: Ophiostomatales and Microascales) and their associated organisms (e.g. Kamgan *et al*. 2008). *Ceratocystis* Ellis & Halst. spp. have been found to sometimes be very aggressive pathogens to hosts trees. *Ceratocystis. tsitsikammensis* Kamgan & Jol. Roux, for example, is a wound infecting fungus, which formed significant lesions when inoculated on its host tree *R. melanophloeos* (Kamgan *et al*. 2008). Regular vectors that transport *Ceratocystis* fungi to wounds on trees include nitidulid beetles (Kamgan Nkuekam *et al*. 2012b; Cease & Juzwik 2001; Moller & Devay 1968) and bark beetles (Harrington & Wingfield 1998; Wingfield *et al*. 1997; Redfern *et al*. 1987). Associations between these organism groups are unfortunately still understudied in the CFR. Although the importance and diversity of *Ceratocystis* in the CFR has received some attention (Kamgan *et al*. 2008), little is known about the diversity and ecology of other ophiostomatoid genera and their associated organisms in these forests.

Ophiostomatoid fungi are represented by genera such as *Ceratocystis*, *Ophiostoma* H. Syd & P. Syd., *Ceratocystiopsis* Upadhyay & Kendrick, *Knoxdaviesia* Wingfield, Van Wyk & Marasas and *Graphium* Corda (De Beer *et al*. 2013). These genera do not form a monophyletic lineage as the genera *Ophiostoma* H. Syd & P. Syd, *Ceratocystiopsis* Upadhyay & Kendrick, *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Raffaelea* Arx & Hennebert and *Leptographium* Lagerb. & Melin belong to the Ophiostomatales, while *Ceratocystis* Ellis & Halst., *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas and *Graphium* Corda resolve in the Microascales (De Beer *et al*. 2013; Spatafora & Blackwell 1994; Hausner *et al*. 1993). Despite this polyphyly, these taxa are often still collectively referred to as ophiostomatoid fungi due to their morphological and ecological similarities. Members of both groups are important tree pathogens (Woolhouse *et al*. 2005; Brasier & Buck 2001; Zhou *et al*. 2001; Roux & Wingfield 1997; Viégas 1960; Ribeiro 1980). *Ophiostoma*, for example, includes several virulent pathogens such as the well-known *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, responsible for the Dutch elm disease pandemics in North America and Europe (Brasier & Buck 2001; Heybroek 1993; Lamb 1979). *Ophiostoma* species are also widely responsible for sapstain on lumber logs and

pulpwood, leading to high economic losses (Zhou *et al*. 2001; Seifert 1993; Lagerberg *et al*. 1927). Some *Raffaelea* species are also serious plant pathogens, including *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva which is responsible for the laurel wilt disease that kills members of the Lauraceae in the southeastern USA (Harrington *et al*. 2008).

A large number of fungi from the Ophiostomatales are associated with bark and ambrosia beetles (Scolytinae and Platypodinae) known to infest trees (Harrington 2005; Six 2003; Whitney 1982). The beetles often gain nutritional benefits from their fungal associates (Eckhardt *et al*. 2004; Klepzig & Six 2004; Six & Paine 1998; Baker & Norris 1968) and in turn vector the fungi from one host tree to the next, suggesting a mutualistic association (Klepzig & Six 2004; Six 2003; Whitney 1982). In some cases the bark beetle associates have evolved specialised spore carrying structures (mycangia) to help maintain and protect the fungi from desiccation and contaminant fungi during transport (Klepzig & Six 2004; Paine & Birch 1983; Barras & Perry 1972; Batra 1963). However, not all ophiostomatoid fungi are associated with bark or other sub-cortical beetles (Hofstetter & Moser 2014; Harrington *et al*. 2008). Nitidulid beetles (Kamgan Nkuekam *et al*. 2012a; 2012b), cerambycid beetles (Jacobs & Kirisits 2003; Jacobs & Wingfield 2001), weevils (Kirisits 2004; Jacobs &Wingfield 2001) and even mites (Klepzig *et al*. 2001a; Roets *et al*. 2007; Moser 1985) have also been implicated as common associates. The association between mites and ophiostomatoid fungi has also proven to sometimes be mutualistic (e.g. Roets *et al*. 2007; Lombardero *et al*. 2003; Klepzig *et al*. 2001a; 2001b; Moser 1985). Again, some mites have evolved specialised flap-like structures to house ascospores of associated fungi when in transit between host plants (Hofstetter & Moser 2014; Roets *et al*. 2007; Moser *et al*, 1995; Moser 1985; Bridges & Moser 1983).

Most knowledge regarding the diversity and arthropod-associations of ophiostomatoid fungi in the Ophiostomatales are confined to the Northern Hemisphere. Most South African studies have focussed on non-native forestry taxa like pines, *Eucalyptus* L'Hér spp. and *Acacia* Mill. spp. (Kamgan Nkuekam *et al*. 2012a; Zhou *et al*. 2006; Zhou *et al*. 2001; De Beer *et al*. 1995). A few studies shifted this focus to also include taxa that are associated with native trees (Kamgan *et al*. 2008; Roets *et al*. 2008; 2006; Marais & Wingfield 2001; 1997; 1994; De Beer *et al*. 1995). More recently it has become apparent that a large number of undescribed taxa are associated with trees

in CFR forests (Musvuugwa *et al*. 2013, Chapters 2, 3 & 4). Only one of these studies investigated the association between arthropods and fungi (Musvuugwa *et al*. 2013, Chapter 2). In the present study we set out to build on these initial studies and document the diversity of ophiostomatoid fungi and their associated arthropods on CFR trees, focussing on those associated with wounds, sub-cortical beetles and mites. As this study will provide a platform for future studies on the ecology of ophiostomatoid fungi in the CFR, we included all available data from previous studies (Musvuugwa *et al*. Chapters 2, 3 & 4) on Afromontane forests.

5.2. Materials and methods

5.2.1. Arthropod sampling

Sampling was conducted in various indigenous forest patches throughout the CFR between 2010 and 2012 (Fig. 1). Bark and wood samples were collected at random from diseased and storm damaged (Fig. 2.) native trees (various taxa) as well as from trees with signs of sub-cortical beetle activity. When present at these sites, samples were also collected from invasive exotic tree species (*Pinus* spp. and *Acacia mearnsii* De Wild). Initial collection of beetles was conducted by inspection of infested trees and wood using a Leica EZ4 dissection microscope (Leica Microsystems, Taiwan) and aseptically placing individual beetles in sterile vials. Plant material that showed signs of beetle activity was placed in insect emergence cages following methods of Musvuugwa *et al*. (2013, Chapter 2). Emergence cages were maintained at room temperature and inspected for beetles every 2-3 days for a period of fifty days. All emerging beetles were collected, assigned to morpho-species, their numbers recorded and stored at 4ºC until further use (but no longer than 5 days). Reference collections of all beetle taxa collected in this study were stored in 70% ethanol.

Collected beetles were inspected for the presence of phoretic mites and when present, these were aseptically removed and stored in eppendorf tubes at 4ºC until further use. In addition, mites were isolated directly from collected bark and wood samples and treated similarly. Reference material of all mite taxa collected in this study were permanently fixed on microscope slides. All reference material was sent for identification by expert mite and beetle taxonomists and is maintained in the Insect Collection of Stellenbosch University (USEC), Stellenbosch, South Africa.

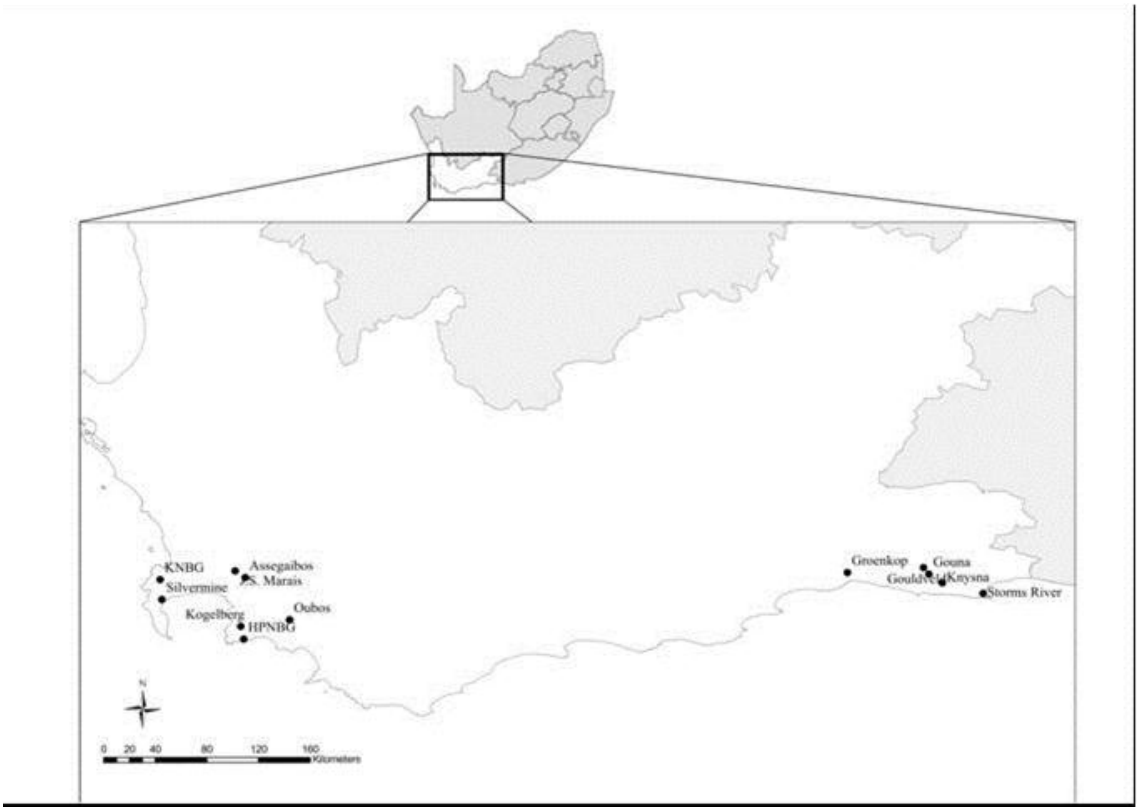

Figure 1. Map showing study sites from which samples were collected for this study. KBG = Kirstenbosch Botanical Garden, HPBG = Harold Porter Botanical Garden, J. Marais = Jan Marais Park

Figure 2. Storm-damaged *Olea capensis* ssp. *macrocarpa* tree from which sub-cortical beetles, bark and wood samples were collected.

5.2.2. Fungal isolation from plant material

A subset of collected wood and bark samples was examined for the presence of ophiostomatoid fungi using a dissection microscope. When no ophiostomatoid fungi were present, collected material was placed in moisture chambers (resealable plastic bags with ddH_2O-moistened paper towels) at room temperature (~23°C) in the dark for up to four weeks to stimulate fungal growth. Where present, masses of ascospores and/or conidia were collected from the apices of sporulating structures using a sterile needle and transferred to 2% malt extract agar (MEA). Isolates were stored in the dark at room temperature and examined daily for fungal growth. Isolates were purified by transferring single hyphal tips from the edges of actively growing fungal colonies to fresh MEA plates.

5.2.3. Fungal isolation from arthropods

Fungi were isolated from sub-cortical beetles and mites following methods of Musvuugwa *et al*. (2013, Chapter 2). The number of individuals per morpho-species used ranged between 3 and 50, depending on availability. When present, a single representative colony of all suspected ophiostomatoid morpho-types growing per isolation plate (one plate per arthropod individual) were randomly chosen and purified. Once purified all cultures were maintained on Petri dishes containing MEA at 4°C until further use.

The mean number of colony-forming units (CFU's) was calculated for each potential ophiostomatoid morpho-type isolated from each arthropod morpho-species. It was assumed that numbers of spores carried by an individual arthropod were correlated to the number of CFU's. In addition, the frequency with which a particular fungus morpho-type was isolated from a particular arthropod species was calculated as follows: F= (NS/NTs) X 100; where F represents the frequency of occurrence (%) of the fungus from each niche, NT represents the total number of samples from which isolations were made and NS represents the number of samples from which fungi were isolated (Yamaoka *et al*. 1997).

5.2.4. Fungal identification

All fungal cultures obtained were grouped according to morpho-type based on cultural and micro-morphological characteristics. Morpho-types originating from different hosts and different arthropod taxa were not cross-referenced and dealt with as discrete units for later molecular identification. In total, 222 isolates were grouped according to morpho-type, of which 80 were selected for molecular characterisation. Representative cultures of all morpho-types collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

For molecular characterization, three or more isolates representing each fungal morpho-type were randomly chosen. For DNA extraction, fungal mycelium was collected from two-week-old actively growing colonies on MEA using a sterile scalpel. Genomic DNA was extracted using a Sigma-Aldrich™ plant extraction kit

(USA) according to the manufacturer's instructions. PCR reaction conditions and methods for DNA amplification followed Musvuugwa *et al*. (2013, Chapters 2 & 3). Amplified PCR products were cleaned using the EXOSAP-IT kit (USB Corporation, Cleveland, Ohio, U.S.A.) following the manufacturer's instructions. Fragments were sequenced using the respective PCR primers and a Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) and analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). The same primers used for PCR amplifications were used and both DNA strands were sequenced. The CLC Genomics Workbench software package (CLC Bio, Cambridge, MA) was used to edit and construct consensus sequences.

Using the ITS sequences, preliminary identification of the isolates was done by performing BLAST (Basic Local Alignment Search Tool) algorithm (searches to compare and find similarities with sequences published on the GenBank sequence database (http://www.ncbi.nlm.nih.gov). The identity of taxa that were not identified in previous studies (Chapters 1, 2 & 3) was determined by comparing generated data to published sequences from other studies on GenBank using BLAST.

5.3. Results

In this section, all authorities for arthropods and fungi are indicated in tables and not in text due to the large number of species discussed.

5.3.1. Sub-cortical beetles and their associated mites

When including data from previous studies Musvuugwa *et al*. (2013, Chapter 2), more than 4500 individuals belonging to at least 15 different genera of sub-cortical beetles (all within the family Curculionidae) were collected from 13 native tree hosts and one exotic *Pinus* sp. host (Table 1, Fig. 3). The *Lanurgus* sp. 1 from *Olea capensis* ssp. *macrocarpa* and *Hapalogenius fuscipennis* from *Virgilia oroboides* were very abundant, with more than 2000 individuals often emerging from single collections of wood. In other cases only 1 individual of a beetle species was collected, as was the case for *Amphiscolytus capensis* associated with *Rhoicissus tomentosa*. Several beetle

species were collected from more than one host tree, while a few (e.g. *Cryphalus* sp. 1, *A. capensis* and Platypodinae sp. 1) were only collected from a single host (Table 1). Three beetle species were only collected from non-native *Pinus* sp. (Table 1). Several host trees (e.g. *R. melanophloeos*, *O. capensis* ssp. *macrocarpa*, *Maytenus acuminata*, *V. oroboides* and *Gonioma kamassi*) were associated with more than one beetle species.

Only a few beetle species were associated with phoretic mites (Table 1). Individuals of *Lanurgus* sp. 1 from *O. capensis* ssp. *macrocarpa* were associated with *Dendrolaelaps quadrisetus* and an *Elattoma* sp. 1 mite. Cryphalinae sp. 1 and *H. fuscipennis* were associated with the same *Elattoma* sp. 1 mite, while the pine beetle *Orthotomicus erosus* was associated with *Elattoma* sp. 2, *Histiogaster* sp. 3 as well as *D. quadrisetus* (Table 1).

5.3.2. Mites from wounds on native trees

In addition to phoretic mites, several non-phoretic mites belonging to 16 different morpho-species were collected from a total of 12 native tree species (Table 2, Fig. 4). *R. melanophloeos* had the highest number of mite species associated with its wounds, with seven mite species collected from this host. A few of the mite species collected were found on more than one host tree species, while others such as Tetranychidae sp. 1, *Uroobovella* sp. 1 and Acaridae sp. 1 seemed to be more specific towards their hosts.

Table 1: Summary of the sub-cortical beetles, their host trees and their phoretic mites collected in this study. Beetle species in bold indicate species that were not encountered in the previous studies (Chapters 2-4). (HPNBG = Harold Porter National Botanical Garden; KNR = Kogelberg Nature Reserve; SNR = Silvermine Nature Reserve)

Beetle species	**Reference numbers**	**Number collected**	**Host tree**	**Site**	**Phoretic mites (reference number)**
Amphiscolytus capensis Schedl	T24; T40	2	*Rhoicissus tomentosa* (Lam.) Wild & Drumm	Keurboomstrand	None observed
Cossoninae sp. 1	T2; T3; T11; T29; T31; T37; T38	9	*Scolopia mundii* (Eckl. & Zeyh.) Warb	Oubos Forest	None observed
Cossoninae sp. 2	T5	2	*Virgilia oroboides*(P. J. Bergius) Salter	HPNBG	None observed
Cossoninae sp. 3	T7	1	*Nuxia floribunda* Benth.	Groenkop Forest	None observed
Cryphalinae sp. 1	N2	200	*Virgilia oroboides*	HPNBG	*Elattoma* sp 1 Mahunka (M6)
***Cryphalus* sp. 1** Erichson	T20	2	Unknown sp. 2	Gouldveld Forest	None observed
Ctonoxylon sp. 1 Hagedorn	T8A;T12, T21; T25; T27; T32,	>350	*Gonioma kamassi (*E. Mey.) *Maytenus acuminata*(L.f.) Loes *Olea*	Gouldveld Forest	None observed

			capensis ssp. *macrocarpa* L.		
Ctonoxylon* sp. 2 *Hagedorn	T28	>300	*Olea* sp.	Gouna Forest	None observed
Hapalogenius fuscipennis Chapuis	N3	>2000	*Virgilia divaricata* Adamson	Storms River	*Elattoma* sp 1(M7)
Hylastes angustatus Herbst	T19	11	*Pinus* sp. L.	Knysna	None observed
Hylurgus ligniperda Fabricius	T16	10	*Pinus* sp. L.	Knysna	None observed
***Hypothenemus* sp**. 1 Westwood	T6	2	Unknown sp. 1	KNR	None observed
Hypothenemus sp. 2 Westwood	T14; T15; T18; T27A; T33	27	*Halleria lucida* L *Maytenus acuminata* *Olinia ventosa* L. Cuford *Rapanea melanophloeos* .	HPNBG	None observed
Lanurgus sp. 1 Eggers	T1; T8; T13; T22; T26; T30; T34; T39	>2000	*Gonioma kamassi* *Olea capensis* ssp. *macrocarpa*	Gouldveld Forest	*Dendrolaelaps quadrisetus* Berlese (M23) *Elattoma* sp. 1 (M34)

Lanurgus sp. 2 Eggers	T23	2	*Rhoicissus tomentosa*	Keurboomstrand	None observed
***Liparthrum* sp.** Knotek	N1	700	*Virgilia oroboides*	SNR	None observed
Orthotomicus erosus Wollaston	T17; T35	>100	*Pinus* sp.	J. Marais Park	*Dendrolaelaps quadrisetus* (M21)
				Knysna	*Elattoma* sp. 2 (M22)
					Histiogaster sp. 3 Berlese (M20)
Platypodinae sp. 1	T43	3	*Rapanea melanophloeos* Mez	Gouldveld Forest	None observed
Xyleborinus aemulus Wollaston	T9, T10, T41	16	*Cunonia capensis* L.		None observed
			Rapanea melanophloeos	Groenkop; HPNBG	
Xyleborinus saxesenii Ratzeburg	T4; T42	117; 5	*Gonioma kamassi*, *Metrosideros*	Assegaaibos;	None observed
			Angustifolia (L.) S.	Gouna Forest	

Figure 3. A selection of sub-cortical beetle species associated with trees in the Cape Floristic Region. A = Cossoninae sp 1, B = *Ctonoxylon* sp. 1, C = *Ctonoxylon* sp. 2, D = *Hylastes angustatus*, E = *Hylurgus ligniperda*, F = *Hypothenemus* sp. 2, G = *Orthotomicus erosus*, H = *Xyleborinus saxesenii*

Table 2: Mites associated with wounds on storm-damaged Afromontane forest trees (HPNBG = Harold Porter National Botanical Garden; KNR = Kolgenberg Nature Reserve; KNBG = Kirstenbosch National Botanical Garden)

Mite species	**Reference number**	**Host**	**Site**
Acaridae sp. 1	M11; M12	*Rapanea melanophloeos*	Groenkop Forest
			Gouldveld Forest
Acaridae sp. 2	M15	*Rapanea melanophloeos*	HPNBG
Dendrolaelaps sp. 1 Halbert	M32	*Trichocladus crinitus* (Thunb.) Pers	Gouna Forest
Histiogaster sp. 1 Berlese	M24	*Olea capensis* ssp. *macrocarpa*	Groenkop Forest
Histiogaster sp .2 Berlese	M30	Climber sp. 2	KNBG
Lasioseius sp. 1 Berlese	M19; T31; T33	*Curtisia dentata* C.A. Sm.	Gouna Forest
		Olea capensis ssp. *macrocarpa*, Climber sp. 1	KNBG
			Gouldveld Forest
Mesostigmata sp. 1	M13; M18	*Curtisia dentata*	Groenkop Forest
		Rapanea melanophloeos	Gouna Forest
Oribatida sp. 1	M16; M27	*Brabejum stellatifolium* L.	Knysna
		Rapanea melanophloeos	Gouna Forest
		Trichocladus crinitus	Assegaaibosch
Oribatida sp. 2	M17; M28	*Curtisia dentata*	Gouna Forest
		Platylophus trifoliatus	Oubos Forest
Paraleius sp. 1 Berlese	M1; M8	*Halleria lucida*	Gouna Forest
		Rapanea melanophloeos	HPNBG

Paraleius sp. 2	M2	Unknown sp. 1	KNR
Proctolaelaps sp. 1	M25; M29	*Olea capensis* ssp. *Macrocarpa*	Groenkop Forest
Berlese			Gouldveld Forest
Proctolaelaps sp. 2 Berlese	M26	*Trichocladus crinitus*	Gouna Forest
Tetranychidae sp. 1	M14	*Rapanea melanophloeos*	KNBG
Trichouropoda sp. 1 Berlese	M3; M4	*Podalyria sericea* (Andrews) R.Br. ex Aiton f.	KNBG
		Virgilia oroboides	
Trichouropoda sp. 2	M10	*Rapanea melanophloeos*	HPNBG
Berlese			
	M9	*Rapanea melanophloeos*	Groenkop Forest
Uroobovella sp. 1 Berlese			
Unidentified sp.	M5	*Virgilia oroboides*	KNBG

5.3.3. Fungi and their host trees

In total, 18 species of ophiostomatoid fungi belonging to five different genera (*Ophiostoma*, *Graphilbum*, *Raffaelea*, *Ceratocystiopsis* and *Graphium*) were collected from 15 different host tree species, including the non-natives *Pinus* sp. and *Acacia mearnsii* (Table 3). The highest number of ophiostomatoid fungal species was collected from *R. melanophloeos* (Table 3). In addition to fungi collected in previous studies (Chapters 2, 3 & 4), six more distinct Operational Taxonomic Units (OTU's) were isolated from diverse hosts in different areas. Blasts searches of their sequences and comparisons with sequences in the GenBank confirmed the identity of some of these species and also confirmed that some are most likely undescribed species. An unknown fungus, identified as *Ophiostoma pluriannulatum*-like, was collected from several native hosts, including *R. melanophloeos*, *O. capensis* ssp. *macrocarpa*, *Curtisia dentata*, *Pterocelastrus* sp., as well as from the non-native *Acacia mearnsii*.

Ophiostoma quercus was also isolated from a variety of native hosts and from *A. mearnsii* (Table 3). Similarly, *Sporothrix fusiforme* was also isolated from both a native host (*Brabejum stellatifolium*) and an exotic host (*A. mearnsii*). Three fungal species were exclusively associated with an exotic *Pinus* species, namely *Ophiostoma ips*, a *Sporothrix* sp. believed to be an undescribed species and *Ceratocystiopsis* sp. 1 that is also mostly likely undescribed based on comparisons with sequences available on GenBank (Table 3).

5.3.4. Arthropod associates

In total, seven ophiostomatoid fungal species were associated with seven species of sub-cortical beetles. Three of the fungal species were associated with *Pinus* sp. and the rest were from two native trees hosts *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* (Table 3). Reproductive propagules of *O. ips* were carried by all three of the beetle species (*O. erosus*, *H. ligniperda* and *H. angustatus*) associated with the *Pinus* species. The two undescribed fungal species (*Sporothrix* sp. and *Ceratocystiopsis* sp.) from the *Pinus* sp. were also associated with *O. erosus* (Table 3). *Sporothrix pallida* was associated with two beetle species, *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 from *O. capensis* ssp. *macrocarpa*, while *S. aemuluphilus* was collected from *X. aemulus* infesting *R. melanophloeos*. *Raffaelea rapanea* and *R. scabbardiae* were associated with Platypodinae sp. 1 from *R. melanophloeos* and *Lanurgus* sp. 1 from *O. capensis* ssp. *macrocarpa*, respectively. The highest mean colony forming unit (CFU) among the beetle-associated *Ophiostoma* species was *S. pallida* (CFU = 10.4), which was associated with the bark beetle *Ctonoxylon* sp. 1. The lowest mean CFU among beetle-associated *Ophiostoma* species was *O. ips* (CFU = 2.8) associated with *H. angustatus* (Table 3). Isolation frequency was the highest for the Platypodinae beetle, of which only three individuals were collected, while isolation frequency was the lowest for *Orthotomicus erosus*.

Several ophiostomatoid fungal species were associated with mite species from 12 different genera (Table 3), including phoretic mite species (*D. quadrisetus*, *Histiogaster* sp. 3, *Elattoma* sp. 1 & 2). In almost all cases, the same fungal species as

that from the host beetle was also isolated from their phoretic mites. For example, *S. pallida* was isolated from both the host beetle (*Lanurgus* sp. 1) associated with *O. capensis* ssp. *macrocarpa* and its phoretic mite *D. quadrisetus*. Interestingly though, three other fungal species from pines (*O. ips*, *Ceratocystiopsis* sp. 1 and *Sporothrix* sp. 1) were also associated with *D. quadrisetus* and its host beetle, *O. erosus*. Some of the fungal-associates were found to be associated with more than one mite species, indicating that the fungi were not specific to one mite-associate only (Table 3). Among the mite-associated *Ophiostoma* species the highest mean CFU was also *S. pallida* (CFU = 10.8), which was associated with the phoretic mite *D. quadrisetus* from the host tree *O. capensis* ssp. *macrocarpa.* The undescribed *Sporothrix* sp. that was cultured from the phoretic mite *D. quadrisetus* from the host tree *Pinus* sp., had the lowest mean CFU (CFU = 1.6) (Table 3). The highest isolation frequency was recorded for *D. quadrisetus* from *O. capensis* ssp. *macrocarpa* and the lowest for *Histiogaster* sp. 3 and *Elattoma* sp. 2 (Table 3).

Table 3: Ophiostomatoid fungi associated with different host trees in Afromontane forests of the CFR, their isolation frequency (F), mean number of colony forming units (CFU'S), and association with sub-cortical beetles and mites.

	GeneBank accession number										
Fungal species	**ITS**	**Bt**	**EF**	**LSU**	**CAL**	**Host plant**	**Arthropod species (B = beetle, M = mite)**	**N**	**F (%)**	**CFUs**	**Site**
Sporothrix sp. 1 Hektoen &Perkins	21	21	NA	NA	21	*Pinus* sp.	*Othortomicus erosus (B)*	50	16	(2-5) 3.5	JS Marais Park
		T222	NA	NA	T222	*Pinus* sp.	*Dendrolaelaps quadrisetus* (M)	20	15	(1-3) 1.6	JS Marais Park
	T223	T223	NA	NA	T223	*Pinus* sp.					JS Marais Park
Sporothrix fusiforme Aghayeva & M. J. Wingf.	22	22	NA	NA	22	*Acacia mearnsii*	Oribatida sp. 1 (M)	19	11	(1-5) 3	Assegaaibosch
	24	24	NA	NA	24	*Brabejum stellatifolium*					Assegaaibosch
	T177	T177	NA	NA	T177	*Acacia mearnsii*					Assegaaibosch
Graphium ilexiense Musvuugwa, LL. Dreyer & F. Roets	33	NA		NA	NA	*Ilex mitis*					Assegaaibosch
	T202	NA	T202	NA	NA	*Ilex mitis*					Assegaaibosch
	T203	NA		NA	NA	*Ilex mitis*					Assegaaibosch
Sporothrix aemuluphilus Musvuugwa, LL. Dreyer & F. Roets	38	38	NA	NA		*Rapanea melanophloeos*	*Xyleborinus aemulus* (B)	6	67	(3-11) 6.25	HPNBG
		T277	NA	NA	T277	*Rapanea melanophloeos*	*Paraleius* sp. 1 (M)	12	17	(3-10) 6.5	HPNBG
		T278	NA	NA		*Rapanea melanophloeos*	*Trichouropoda* sp. 2 (M)	50	34	(4-16) 8.5	HPNBG
Sporothrix reniformis Musvuugwa, LL. Dreyer & F. Roets	39	39	NA	NA		*Rapanea melanophloeos*					HPNBG
	T197	T197	NA	NA		*Rapanea melanophloeos*					HPNBG
	NO	T198	NA	NA		*Rapanea melanophloeos*					HPNBG

Ophiostoma stenoceras (Robak) Nannf.	T1	T1	NA	NA	T1	*Rapanea melanophloeos*					Weza Forest KZN
	T10	T10	NA	NA	T10	*Rapanea melanophloeos*					Weza Forest KZN
		T187	NA	NA	T187	*Rapanea melanophloeos*					Weza Forest KZN
Ophiostoma pluriannulatum (Hedgc.) Syd. -like	T102		NA	NA	NA	*Olea capensis*					
	T33		NA	NA	NA	*Pterocelastrus* sp.					
	T41	T41	NA	NA	NA	*Rapanea melanophloeos*					
	T50		NA	NA	NA	*Olea capensis*	Acaridae sp. 1 (M)	15	33	(3-10) 5.5	Storms River
	T51		NA	NA	NA	*Pterocelastrus* sp					Gouna Forest
	T56	T56	NA	NA	NA	Rapanea *melanophloeos*					Gouldveld Forest
	T63		NA	NA	NA	*Curtisia dentata*	*Mesostigmata* sp. 1 (M)	8	25	(4-9) 7	Gouldveld Forest
	T63b		NA	NA	NA	*Curtisia dentata*					Gouna Forest
	T64		NA	NA	NA	*Rapanea melanophloeos*					Groenkop Forest
	T64b		NA	NA	NA	*Rapanea melanophloeos*	*Uroobovella* sp. 1 (M)	15	20	(3-9) 5.3	Gouna Forest
	T77		NA	NA	NA	*Acacia mearnsii*					Gouna Forest
	T78	T78	NA	NA	NA	*Rapanea melanophloeos*					Groenkop Forest
	T99	T99	NA	NA	NA	*Olea capensis*					Groenkop Forest
											Knysna
											Gouna Forest
											Knysna
Sporothrix noisomeae Musvuugwa, LL. Dreyer &F. Roets	T3	T3	NA	NA	-	*Rapanea melanophloeos*					Weza KZN
	T11	T11	NA	NA	T11	*Rapanea melanophloeos*					Weza KZN
	T12		NA	NA	T12	*Rapanea melanophloeos*					Weza KZN
	T13	T13	NA	NA	T13	*Rapanea melanophloeos*					Weza KZN
Sporothrix lunateae Musvuugwa, LL. Dreyer & F. Roets	T124	T124	NA	NA	T124	*Rapanea melanophloeos*					Weza KZN
	T125	T125	NA	NA	T125	*Rapanea melanophloeos*					Weza KZN
	T6	T6	NA	NA	T6	*Rapanea melanophloeos*					Weza KZN
Ceratocystiopsis sp.1 Upadhyay & Kendrick	T142	T142	NA	T142	NA	*Pinus* sp.	*D. quadrisetus* (M)	20	10	(1-4) 2.5	Kysna
	T143	T143	NA	T143	NA	*Pinus* sp.	*O. erusus* (B)	50	14	(2-6) 3.2	Kysna
	T97	T97	NA	T97	NA	*Pinus sp.*					Knysna
Sporothrix rapaneae Musvuugwa, LL. Dreyer & F. Roets		T148	NA	NA	T148	*Rapanea melanophloeos*					Groenkop Forest
	T149	T149	NA	NA		*Rapanea melanophloeos*					Groenkop Forest
	T60	T60	NA	NA	T60	*Rapanea melanophloeos*					Groenkop Forest

Sporothrix capensis Musvuugwa, LL. Dreyer & F. Roets	T206	T206	NA	NA		*Olea capensis*					Goudeveld Forest
	-	T207	NA	NA		*Olea capensis*					Goudeveld Forest
	T39	T39	NA	NA		*Olea capensis*					Gouldveld Forest
Ophiostoma stenoceras	T25	T25	NA	NA	T25	*Virgilia oroboides*					KNBG
	T252	T251	NA	NA	T252	*Virgilia oroboides*					KNBG
	T253	T253	NA	NA		*Virgilia oroboides*					KNBG
Raffaelea scabbardiae Musvuugwa, LL. Dreyer & F. Roets	T28	T28	NA	T28	NA	*Olea capensis*					Goudeveld Forest
	T32	T32	NA	T32	NA	*Olea capensis*	*Lanurgus* sp. 1				Gouldveld Forest
	T43	T43	NA	T43	NA	*Olea capensis*		50	59	(6-17) 9.2	Gouldveld Forest
	T44	T44	NA	-	NA	*Olea capensis*					Gouldveld Forest
Sporothrix pallida (Tubaki) Matsushima	T35	T35	NA	NA		*Olea capensis*	*Cytonoxylon* sp. 1 (B)	25	60	(5-19) 10.4	
	T57	T57	NA	NA							
	T76	T7	NA	NA	T76	*Olea capensis*	*Lanurgus* sp. 1 (B)	50	68	(8-27) 16.4	Goudeveld Forest
							D. quadrisetus (M)	50	64	(5-24) 10.8	
							Elattoma sp. 1 (M)	20	45	(3-15) 10	Groenkop Forest
											Gouldveld Forest
Ophiostoma ips (Rumbold) Nannf.	T241	T241	T241	NA	NA	*Pinus* sp.	*O. erosus* (B)	50	58	(1-12) 4.4	Knysna
	T98	T98	T98	NA	NA	*Pinus* sp.	*H. ligniperda* (B)	10	40	(1-9) 4.4	Knysna
	T96	T96	T96	NA	NA	*Pinus* sp.	*H. angustatus* (B)	11	45	(1-6) 2.8	Knysna
							D. quadrisetus (M)	20	25	(1-7) 4.1	
							Histiogaster sp. 3 (M)	15	6	2	
							Elattoma sp. 2 (M)	30	6	(2-4) 3	

Ophiostoma quercus (Georgévitch)	T47	T47		NA	NA	*T. crinitus*	Oribatida sp. 1 (M)	30	30	(2-10) 7.3	Gouna Forest
Nannf.	T58			NA	NA	*R. melanophloeos*					Groenkop Forest
	T61	T61	T61	NA	NA	*R. melanophloeos*					Groenkop Forest
	T62	T62	T62	NA	NA	*Olea capensis*					Gouldveld Forest
	T65	T65		NA	NA	*T. crinituss*	*Proctolaelaps* sp. 2 (M)	17	29	(4-11) 6.4 (4-	Gouna Forest
	T66		T66	NA	NA	*Curtisia dentata*	Oribatida sp. 2 (M)	50	44	15) 7.6	Gouna Forest
	T67			NA	NA	*Acacia mearnsii*					Knysna
	T69			NA	NA	*Curtisia dentata*					Gouna Forest
	T70	T70		NA	NA	*T. crinitus*	*Dendrolaelaps* sp. 1 (M)	9	56	(3-12) 7.2	Gouna Forest
Graphilbum roseus Musvuugwa, LL.	T46		NA	T46	NA	*Curtisia dentata*	Oribatida sp. 2 (M)	50	38	(4-10) 6.4	Gouna Forest
Dreyer & F. Roets	T72		NA	T72	NA	*Curtisia dentata*	*Lasioseius* sp. 1 (M)	12	28	(2-7) 4.1	Gouna Forest
	T79		NA	T79	NA	*R. melanophloeos*					Groenkop Forest
	T81		NA		NA	*Curtisia dentata*	Mesostigmata sp. 1 (M)	6	50	(3-7) 5.3	Gouna Forest
	T82		NA	T82	NA	*Halleria lucida*					Gouna Forest
Raffaelea rapaneae Musvuugwa, LL.	T92	T92	NA		NA	*R. melanophloeos*					Gouna Forest
Dreyer & F. Roets	T93	T93	NA		NA	*R. melanophloeos*	Platypodinae sp. 1 (B)	1	100	5	Gouna Forest
	T110	T110	NA		NA	*R. melanophloeos*					Gouna Forest

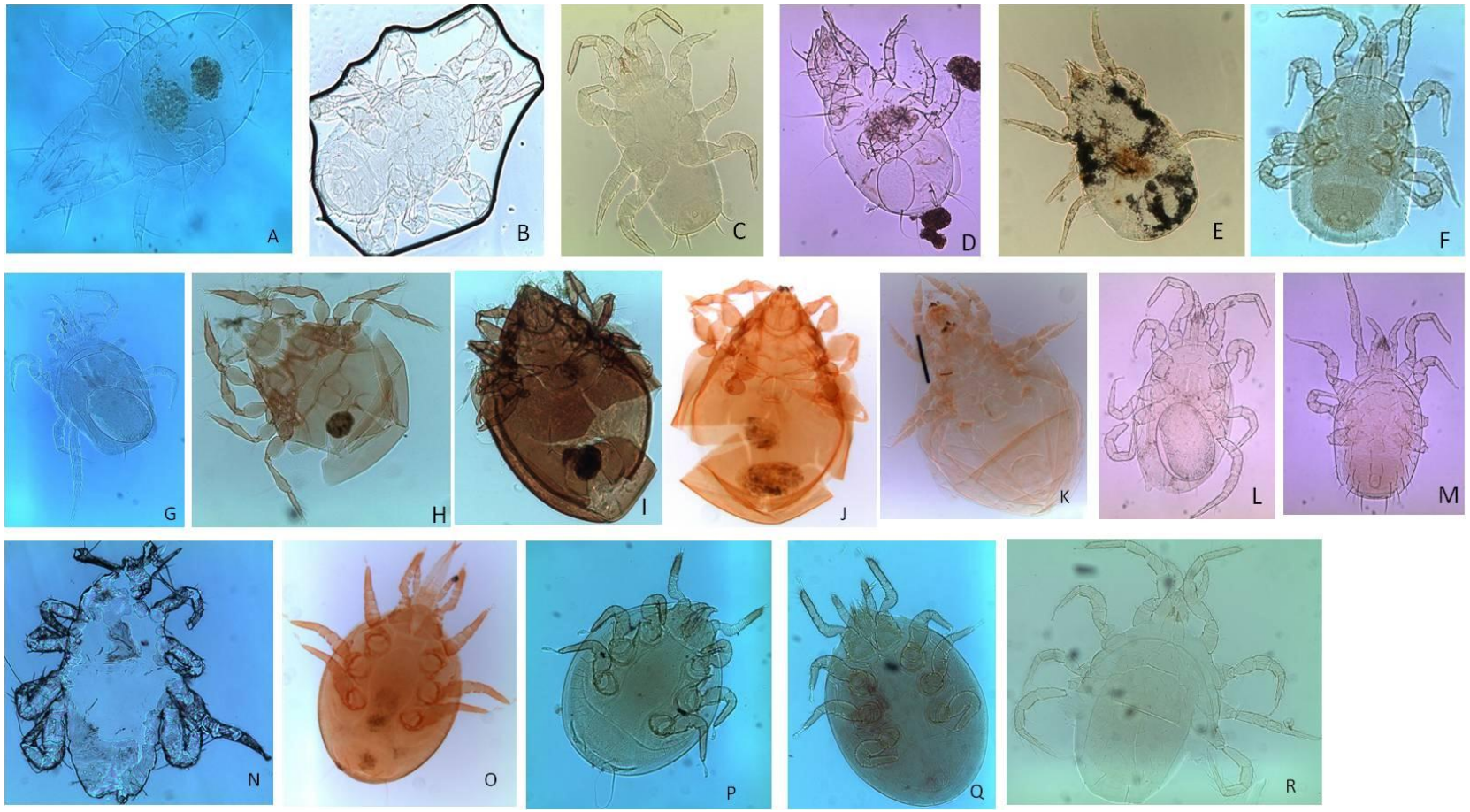

Figure 4. Mites associated with wounds on trees in the Cape Floristic Region, A = Acaridae sp. 1; B = Acaridae sp. 2; C = *Dendrolaelaps* sp.1; D = *Histiogaster* sp.1; E= *Histiogaster* sp. 2; F = *Lasioseius* sp.1; G = *Mesostigmata* sp. 1; H = Oribatida sp. 1; I = Oribatida sp. 2; J = *Paraleius* sp. 1; K = *Paraleius* sp.2; L = *Proctalaelaps* sp. 1; M = *Proctalaelaps* sp. 2; N = Tetranychidae sp. 1; O = *Trichouropoda* sp. 1; P = *Trichouropoda* sp. 2; Q = *Uroobovella* sp. 1; R = Unknown sp. 1

5.4. Discussion

This study represents one of only a few studies on the ecology and biodiversity of ophiostomatoid fungi associated with native trees in the Afromontane forests of South Africa. Results from this study indicate that these Afromontane forests are home to a high diversity of ophiostomatoid fungi. Fifteen species were isolated from only nine native hosts. Many of these were found to be fairly specific towards their hosts. Considering that there are more than 50 tree species in these forests, it is reasonable to assume that the diversity of these fungi is still poorly documented. Many taxa probably still await discovery. Except for the new *Graphium* species, all fungal taxa collected here belong to the Ophiostomatales (De Beer *et al*. 2013). These genera have largely been overlooked in previous studies in the CFR, as the emphasis of these studies was on pathogenic species in the Microascales, especially in the genus *Ceratocystis* (Kamgan *et al*. 2008). Our results therefore provide an important first step towards uncovering the diversity and importance of fungi in the Ophiostomatales from CFR forests.

Although ophiostomatoid fungi have been studied best in plantation forestry trees (e.g. *Pinus* spp.) in South Africa, new taxa are still being discovered on forestry trees that have escaped to native vegetation. We isolated a *Ceratocystiopsis* species that is probably new, and a new *Sporothrix* species from pines. Both of these were associated with the well-known forestry pest *O. erosus* and its phoretic mite *D. quadrisetus*. The origin of these fungal taxa remains uncertain, but it is likely that they were introduced from outside of South Africa. This is especially true for the *Ceratocystiopsis* species, as most of the members of this genus are only known as pine-associates (Zhou *et al*. 2004; 2001; Hsiau & Harrington 1997) and no *Pinus* species are native to the African continent. *Orthotomicus erosus* is known as an associate of pines in Europe, the Mediterranean region and the Middle East (Ferreira & Ferreira 1986). *Dendrolaelaps quadrisetus* is most likely of European and North Amerian origin, as it has been recorded in Europe in a number of studies, and has been found to be especially associated with bark beetles such as *Dendroctonus rufipennis* (Fernandez *et al*. 2013; Gwiazdowicz *et al.* 2012; Gwiazdowicz et *al.* 2011; Cardoza *et al*. 2008).

In total, 10 ophiostomatoid species were collected from *Rapanea melanophloeos*, by far the most collected from any host. Most of these species were associated with wounds caused by storm damage. The pathogenicity of these fungi towards this host was not determined in this

study, but recent studies showed it to be very vulnerable towards some of these fungal taxa (Chen *et al*. 2013, Kamgan *et al*. 2008). The thick, sap-rich bark of this tree represents an ideal habitat for many wound infecting fungal taxa. It is considered a forest pioneer with a relatively short life-span which may be partially explained by this elevated susceptibility to fungal attack. Testing of the pathogenicity of the fungi associated with *R. melanophloeos* should be prioritized in future studies.

Three fungal species were collected from both native and exotic hosts. *Ophiostma quercus* and the *O. pluriannulatum* -like fungus were collected from several native hosts and from exotic *A. mearnsii*, while *Sporothrix fusiforme* was isolated from *B. stellatifolium* and from *A. mearnsii* that was growing in close proximity to the other hosts. *Ophiostoma quercus* is a well-known fungus in both the Southern and Northern Hemispheres, and is known to colonise many hardwood species (De Beer *et al*., 2003; Harrington *et al*., 2001). It was therefore not surprising to have collected it from several native hosts as well as from *A. mearnsii*. Previously in South Africa, it has also been isolated from *Terminalia sericea* (Roxb.) Wight & Arn., *Olinia* sp. Thunberg. *Quercus robur* L., *Virgilia oroboides* and the exotic *Eucalyptus grandis* W.Hill ex Maiden (Kamgan *et al.* 2008; De Beer *et al*. 1995). *Ophiostoma pluriannulatum* belongs to the *Ophiostoma pluriannulatum* complex, along with several other species (De Beer *et al*. 2013). It has previously been isolated widely from other hardwood and conifer hosts in North America (Appel *et al*. 1990), Japan (Aoshima 1965), Europe (Romón *et al*. 2007) and South Africa Zhou *et al*. 2006) although it was mostly based on morphology and a single genetic marker (ITS). The taxon collected in this study may therefore represent a distinct species. *Sporothrix fusiforme* was first described from *Populus nigra* L., *Castanea sativa* Mill., *Quercus petraea* (Mattuschka) Liebl and *Larix decidua* Mill from Azerbaijan and Austria (Aghayeva *et al*. 2004). Increasing globalisation of trade in plants, as well as flaws in the international protocols of plant biosecurity, has led to increased events of organisms utilising newly encountered hosts (Brasier & Webber 2010) and may have led to the isolation of this fungus in South Africa. It is not known whether *S. fusiforme* is pathogenic towards its hosts in its native ranges. However, its movement to CFR native hosts may be problematic, even if it is not pathogenic to these newly encountered hosts, as it may be able to outcompete native fungi and disrupt normal ecosystem processes. Also, the dispersal ecology of this fungus is unknown, but may include vectoring by mites, as it was also isolated from an Oribatidae mite species in the present study.

Although most of the fungal species collected from native hosts were only specific to one native host, a couple of wound-associated fungal species were found on more than one native host. These were *S. pallida*, *Graphilbum roseus*, *O. quercus* and the *O. pluriannulatum*- like fungus. Interestingly, all of these species were also associated with several mite species. This may explain why they were found on several host trees, as their associations with mites may aid their transportation between host species. *Ophiostoma pluriannulatum* has previously been isolated from several wood- and bark-infesting insects, including bark beetles and nitidulids (Romón *et al*. 2007; Zhou *et al*. 2006; 2004; Appel *et al*. 1990). However, since the fungus was identified only morphologically or through the use of a single genetic marker (ITS), the insect–fungus interactions of this species cannot be confirmed with confidence (Zanzot *et al*. 2010). *Sporothrix pallida* was also isolated from the beetles *Ctonoxylon* sp. 1 and *Lanurgus* sp. 1 (Musvuugwa *et al*. 2013, Chapter 2), a host to the mite species (*D. quadrisetus*) that also carried this fungus. As highlighted before (Musvuugwa *et al*. 2013; Chapter 2), this is the first report of *S. pallida* forming associations with Scolytinae beetles. We are also unaware of any other reports of associations between this fungus and any mite species.

The beetle-associated fungi were fairly host and vector specific compared to the wound-associated fungi. Of the seven beetles associated with ophiostomatoid fungi, five were associated with a single fungal species. This difference in host specificity between wound associated-fungi and beetle-associated fungi may again be attributed to the mites associated with the wound-associated fungi. In some cases more than one mite species were associated with a single fungal species. This suggests that the fungi may be able to rely on many taxa for dispersal to other hosts. Although not tested, these wound-associated fungi may also be palatable to the mite species associated with them.

Including data from Musvuugwa *et al*. 2013 (Chapter 2), many arthropods have been identified as associates of *ca*. 50% of the fungal species collected. Beetles and mites are well-known associates of ophiostomatoid fungi (Hofstetter *et al*. 2013), and in some cases they share mutualistic relationships (Harrington 2005; Kirisits 2004; Six 2003; Moser *et al*. 1995; Moser 1985; Whitney 1982). The southern pine beetle *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae), its phoretic mites and their associate fungi provide a very good example of such mutualistic relationships (Klepzig *et al*. 2001b; Price *et al*. 1992; Smiley & Moser 1974). The arthropods vector *Ophiostoma minus* (Hedgcock) H. & P. Sydow, and in turn benefit by feeding and reproducing on the fungal hyphal growth (Klepzig *et al*. 2001b).

There is a distinct possibility that some of the fungal taxa collected in this study may also have mutualistic relationships with beetles and/or mites they are associated with. The exact nature of these associations still needs to be determined, and may provide information on why some fungal species seem to be host specific, while others have wide host ranges.

Four phoretic mite species were collected in this study. Interestingly the phoretic mite *D. quadrisetus* was phoretic on two different beetle species, *Lanurgus* sp. 1 from the native *O. capensis* ssp. *macrocarpa* and *O. erosus* from the exotic *Pinus* species. As explained earlier, both *D. quadrisetus* and *O. erosus* are not-native to southern Africa. When arthropods such as conifer-infesting bark beetles are introduced into new habitats, they are thought to carry associated fungi (and their phoretic mites and/or other associated biotic agents) (Bridges & Moser 1983; Brasier 1978). Therefore, these taxa were likely introduced into South Africa on *Pinus* spp. used in plantation forestry and *D. quadrisetus* was able to move from its natural associates (both plant and beetle) to form a new association with *Lanurgus* sp. 1 that is only known from native trees. While it has been demonstrated that when introduced bark beetles come into contact with native bark beetles, their associated organisms may form new relationships with the new host beetle, this phenomenon is known for some fairly closely related host taxa only (Wingfield *et al*. 2013). Also, *D. quadrisetus* is a predator that feeds on eggs and larvae of its phoront beetle (Kinn 1967) and also in some cases feeds on nematodes. It is possible that the egg and larvae of *Lanurgus* sp. 1 beetle might be palatable to *D. quadrisetus* or that the beetle actually carries some nematodes that are palatable to the mite. This could have facilitated the movement of this mite from *O. erosus* to *Lanurgus sp*. 1. These associations merit more focussed studies.

5.5. Conclusion

This study reported a very high diversity of ophiostomatoid fungi, sub-cortical beetles and mites associated with trees in the Afromontane forests of the CFR. Some of the fungal species were arthropod-associated and most of the beetle-associated species were only specific to one host and one vector. However, several other fungal taxa, and a few of their associated arthropods, were not specific towards any particular host, including some non-native taxa. Many ophiostomatoid fungi are virulent pathogens to their hosts and rely on their associated organisms for dispersal. This study therefore highlights the need for future studies

to unravel the nature of these associations so that potential ecological interferences from exotic organisms can be identified and hopefully controlled.

References

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W. and Kirisits, T. 2004. Two new *phiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* 96: 866–878.

Aoshima, K. 1965. Studies on wood-staining fungi of Japan. [In Japanese with English summary]. PhD thesis, University of Tokyo, Tokyo.

Appel, D.N., Kurdyla, T., and Lewis, J.R. 1990. Nitidulids as vectors of the oak wilt fungus and other *Ceratocystis* spp. in Texas. *European Journal of Plant Pathology* 20: 412–417.

Baker, J.M. and Norris, D.M. 1968. A complex of fungi mutualistically involved in the nutrition of the ambrosia beetle *Xeleborus ferrugineus*. *Journal of Invertebrate Pathology* 11: 246–250.

Barras, S.J. and Perry, T.J. 1972. Fungal symbionts in the prothoracic mycangium of *Dendrocfonus frontalis*. *Zeitschrift fiir angwandefe Enfomologie* 71: 95-104.

Batra, L.R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science* 66: 213-236.

Brasier, C.M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57: 792–808.

Brasier, C.M. 1978. Mites and reproduction in *Ceratocystis ulmi* and other fungi. *Transactions of the British Mycological Society* 70: 81-89.

Brasier, C. and Webber, J. 2010. Sudden larch death. An aggressive and unpredictable fungal pathogen is devastating larch plantations in Britain. Its remarkably broad host range, and the possibility of further geographical spread, give heightened cause for concern. *Nature* 466: 824-825.

Brasier, C.M. & Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Bridges, J.R. and Moser, J.C. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* 8: 9–12.

Cardoza, Y.J, Moser, J.C., Klepzig, K.D. and Raffa, K.F. 2008. Multipartite symbioses among fungi, mites, nematodes, and the spruce beetle, *Dendroctonus rufipennis*. *Environmental Entomology* 37: 956-963.

Castello, J.D., Leopold, D.J. and Smallidge, P.J. 1995. Pathogens, patterns and processes in Forest Ecosystems. *BioScience* 45: 16-24.

Cease, K.R. and Juzwik, J. 2001. Predominant nitidulid species (Coleoptera: Nitidulidae) associated with spring oak wilt mats in Minnesota. *Canadian Journal of Forest Research* 31:635–643.

Chen, S.F., Wingfield, M.J., Roets, F., and Roux, J. 2013. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea* in South Africa. *Plant Pathology* 62: 667-678.

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., de Beer, Z.W., Wingfield, M.J., Hawksworth, D.L., 2006. How many species of fungi are there at the tip of Africa? *Studies in Mycology* 55: 13–33.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013. A nomenclature for ophiostomatoid genera and species in the *Ophiostomatales* and *Microascales*. In : *Ophiostomatoid fungi: expanding frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.). CBS Biodiversity Series 12, pp. 245-322.

De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2003. The *Ophiostoma piceae* complex in the southern hemisphere: a phylogenetic study. *Mycological Research* 107: 469-476.

De Beer, Z.W., Wingfield, M.J. and Kemp, G.H.J. 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* 91: 6.

Doidge, E.M. and Bottomley, A.M. 1931. A revised list of plant diseases in South Africa. *Memoirs of the Botanical Survey of South Africa* 11:1-78.

Eckhardt, L.G., Goyer, R.A. Klepzig, K.D. and Jones, J.P. 2004. Interactions of *Hylastes* species (Coleoptera: Scolytidae) with *Leptographium* species associated with Loblolly pine decline. *Journal of Economic Entomology* 97: 468–474.

Ferna´ndez, M., Diez, J. and Moraza, L.M. 2013. Acarofauna associated with *Ips sexdentatus* in northwest Spain. *Scandinavian Journal of Forest Research* 28: 358-362.

Geldenhuys, C.J. 2010. Distribution, size and ownership of forests in the Southern Cape. *South African Forestry Journal* 158: 51-66.

Geldenhuys, C.J. 1997. Composition and biogeography of forest patches on the inland mountains of the Southern Cape. *Bothalia* 27: 57-74.

Goldblatt, P. and Manning, J. 2000. Cape plants: A conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria, South Africa; Missouri Botanical Garden Press, St. Louis, Missouri, USA.

Gwiazdowicz, D.J., Kamczyc, J., Teodorowicz, E. and Błoszyk, J. 2012. Mite communities (Acari, Mesostigmata) associated with *Ips typographus* (Coleoptera, Scolytidae) in managed and natural Norway spruce stands in Central Europe. *Central European Journal of Biology* 7: 910-916.

Gwiazdowicz, D.J., Kamczyc, J. and Bloszyk, J. 2011. The diversity of phoretic Mesostigmata on *Ips typographus* (Coleoptera: Scolytinae) caught in the Karkonosze forest. *European Journal of Entomology* 108: 489-491.

Harrington, T.C. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: *Insect-Fungal Associations: Ecology and Evolution* (Vega, F.E. and Blackwell, M. eds.), Oxford University Press, New York, pp. 1–22.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* 73: 1123-1129.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D. N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* l104: 399-404.

Harrington, T.C., Farrell, R., Hofstra, D., McNew, D. and Steimel, J. 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* 93: 111-136.

Harrington, T.C. and Wingfield, M.J. 1998. The *Ceratocystis* species on conifers. *Canadian Journal of Botany* 76:1446–1457.

Hausner, G., Reid, J. and Klassen, G.R. 1993. On the subdivision of *Ceratocystis s.l.*, based on partial ribosomal DNA sequences. *Canadian Journal of Botany* 71: 52-63.

Heiniger, U. and Rigling, D. 1994. Biological control of chestnut blight in Europe. *Annual Review of Phytopathology* 32: 581-599.

Heybroek, H.M. 1993. Why bother about the elm? In: *Dutch Elm Disease Research. Cellular and Molecular Approaches*, (Sticklen, M.B. and Sherald, J.L. eds.), Springer-Verlag, New York, pp 1–8.

Hofstetter, R.W. and Moser, J.C. 2014. The role of mites in insect-fungus associations. *Annual Review of Entomology* 59: 537-557.

Hofstetter, R.W., Moser, J.C. and Blomquist, S.R. 2013. Mites associated with bark beetles and their hyperphoretic Ophiostomatoid fungi. In: *Ophiostomatoid Fungi: Expanding Frontiers* (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J., eds). CBS Biodiversity Series 12: 165-174.

Hsiau, P.T.W. and Harrington, T.C. 1997. *Ceratocystiopsis brevicomi* sp. nov., a mycangial fungus from *Dendroctonus brevicomis* (Coleoptera: Scolytidae). *Mycologia* 89: 661-669.

Jacobs, K. and Kirisits, T. 2003. *Ophiostoma kryptum* sp. nov. from *Larix deciduas* and *Picea abies* in Europe, similar to *O. minus*. *Mycological Research* 107: 1231-1242.

Jacobs, K. and Wingfield, M.J. 2001. *Leptographium* species: Tree pathogens, insect associates and agents of blue stain. American Phytopathological Society, St. Paul.

Kamgan Nkuekam, G., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2012a. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533.

Kamgan Nkuekam, G., Wingfield, M.J., Mohammed, C., Carnegie, A.J., Pegg, G.S. and Roux, J. 2012b. *Ceratocystis* species, including two new species associated with nitidulid beetles, on eucalypts in Australia. *Antonie van Leeuwenhoek* 101: 217-241.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Klepzig, K.D and Six, D.L. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis* 37: 189-205.

Klepzig, K.D., Moser, J.C., Lombardero, M.J., Ayres, M.P., Hofstetter, R.W. and Walkinshaw, C.J. 2001a. Mutualism and antagonism: Ecological interactions among bark beetles, mites and fungi. In Biotic Interactions In Plant-Pathogen Associations. (Jeger, M.J. and Spence, N.J. eds) CAB International, New York, U.S.A, pp. 237–267.

Klepzig, K.D., Moser, J.C., Lombardero, F.J, Hofstetter, R.W. and Ayres, M.P. 2001b. Symbiosis and competition: Complex interactions among beetles, fungi and mites. *Symbiosis* 30: 83–96.

Kirisits, T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: *Bark and wood boring insects in living trees in Europe, a synthesis*, (Lieutier, F., Day, K.R., Battistis, A., Gregoire, J.C. and Evans, H.F. eds.), Kluwer, The Netherlands, pp. 181-235.

Lagerberg, T., Lundberg, G and Melin, E. 1927. Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvårdsföreningens Tidskrift* 25: 145-272.

Lamb, R .1979. World without Trees. Wildwood House, London.

Lombardero, M..J., Ayres, M.P., Hofstetter, R.W., Moser, J.C. and Klepzig, K.D. 2003. Strong indirect interactions of *Tarsonemus* mites (Acarina: Tarsonemidae) and *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Oikos* 102: 243–252.

Marais, G.J. and Wingfield, M.J. 2001. *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. *Mycological Research* 105: 240-246.

Marais, G.J. and Wingfield, M.J. 1997. *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. *Canadian Journal of Botany* 75: 362-367.

Marais, G.J. and Wingfield, M.J. 1994. Fungi associated with infructescences of *Protea* species in South Africa, including a new species of *Ophiostoma*. *Mycological Research* 98: 374-396.

Mendel, Z. 1983. Seasonal history of *Orthotomicus erosus* (Coleoptera: Scolytidae) in Israel. *Phytoparasitica* 11: 13-24.

Morgenthal, T.L. and Cilliers, S.S. 2000. Species composition and phytogeographical significance of an afromontane forest fragment in the Mpumalanga province, South Africa. *The Southern African Forestry Journal* 189: 81-102.

Moller, W.J. and Devay, J.E. 1968. Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* 58:1499–1507.

Moser, J.C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. *Transactions of the British Mycological Society* 84: 750–753.

Moser, J.C., Perry, T.J., Bridges, J.R., and Yin, H.-F. 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus*, a mycangial fungus of the southern pine beetle. *Mycologia* 87: 84-86.

Myers, N., Mittelmeier, R.A., Mittelmeier, C.G., da Fonseca, G.A.B. & Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Paine, T.D. and Birch, M.C. 1983. Acquisition and maintenance of mycangial fungi by *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae). *Environmental Entomology* 12: 1384-1386.

Pipe, N.D., Brasier, C.M. and Buck, K.W. 2000. Evolutionary relationships of the Dutch elm disease fungus *Ophiostoma novo-ulmi* to other *Ophiostoma* species investigated by restriction fragment length polymorphism analysis of the rDNA region. *Journal of Phytopathology* 148:533–539.

Price, T.S., Doggett, C., Pye, J.L. and Holmes, T.P. 1992. A history of southern pine beetle outbreaks in the southeastern United States. Sponsored by the Souhtern Forest Insect Workshop Conference. The Georgia Forestry Commission, Macon, GA, p. 65.

Ribeiro, I.J.A. 1980 Seca da mangueira. Agentes causais e estudo da molestia. In: *Anais do I Simposio Brasiliero Sobre a Cultura de Mangueira.* Sociedade Brasileira de Fruticultura, Jaboticabal, pp. . 123–130.

Redfern, B.D., Minter, D.W., Stoakley, J.T. and Steele. H. 1987. Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* 36:467–480.

Roets, F., De Beer, Z.W., Wingfield, M.J. Crous, P.W and Dreyer, L.L. 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* 100: 496-510.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Roets, F., De Beer, Z. W., Dreyer, L.L., Zipfel, R., Crous, P.W. and Wingfield, M.J. 2006. Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. *Studies in Mycology* 55: 199-212.

Romón, P., Zhou, X., Iturrondobeitia, J.C., Wingfield, M.J. and Goldarazena, A. 2007. *Ophiostoma* species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain. *Canadian Journal of Microbiology* 53:756–767.

Roux, J. and Wingfield, M.J. 1997. Survey and virulence of fungi occurring on diseased *Acacia mearnsii* in South Africa. *Forest Ecology and Management* 99: 327-336.

Seifert, S.A., Wingfield, M.J. and Kendrick, W.B. 1993. A nomenclature for described species of *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis*, *Ceratostomella* and *Sphaeromella*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*, (Wingfield, M.J., Seifert, K.A. and Webber, J., eds.). American Phytopathological Society, St. Paul, Minnesota, U.S.A, pp. 269-287.

Six, D.L. 2003. Bark beetle-fungus symbioses. In: *Insect Symbioses*, (Bourtzis, K. and Miller, T. eds.) CRS press, Boca Raton, FL, USA, pp. 97-114.

Six, D.L. and Paine, T.D. 1998. Effects of mycangial fungi on host tree species progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology* 27: 1393–1401.

Smiley, R.T. & Moser, J.C. 1974. New Tarsonemids associated with bark beetles (Acarina: Tarsonemidae). *Annals of the Entomological Society of America* 69: 713-715.

Spatafora, J.W. and Blackwell, M. 1994. The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* 98: 1-9.

Viegas AP. 1960. Mango blight. *Bragantia* 19: 163–182.

Whitney, H.S. 1982. Relationships in bark beetles and symbiotic organisms. In: *Bark Beetles in North America conifers*, (Mitton, J.B. & Sturgeon, K.B. eds.). University of Texas, Austin, pp. 183-211.

Wingfield, M.J., Roux, J., Wingfield, B.D. and Slippers, B. 2013. *Ceratocystis* and *Ophiostoma*: International spread, new associations and plant health. In : *Ophiostomatoid Fungi: Expanding Frontiers*, (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp 191-200.

Wingfield, M.J., Coetzee, M. P.A., Crous, P.W., Six, D. and Wingfield, B.D. 2010. Fungal phoenix rising from the ashes? *International Mycological Association Fungus* 1: 149–153.

Wingfield, M.J., Harrington, T.C. and Solheim, H. 1997. Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* 75:827–834.

Woolhouse, M.E.J., Haydon, D.T and Antia, R. 2005. Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology and Evolution* 20: 238-244.

Zhou, X.D., De Beer, Z.W. and Wingfield, M.J. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.

Zhou, X.D., De Beer, Z.W., Cibrian, D., Wingfield, B.D. and Wingfield, M.J. 2004. Characterisation of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research* 108:690–698.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* 53: 290-300.

Chapter 6: The danger posed by ophiostomatoid fungi when encountering new hosts

Abstract

The ophiostomatoid fungi (Ophiostomatales) contain many virulent tree pathogens that pose a significant threat to forestry industries worldwide. Increased global trade of plants increases encounters with potential novel hosts, which may not only have devastating ecological consequences, but can also have huge economic implications. In this study we assess the potential danger posed by the movement of ophiostomatoid fungi between different hosts by testing the pathogenicity of five species collected from native South African trees (*Graphilbum roseus*, *O. pluriannulatum*-like, *Raffaelea rapaneae*, *Sporothrix pallida* and *Sporothrix rapaneae*) and three exotic (*Ceratocystiopsis* sp. 1, *Ophiostoma ips* and *Sporothrix* sp. 1) species on three native (*Curtisia dentata*, *Olea capensis* ssp. *Macrocarpa* and *Rapanea melanophloeos*) and three exotic plantation trees (*Acacia mearnsii*, *Eucalyptus grandis* and *Pinus radiata*). We also evaluate the potential for taxa to move to new hosts by testing the strength of their associations with the organisms involved in their dispersal. Results indicate that some of these fungi can be virulent pathogens on distantly-related, newly encountered hosts. For example, *Graphilbum roseus* was pathogenic towards both native (*R. melanophloeos* and *C. dentata*) and exotic (*A. mearnsii* and *E. grandis*) trees, while *O. pluriannulatum*-like was pathogenic to *R. melanophloeos* and *A. mearnsii*. High host specificity of the vectors of beetle-associated species and the specificity of mites dispersed by these beetles makes encounters with new hosts unlikely for many taxa. However, taxa that are associated with numerous mite species and with wounds on a multitude of different native tree taxa (e.g. *Graphilbum* sp. 1 and *O. pluriannulatum*-like) are most likely to encounter new hosts and may pose a threat to the health of these hosts.

Key words: Inoculations, vectors, mites

6.1. Introduction

Ophiostomatoid fungi (Ophiostomatales) are best known as associates of beetles (Coleoptera: Curculionidae, Scolytinae) (Jacobs *et al*. 2003; Jacobs & Wingfield 2001; Harrington 1987). In most cases these associations are mutualistic, whereby the fungi are believed to provide nutritional benefits to the beetles (Klepzig *et al*. 2001) and the beetles aid in transporting fungi to new host plants (Six 2003; Paine *et al*. 1997). However, some members are not associated with scolytine beetles and have been isolated from diverse habitats including wounds on trees, soil, air, other fungi and wood of dead trees (Musvuugwa *et al*. 2013, Chapters 3 & 4; Cruywagen *et. al* 2010; de Meyer *et al*. 2008; Kamgan *et al*. 2008) and may be associated with many other arthropods such as Cerambycidae beetles, mites and nitidulid beetles (Roets *et al*. 2007; Lombardero *et al*. 2003; Jacobs & Wingfield 2001; Klepzig *et al*. 2001; Moser 1985; Bridges & Moser 1983). In fact, all members of the Ophiostomatales are adapted for arthropod mediated dispersal as they produce sticky spores at the tips of elongated structures that are not easily distributed via other mechanisms such as air (Malloch & Blackwell 1993). The dispersal biology of the non-scolytinae taxa are far less well-studied, but it is becoming clear that mites may play central roles in the dispersal of many taxa. Mites are known to have associations with Ophiostomatales associated with many other environments such as the association with taxa from *Protea* flower heads (Roets *et al*. 2007) and wounds on the bark of trees (for example Musvuugwa *et al*. 2013, Chapter 5).

Importantly, mites also play a very important role in the dispersal of some Ophiostomatales associated with Scolytinae beetles (Hofstetter *et al*. 2013). For example, *Tarsonemus ips* Lindquist and *T. krantzii* Smiley & Moser are associated with the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Klepzig *et al*. 2001b; Price *et al*. 1992; Smiley & Moser 1974) and both are responsible for vectoring spores of *Ophiostoma minus* (Hedgcock) H. and P. Sydow (Klepzig *et al*. 2001b). Just as in the case of some beetles that have specialised spore carrying structures (mycangia), mites may have mutualistic relationships with the fungi they carry and some also carry fungi in such structures (sporothecae) (Moser *et al*. 1995; Bridges & Moser 1983). These relationships can be just as specialised as with the beetles, as it has been shown that some mites feed and reproduce exclusively on their fungal associates (Roets *et al*. 2007; Klepzig & Six 2004).

Many ophiostomatoid fungi are well-known pathogens of both native and exotic trees (Six and Wingfield 2011; Klepzig *et al*. 2001). They have caused major financial losses in many

forestry and agricultural sectors. For example, *Raffaelea quercivora* Kubono et Shin. Ito (Ophiostomatales) together with its associate beetle, *Platypus quercivorus* (Murayama) were responsible for the catastrophic mass mortality and die-back of Japanese oak trees (Kubono & Ito 2002) and in agriculture, *Ceratocystis fimbriata* Ellis & Halst. *s.s.* (Microascales) is a well-known pathogen responsible for causing black rot of sweet potatoes and canker on coffee (Baker 2005; Kile 1993), while *C. paradoxa* (Dade) Moreau (Microascales) is responsible for causing the rotting of pawpaw and pineapple leaves and pineapple fruit (Kile 1993). Numerous taxa can also cause substantial financial losses as they contribute to blue-staining of conifer timber (Seifert 1993). For example the blue-stain fungus *Ophiostoma minus* (Hedgcock) H. & P. Sydow and *O. pluriannulatum* (Hedgcock) H. & P. Sydow are known to reduce the commercial value of timber (Harrington 2005; Seifert 1993).

In many cases, accidental introductions of the fungal species and their associated vectors into new environments can cause disease outbreaks with serious consequences (Loo 2009; Brasier 2008). These accidental introductions happen due to increased globalisation of trade in plants, and flaws in the international protocols of plant biosecurity (Brasier & Webber 2010). In some cases the fungi can move from one host species to another, often aided by their vectors (Woolhouse *et al*. 2005; Wingfield 2003) and the limited resistance offered by the new hosts often leads to excessive aggressiveness by the introduced pathogen (Brasier & Buck 2001). Well-known examples include the Dutch elm disease epidemics that were responsible for catastrophic losses of American elm trees, *Ulmus americana* L. (Brasier & Buck 2001; Heybroek 1993; Lamb 1979). This was due to the introduction of *Ophiostoma* species, *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier together with their vector, the European elm bark beetle (*Scolytus multistriatus* Marsham) (Webber 1990) from their host tree, the European elm tree, through the importation of infested elm timber from Europe to America (Brasier 1990; Peace 1960). The fungi shifted host to the indigenous American elm trees, causing the Dutch elm disease (Brasier & Buck 2001; Heybroek 1993; Lamb 1979). *Raffaelela lauricola* T.C. Harr., Fraedrich & Aghayeva along with its vector beetle, the invasive exotic red bay ambrosia beetle *Xyleborus glabratus* Eichhoff, were introduced into the southeastern USA from Asia on solid wood packing material (Harrington *et al*. 2008). The fungi shifted host onto native members of the Lauraceae including avocado trees, causing the Laurel wilt disease that killed most of these new hosts (Harrington *et al*. 2008). In some cases ophiostomatoid fungi have shifted from native hosts to introduced hosts with negative consequences. For example, *Ceratocystis albifundus* De Beer, Wingfield & Morris,

which is believed to be native to South Africa, is found on several native hosts including *Protea* sp. (Morris *et al*. 1993; Gorter 1977), *Acacia caffra* Willd, *Burkea africana* Hook, *Combretum molle* R.Br. ex G. Don, *Faurea saligna* Harv, *Ozoroa paniculosa* (Sond.) R. Fern. and *Terminalia sericea* Cambess (Roux *et al*. 2007). This species has moved onto introduced *A. mearnsii* trees (Roux & Wingfield 1997) on which it is extremely virulent and now threatens the cultivation of *A. mearnsii* in South Africa (De Beer 1994). It has already led to significant economic losses in the *A. mearnsii* plantation industry in this country (Roux *et al*. 1999).

Like other ecosystems around the world, the Cape Floristic Region (CFR) of South Africa is under increased threat from anthropogenic influences. Numerous economically important exotic plant species and their associated organisms have been introduced and are now invasive within natural CFR habitats. This is especially true for some of the most important South African plantation forestry taxa such as *Acacia mearnsii*, *Eucalyptus* spp. and *Pinus* spp. (Moran & Hoffman 2012). These taxa occupy vast areas of natural CFR vegetation and are ideal organisms to study the possibility and consequences of fungi moving to newly encountered hosts, as they are in close contact with many native tree taxa. The aim of this study was firstly to identify possible consequences when fungi from the Ophiostomatales move to newly encountered hosts by testing their pathogenicity on various usual hosts and on possible newly encountered hosts. The likelihood that fungi would move to newly encountered hosts was further assessed by identifying known host ranges of the fungi and their vector organisms, and also by testing the specificity of various mites towards their natural beetle vectors and those that they would not normally associate with.

6.2. Materials and methods

6.2.1 Study organisms

In the present study we focussed on three important plantation forestry tree species (*Acacia mearnsii*, *Eucalyptus grandis* W.Hill ex Maiden and *Pinus radiata* D. Don) that are invasive in natural CFR vegetation (Moran & Hoffman 2012; Lowe *et al* 2008;). These taxa often grow in dense stands and are often in close association with native trees found in the Afromontane forest patches scattered throughout the CFR. Recent surveys of members of the Ophiostomatales from the CFR revealed that there are many undescribed or newly described taxa present on native trees within Afromontane forests (Musvuugwa *et al*. 2013, Chapters 2,

3 & 4). Some taxa appeared to be host specific, while others had very wide host ranges. These surveys also revealed the presence of some members of the Ophiostomatales from exotic species (Musvuugwa *et al*. 2013, Chapter 5). Numerous taxa were associated with sub-cortical beetles, their phoretic mites and/or mites from wounds on storm damaged trees (Musvuugwa *et al*. 2013, Chapter 5). Here we selected fungal species that differ in their dispersal ecology and host ranges in order to assess the possible consequences and likelihood of moving to new hosts (Table 1). This study was prompted by recent problems caused by a native ophiostomatoid fungus (Microascales: *C. albifundus*) on exotic plantation forestry trees (*A. mearnsii*) (De Beer 1994; Roux & Wingfield 1997) and after the collection of a exotic phoretic mite (*Dendrolaelaps quadrisetus* Berlese) on both a non-native sub-cortical beetle (*Orthotomicus erosus* Wollaston) from *Pinus radiata* and a native species (*Lanurgus* sp. 1 Eggers) from a native host, *Olea capensis* ssp. *macrocarpa* (Musvuugwa et al. 2013, Chapter 5, Table 1).

Dendrolaelaps quadrisetus was found to be associated with three exotic Ophiostomatales species (*Ophiostoma ips*, *Sporothrix* sp. 1 and *Ceratocystiopsis* sp. 1) on *P. radiata* (all associated with exclusively pine-associated bark-beetles) and *O. pallida* that was isolated from *Olea capensis* spp. *macrocarpa* (Musvuugwa *et al*. 2013, Chapter 5). *Sporothrix pallida* was also isolated from the beetle *Lanurgus* sp. 1, the native phoretic partner of *D. quadrisetus* (Musvuugwa *et al*. 2013, Chapter 2). It is therefore expected that the fungi associated with this mite can move between host plants and that the mite is not very specific towards its vectors. *Ophiostoma pallida* was also found to be associated with the native subcortical beetle taxa *Cytonoxylon* sp. 1 (Musvuugwa *et al*. 2013, Chapter 2, Table 1). This fungus is therefore not very host and/or vector specific and can easily move from one host to the next.

Ophiostoma pluriannulatum and *Graphilbum roseus* are associated with numerous mite taxa (none known to be phoretic) and with wounds on diverse native trees (Musvuugwa *et al*. 2013, Chapter 5). These are expected to be able to easily move between various hosts by using these mites as primary vectors. These fungi have, however, not yet been isolated from wounds on exotic trees, which raises the question of whether they are able to grow on these hosts. *Sporothrix rapaneae* is only currently known from wounds on one host tree species (*Rapanea melanophloes*). Its associated organisms are unknown, but most likely include mites. Like with *O. pluriannulatum* and *Graphilbum roseus*, it may therefore be able to move easily between different hosts. *Raffaelea rapaneae* is an ambrosial fungus associated with a Platypodinae beetle. The nature of this association is probably very specific (Henriques *et al*.

2006) and the dispersal of this species to tree taxa other than *R. melanophloeos* is therefore less likely.

Table 1. Study organisms and their associated host tree species used in growth and vector studies.

Fungal species	Host tree	Beetle associates	Mite associates
Ceratocystiopsis sp. 1	*Pinus radiata*	*Orthotomicus erosus*	*Dendrolaelaps quadrisetus*
Ophiostoma ips	*Pinus radiata*	*Orthotomicus erosus*	*Dendrolaelaps quadrisetus*
Sporothrix sp. 1	*Pinus radiata*	*Orthotomicus erosus*	*Dendrolaelaps quadrisetus*
Raffaelea rapaneae	*Rapanea melanophloeos*	Platypodinae sp. 1	None known
Sporothrix pallida	*Olea capensis ssp. macrocarpa*	*Lanurgus* sp. 1, *Ctonoxylon* sp. 1	None known
Graphilbum roseus	*Curtisia dentata, Halleria lucida, Pterocelastrus tricuspidatus, Trichocladus crinitus and O. capensis ssp. macrocarpa*	None known	Oribatida sp. 2 *Lasioseius* sp. 1 Mesosigmata sp. 1
Ophiostoma pluriannulatum-like	*Olea capensis, Rapanea melanophloeos, Pterocelastrus sp., Curtisia dentata, Acacia mearnsii*	None known	Acaridae sp. 1 Mesostigmata sp.1 *Uroobovella* sp. 1
Sporothrix rapaneae	*Rapanea melanophloeos*	None known	None known

6.2.1. Growth studies

The growth of various members of the Ophiostomatales from the CFR was tested by inoculating them onto various hosts including those that they are not usually associated with (Table 1). Three native tree species (*Curtisia dentata* (Burm.f.) C.A.Sm., *Olea capensis* ssp. *macrocarpa* and *Rapanea melanophloeos* and three exotic tree species (*Acacia mearnsii*, *Eucalyptus grandis*, *Pinus radiata*) were selected for these experiments. These experiments aimed to determine how host specific these fungi are (i.e. can they grow on possible newly encountered hosts) and if so, whether they could be pathogenic to these newly encountered hosts. Three isolates of each of the eight fungal species assessed were used for inoculation experiments. A cork borer (8 mm diameter) was used to make wounds on branches (*ca*. 15 mm diameter) of trees by opening the cambium. Mycelial plugs of the same size were taken from edges of actively growing fungal colonies (1-2 weeks old) on Malt Extract Agar (MEA; Biolab, Midrand, South Africa) and inserted onto the wounds with mycelium facing the cambium, whereafter wounds were sealed with masking tape to prevent desiccation. As a control, wounds were inoculated with sterile MEA plugs. After a period of six weeks lesion lengths resulting from the inoculations were measured. Re-isolations were made from the front of the lesions in order to confirm the identity of the lesion-causing fungi. Re-isolations were made by surface sterilising wounds with 70% ethanol and removing pieces of inner wood (2 mm^2). These were placed on MEA in Petri dishes and incubated in the dark at room temperature (*ca*. 23°C). Pure cultures of the fungi were identified based on morphological characters.

Differences in mean lesion length caused by the various fungal isolates and the control on each of the host trees were determined using One-way ANOVA in Statistica 11 (Statsoft Corporation, USA). Means were separated by Dunnett Post-hoc test Statistica 11 (Statsoft Corporation, USA). We assumed that when the mean lesion length for an isolate was higher than the upper standard error of the control, the fungal isolate was able to grow on that particular host. We further assumed that a particular isolate was pathogenic to the host when its mean was statistically different from that of the control.

6.2.2. Ability of phoretic mites to distinguish between vector and non-vector beetles

To determine the level of specificity between phoretic mites and their beetle vectors, we tested the ability of mites to distinguish between vector and non-vector beetles. Three mites that are known to be vectored by beetles were selected based on availability. These included *D. quadrisetus* known from bark beetles on *Pinus* spp. and *Olea capensis* ssp. *macrocarpa* (Table 1), and two species (*Proctalaelaps vandenbergi* Ryke and *Trichouropoda* sp. 3 Berlese) that are only known as associates of native beetles (*Genuchus hottentottus* (F.) and *Trichostetha fascicularis* L., both in the Scarabaeidae, collected from *Protea* spp. (Roets *et al*. 2009). *Dendrolaelaps quadrisetus* is a predatory mite that feeds on eggs and larvae of its phoront beetle (Kinn 1967) and also in some cases feeds on nematodes, but it often carries spores of members of the Ophiostomatales (Musvuugwa *et al*. 2013, Chapter 5; Cardoza *et al*. 2008). *Trichauropoda* sp. 3 has a close mutualistic association with members of the Ophiostomatales (Roets et al 2007), while *Proctolaelaps vandenbergi* is a pollen and nectar feeder (Coetzee *et al*. 1986) that also often carries spores of the Ophiostomatales (Roets *et al* 2007). Mites were allowed to associate with three other beetle taxa besides their natural vector. *Dendrolaelaps quadrisetus* was allowed to associate with one of its normal vectors (*Lanurgus* sp. 1 from host *Olea capensis* ssp. *macrocarpa*) and the non-usual vectors *Lasioderma serricorne* Fabricius (Anobiidae) (from contaminated stored products), Cryphalinae sp. 1 (from *Virgilia oroboides* P. J. Bergius) and *Ctonoxylon* sp. 2 (from *Olea* sp. 1 L.). *Lasioderma serricorne* was chosen as this beetle has no known mite associates and is not found in natural areas in the CFR. Both *P. vandenbergi* and *Trichauropoda* sp. 3 were allowed to associate with their normal vector (*G. hottentottus*) and the non-usual vectors *Lanurgus* sp. 1, *L. serricorne* and Cryphalinae sp. 1. Vector studies were carried out testing two different scenarios, a no-choice experiment and a choice experiment.

In the no-choice experiments, five individuals of each tested mite species were allowed to associate with a single individual of a beetle species in a sealed Petri dish (60 mm diameter). The experiment was replicated five times for all the different beetle species individually. Beetles were monitored for 24 hours and the total number of mites found on the individual beetles was counted at the end of the experiment. In the choice experiment, 20 individuals of each mite species selected for this study were allowed to associate with one individual of each of the tested beetles in a single Petri dish. The experiment was replicated five times for each mite species tested. These experiments were also monitored for 24 hrs and the number of mites found on each beetle was recorded at the end of the experiment.

6.3. Results

5.3.1 Growth studies

After a period of six weeks all fungal species had at least two isolates that were able to grow on *Pinus radiata* (Fig. 1A). However, none of the fungal species used in inoculations proved to be pathogenic to this tree species (Fig. 1A). All fungal taxa tested were able to grow on *A. mearnsii*, except for the pine associated *O. ips* (Fig. 1B). Only *Graphilbum roseus* and *O. pluriannulatum* were pathogenic to this species, producing distinct significant lesions as compared to the control, ($df = 24$; $f = 6.8$; $p < 0.001$) (Fig. 1B). All fungal taxa tested were able to grow on *Eucalyptus grandis*, except for *S. rapaneae*, *S. pallida*, *O. pluriannulatum* and *Ceratocystiopsis* sp. 1 (Fig. 1C). The native fungi *R. rapaneae*, *G. roseus* and the non-native *Sporothrix* sp. 1 and *O. ips* were pathogenic on *E. grandis*, ($df = 24$; $f = 3.7$; $p = 0.000003$) (Fig. 1C).

All isolates of all tested fungal species were able to grow on *R. melanophloeos* (Fig 2A). Taxa that proved to be pathogenic to this species included *S. rapaneae*, *G. roseus*, *S. pallida*, *O. pluriannulatum* and the non-native *O. ips*, ($df = 24$; $f = 4.1$; $p < 0.001$) (Fig. 2A). Very few isolates of the Ophiostomatales tested were able to grow on *O. capensis* ssp. *macrocarpa* and none proved to be pathogenic to this tree, ($df = 24$; $f = 0.81$; $p = 0.72$) (Fig. 2B). All fungal species tested on *Curtisia dentata* were able to grow on this species (Fig. 2C). Five of these fungal species produced distinct lesions and were thus considered pathogenic on this host, ($df = 24$; $f = 20.2$; $p < 0.001$). These were *S. rapaneae*, *R. rapaneae*, *G. roseus*, *S. pallida* and the non-native *Ceratocystiopsis* sp.1 (Fig. 2C).

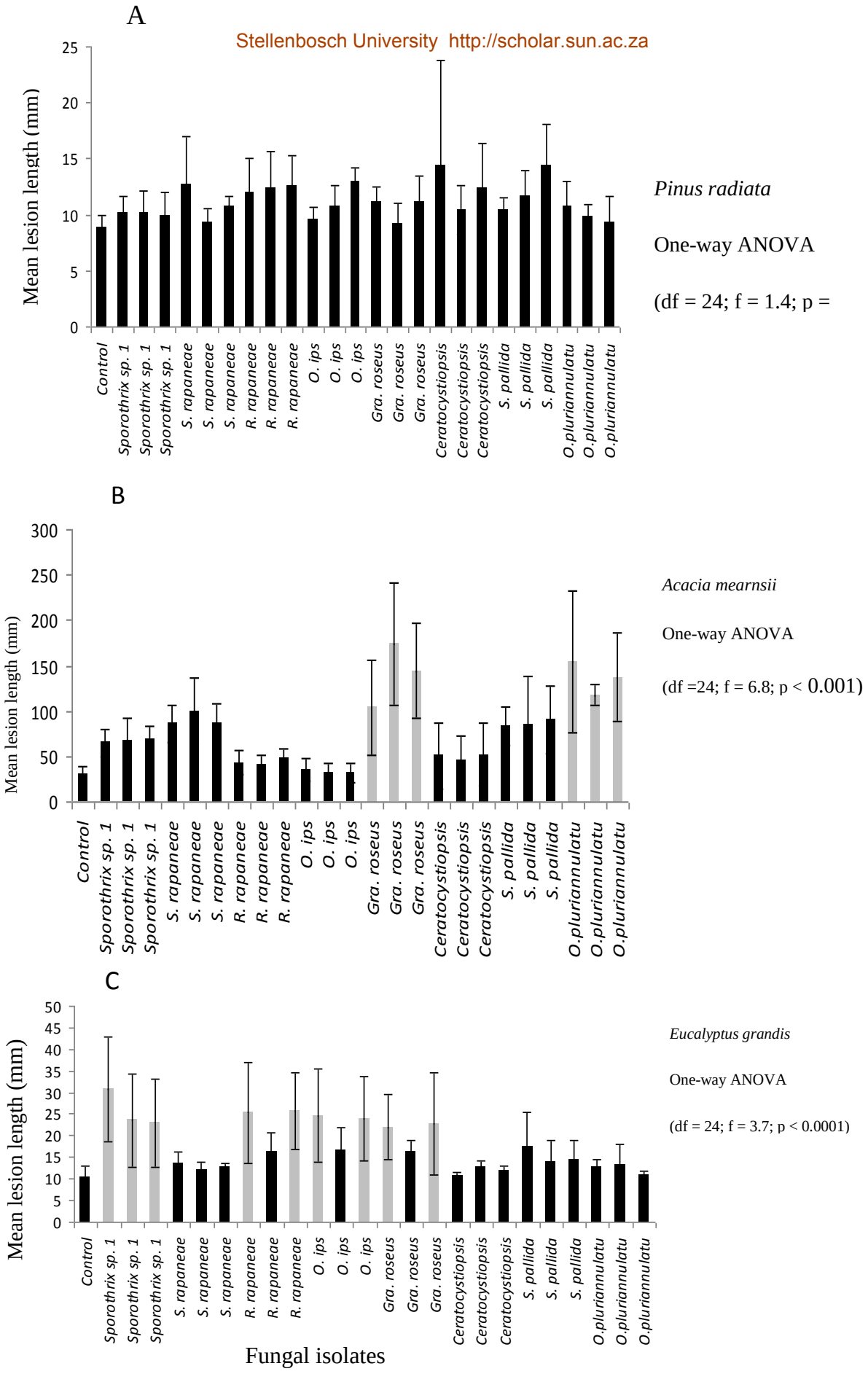

Figure 1. Mean lesion lengths (+/- SE) caused by various members of the Ophiostomatales on the non-native taxa (A) *Pinus radiata*, (B) *Acacia mearnsii* and (C) *Eucalyptus grandis*

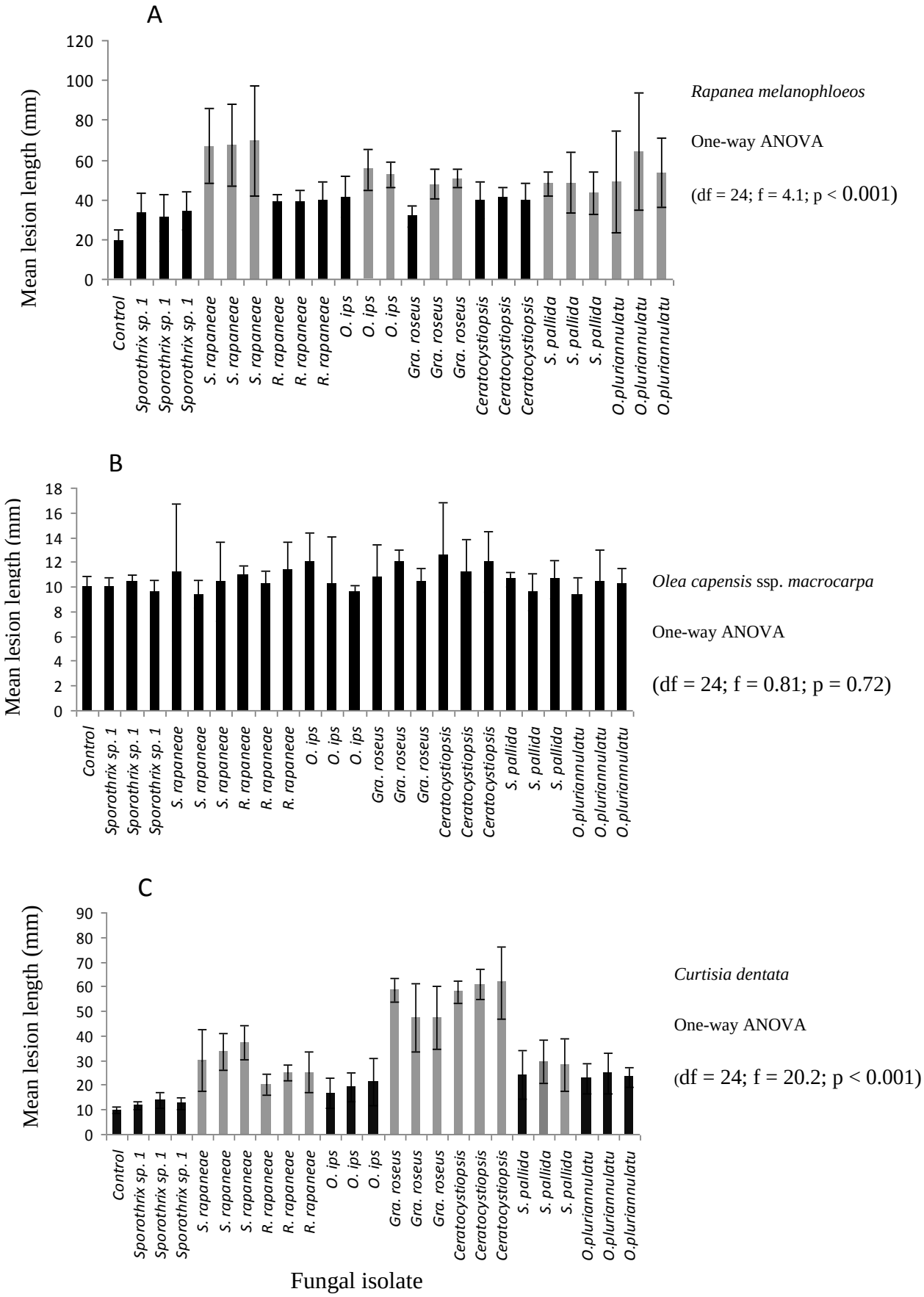

Figure 2. Mean lesion lengths (+/- SE) caused by various members of the Ophiostomatales on the native taxa (A) *Rapanea melanophloeos* trees, (B) *Olea capensis* ssp. *macrocarpa* and (C) *Curtisia dentata*

6.3.2. Ability of mites to distinguish between vector and non-vector beetles

In the no-choice experiments, all the phoretic mite species could only associate with their natural vector beetles. *Dendrolaelaps quadrisetus* only associated with its vector beetle, *Lanurgus* sp. 1 (Fig 3A), H (df = 3, N = 20) = 18.5547, p = 0.0003), *Trichouropoda* sp. 3 only chose to climb onto their native *Protea* beetle vector, *Genuchus hottentottus* (Fig. 4A), H (df = 3, N = 20) = 18.6275, p = 0.0003, while the *Protea*-associated *Proctalaelaps vandenbergi* were also specific on their native vector beetle, *Genuchus hottentottus* (Fig. 5A), H (df = 3, N = 20) = 18.6031, p = 0.0003).

Similarly, in the choice experiments, all the phoretic mite species only associated with their natural vector beetles. The *Olea*-associated *D. quadrisetus* only associated with *Lanurgus* sp. 1 (Fig 3B), H (df = 3, N = 20) = 18.5547, p = 0.0003, *Trichouropoda* sp. 3 only associated with their natural phoront, *G. hottentottus* (Fig. 4B), H (df = 3, N = 20) = 18.7500, p = 0.0003 and *P. vandenbergi* only climbed onto *Genuchus hottentottus* (Fig. 5A), H (df = 3, N = 20) = 18.6275, p = 0.0003.

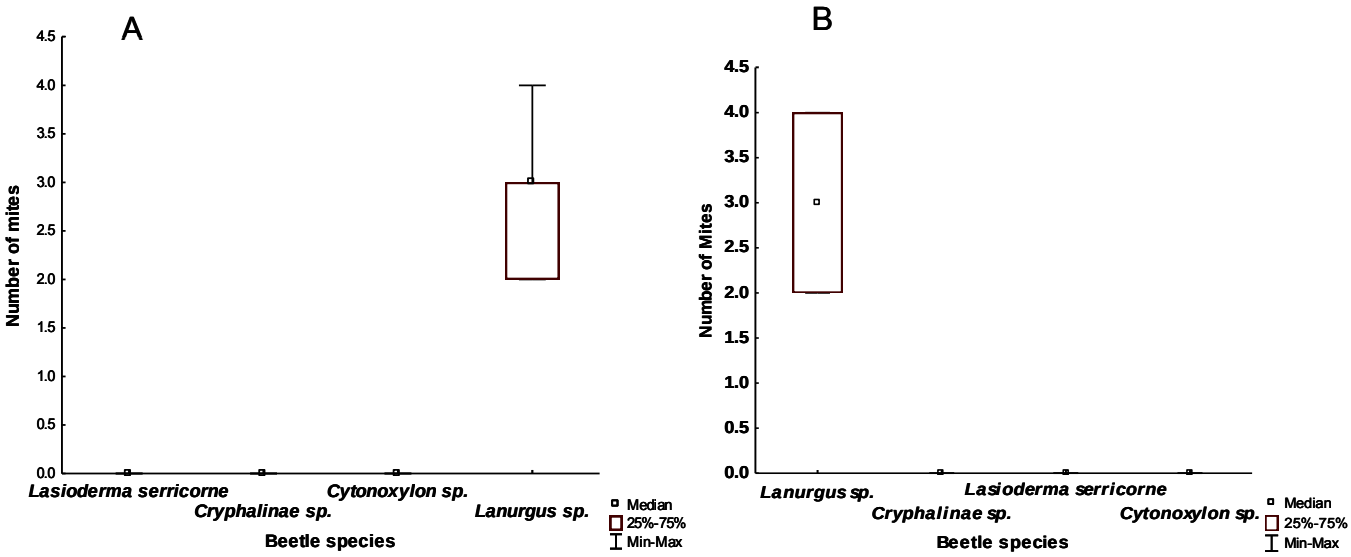

Figure 3. Mean number of *Dendrolaelaps quadrisetus* mites on four different beetle after a period of 24 hours in the no-choice experiment (A), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.5547, p = 0.0003 and the choice experiment (B), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.5547, p = 0.0003.

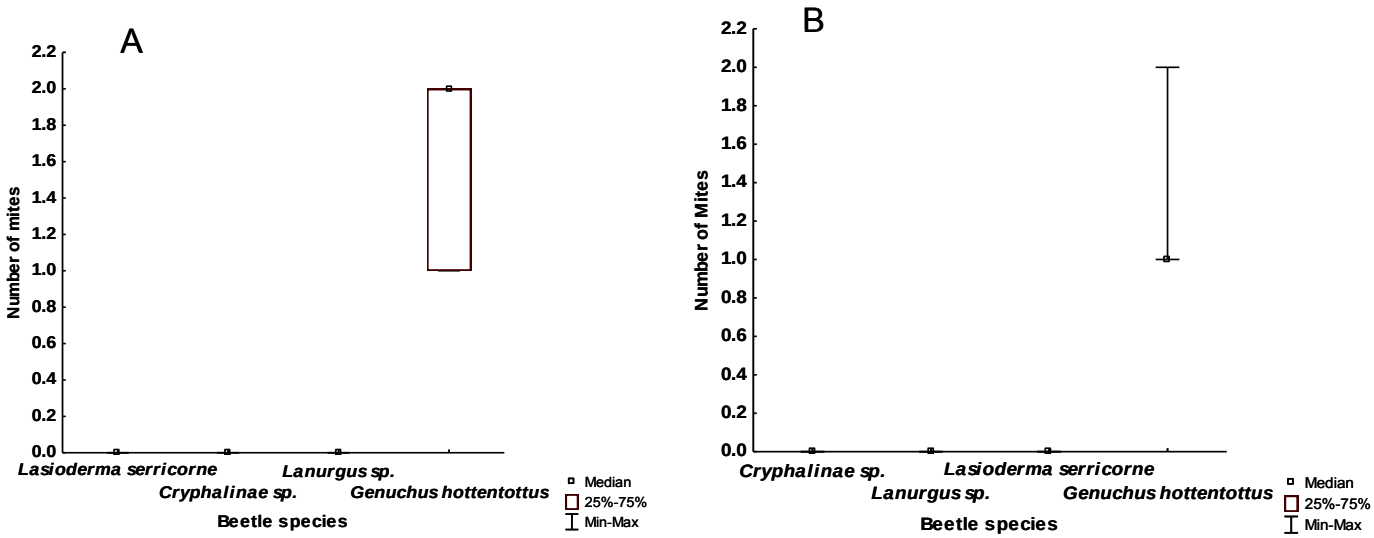

Figure 4. Mean number of *Protea*-associated *Trichouropoda* sp. 3 mites on four different beetle species after a period of 24 hours in the no-choice experiment (A), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.6275, p = 0.0003 and the choice experiment (B), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.7500, p = 0.0003

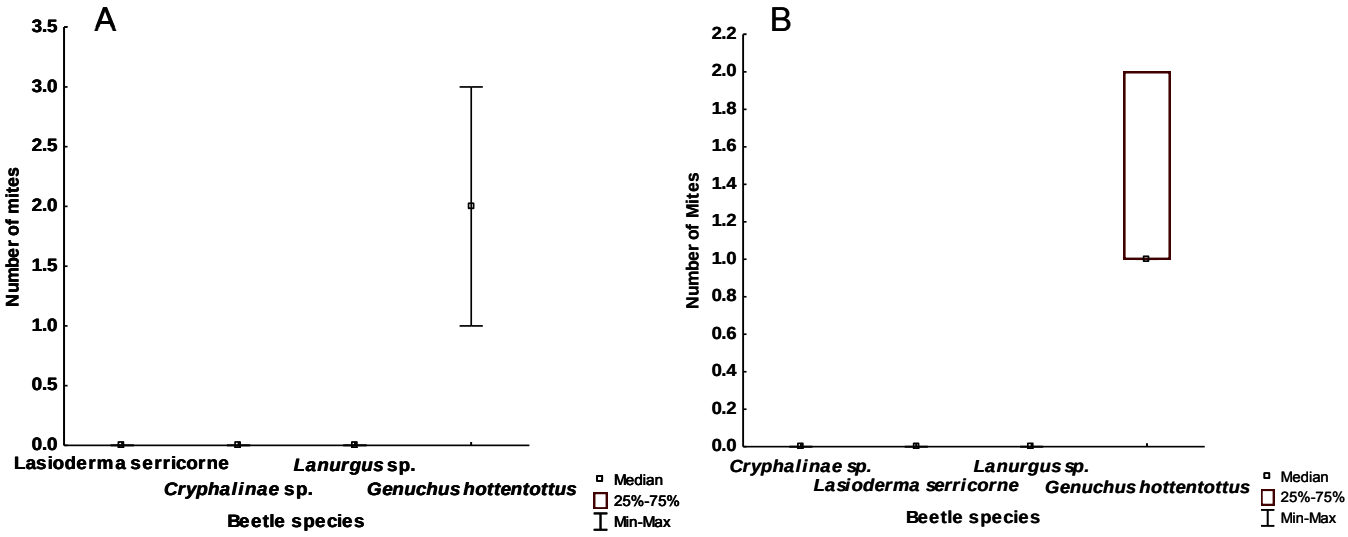

Figure 5. Mean number of *Proctalaelaps vandenbergi* mites on four different beetle species after a period of 24 hours in the no-choice experiment (A), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.6031, p = 0.0003 and the choice experiment (B), Kruskal-Wallis ANOVA, H (df = 3, N = 20) = 18.6275, p = 0.0003

6.4. Discussion

Results from this study reveal the possible effects and dangers posed by various members of the Ophiostomatales when they encounter new host trees in the CFR. This is important, as it has been shown that when fungal species encounter new environments, they can cause disease outbreaks with serious consequences (Loo 2009; Brasier 2008;). When they move from one host species to another, usually through their vectors (Woolhouse *et al*. 2005; Wingfield 2003,), there can experience limited resistance by the new hosts, which can lead to excessive aggressiveness by the introduced pathogen (Brasier & Buck 2001). At least two fungal species were able to grow on each of the host trees they were tested on and at least two fungal species were pathogenic on all the trees species they were tested on except on *Olea capensis* ssp. *macrocarpa* and *Pinus radiata*. Also presented in this study is the relationship between phoretic mites, their natural beetle vectors and other beetles they are most likely not to encounter in the field. Results showed that there is a high level of specificity between the mites and their natural vectors. This study provides an initial step into understanding the possible consequences that may result if different Ophiostomatales fungi shift hosts within the CFR.

None of the fungi tested was pathogenic on *Pinus radiata* or *Olea capensis* ssp. *macrocarpa*. The reason for this is not known, but it is possible that these tree species are highly resistant to attack by most pathogens. Defences could be through the formation of physical barriers that will block the fungi from entering and spreading through the plant or through the production of chemicals that are toxic to the fungi or that inhibit growth of the fungi in the plant (Shigo *et al*. 1984). *Olea capensis* ssp. *macrocarpa* has never before been recorded to be attacked by or associated with any pathogenic fungus. Therefore it is not surprising that any of the fungal species tested were non-pathogenic on this tree species as it is most likely highly resistant to attack by pathogens. However, *P. radiata* has been recorded to be associated with members of the Ophiostomatales, some of which cause sapstain in this host (Reay *et al*. 2002). Major losses of *P. radiata* caused by non-ophiostomatoid fungi have been recorded in South Africa. Examples include *Arnylostereurn areolatum* (Fr.) Boid. and *A. chailletii* (Pers.: Fr.) Boid vectored by the woodwasp, *Sirex noctilio* Fabricus (Hurley *et al*. 2007; Madden 1988).

The wound-associated species *Graphilbum roseus* and *Ophiostoma pluriannulatum*-like were pathogenic on several tree species. *Graphilbum roseus* was pathogenic on the non-native

Acacia mearnsii and *Eucalyptus grandis* as well as on the native *Rapanea melanophloes* and *Curtisia dentata*. This species was recently isolated from several native hosts, including *Curtisia dentata*, *Halleria lucida*, *Pterocelastrus tricuspidatus*, *Trichocladus crinitus* and *Olea capensis* ssp. *macrocarpa* (Musvuugwa *et al*. 2013, Chapter 4). Interestingly it is also associated with several wound associated mites such as Oribatida sp. 2, *Lasioseius* sp. 1 and Mesostigmata sp. 1 (Musvuugwa *et al*. 2013, Chapter 5). These mites may aid in the transport of this fungus to many hosts, especially since these hosts grow in close proximity to each other. Since it is possible that this fungus is vectored by its associated mites (Musvuugwa *et al*. 2013, Chapter 5), it is likely that it can at some point be transported to these exotic hosts and pose a danger to South Africa's forestry industry. This scenario has occurred at least once in South Africa where *Ceratocystis albifundus* shifted hosts from native taxa to the non-native *A. mearnsii*, causing a serious wilt disease that led to financial losses in the forestry industry (Roux *et al*. 1999; Roux & Wingfield 1997; De Beer 1994). Similarly, *Ophiostoma pluriannulatum*-like was also isolated from various hosts, including *A. mearnsii* and *R. melanophloes* and is also associated with several mite species (Musvuugwa *et al*. 2013, Chapter 5). It has now been shown that it can be pathogenic on *A. mearnsii* and *R. melanophloes*. It may therefore pose a significant threat to *A. mearnsii* should it move into plantation forestry areas. The current distribution of this fungus is unknown, but as *A. mearnsii* has invaded much of coastal South Africa, it may well be able to extend its range rapidly eastwards to where *A. mearnsii* is commercially planted. The other wound-associated fungus, *Sporothrix rapaneae*, was pathogenic on its host *R. melanophloeos* and on *Curtisia dentata*. Currently the fungus is only known from one host and its associated arthropods are not known, although it is highly likely to be mites. However, its vector species may to some extent be specific to this fungus and/or its host, since this fungus has thus far been collected from only one host tree.

Three of the fungal species from pine trees, associated with *Dendrolaelaps quadrisetus* and *Orthotomicus erosus*, were also pathogenic on some of the tree species tested. Although these fungi were able to grow on almost all the tree species they were tested on, they were only pathogenic on a few. *Sporothrix* sp. 1 was pathogenic on *Eucalyptus grandis*, the *Ceratocystiopsis* species was pathogenic on *Curtisia dentata*, and *O. ips* was pathogenic on *E. grandis* and *R. melanophloeos*. These species may be able to move to *E. grandis* and native hosts in areas where these occur in close proximity using *D. quadrisetus* as a carrier. This notion is aided by the recent collection of this mite from both native and exotic bark

beetles and hosts (Musvuugwa *et al*. 2013, Chapter 5). A possible reason why none of these fungi have been found on native hosts yet is that this mite has moved from pine-associated beetles to a beetle associated with *O. capensis* ssp. *macrocarpa* (Musvuugwa *et al*. 2013, Chapter 5), a host that is very resistant to ophiostomatoid fungi. However, this beetle may not be very host specific as it is also known from *Gonioma kamassi* E. Mey. (Musvuugwa *et al*. 2013, Chapter 5). Future exchanges of ophiostomatoid fungi between usual and non-usual hosts cannot be ruled out.

The beetle-associated fungi from native hosts, *Sporothrix pallida* and *Raffaelea rapaneae*, were able to grow on all tree species and were pathogenic on some. Both species were pathogenic on *Curtisia dentata*, while *S. pallida* was also pathogenic on *Rapanea melanophloeos* and *Raffaelea rapaneae* was also pathogenic on *Eucalyptus grandis*. *Sporothrix pallida* is associated two beetle species, *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1, as well as with the phoretic mite of *Lanurgus* sp. 1 (*D. quadrisetus*) (Musvuugwa *et al*. 2013, Chapter 2 & 5). The fact that this fungus is associated with three different arthropods may aid its dispersal to new hosts on which it may be pathogenic. In fact, this species may not be very vector specific at all, as it is known from many different environments including water sediments, soil, and the sporophore of a slime-mould and even from a human corneal ulcer (de Meyer *et al*. 2008). Conversely, *Raffaelea rapaneae* is currently only known as an associate of one Platypodinae beetle species (Musvuugwa *et al*. 2013, Chapter 2). Since it was only isolated from this beetle species, it may be highly specific to it, which would reduce the chances of it being vectored to another tree host.

Conclusion

It has clearly been shown that many of the fungal species used in this study may threaten new hosts that they may encounter. This is especially true for those species found on wounds and those associated with several arthropod taxa. These threats are less severe for fungal species associated with bark beetles, unless these carry mites that are less vector-specific. The virulence of pathogenic fungi identified here is not known at this point, but some taxa my be able to kill newly encountered hosts. Even if they cannot kill their hosts, they could still pose a great ecological (when exotic fungi compete with native fungi) or economic threat (e.g. blue staining of lumber).

References

Brasier, C.M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57: 792–808.

Brasier, C.M. 1990. China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* 39: 5–16.

Brasier, C. and Webber, J. 2010. Sudden larch death. An aggressive and unpredictable fungal pathogen is devastating larch plantations in Britain. Its remarkably broad host range, and the possibility of further geographical spread, give heightened cause for concern. *Nature* 466: 824-825.

Brasier, C.M. & Buck, K.W. 2001. Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions* 3: 223–233.

Bridges, J.R. and Moser, J.C. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* 8: 9–12.

Cardoza, Y.J, Moser, J.C., Klepzig, K.D. and Raffa, K.F. 2008. Multipartite symbioses among fungi, mites, nematodes, and the spruce beetle, *Dendroctonus rufipennis*. *Environmental. Entomology* 37: 956-963.

Cruywagen, E.M., de Beer Z.W., Roux, J. and Wingfield M.J. 2010. Three new *Graphium* species from baobab trees in South Africa and Madagascar. *Persoonia* 25: 61–71.

Coetzee, J.H., Rust, D.J. and Latsky, L.M. 1986. Mites (Acari) on Proteas. *Acta Horticulturae* 185.

De Beer, C. 1994. *Ceratocystis frimbiata* with special reference to its occurrence as a pathogen on *Acacia Mearnsii* in South Africa. M.Sc. Thesis. University of the Orange Free State. Bloemfontein. South Africa, pp 42-88.

De Meyer, E.M., De Beer, Z.W., Summerbell, R.C., Moharram, A.M., De Hoog, G.S., Vismer, H.F. and Wingfield, M.J. 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* 100:647–661.

Gorter, G.J.M.A. 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Science Bulletin no. 392. Pretoria, South Africa: Plant Protection Research Institute, Department of Agricultural Technical Services.

Harrington, T.C. 1987. New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. *Mycotaxon* 28: 39-43.

Harrington, T.C., Fraedrich, S.W. and Aghayeva, D.N. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon* 104: 399-404.

Henriques, J., de Lurdes Inacio, M. and Sousa, E. 2006. Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. *Revista Iberoamericana de Micologia* 23: 185-188.

Heybroek, H.M. 1993. Why bother about the elm? In: *Dutch Elm Disease Research. Cellular and Molecular Approaches*, (Sticklen, M.B. and Sherald, J.L. eds.), Springer-Verlag, New York , pp 1–8.

Hurley, B.P., Slippers B. and Wingfield, M.I. 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. *Agriculture and Forest Entomology* 9:159-171.

Hofstetter, R.W., Moser, J.C. and Blomquist, S.R. 2013. Mites associated with bark beetles and their hyperphoretic Ophiostomatoid fungi. In: *Ophiostomatoid Fungi: Expanding Frontiers* (Seifert, K.A., De Beer, Z.W. and Wingfield, M.J., eds). CBS Biodiversity Series 12: 165-174

Jacobs, K., Seifert, K.A., Harrison, K.J. and Kirisits, T. 2003. Identity and phylogenetic relationships of ophiostomatoid fungi associated with invasive and native *Tetropium* spp. (Coleoptera: Cerambycidae) in Atlantic Canada. *Canadian Journal of Botany* 81: 316-29.

Jacobs, K. and Wingfield, M.J. 2001 *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. APS press, St Paul, Minnesota, U.S.A.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Klepzig, K.D. and Six D.L. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis* 37: 189-205.

Klepzig, K.D., Moser, J.C., Lombardero, M.J, Hofstetter, R.W. and Ayres, M.P. 2001. Symbiosis and competition: Complex interactions among beetles, fungi and mites. *Symbiosis* 30: 83–96.

Kubono, T. and Ito, S. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Lamb, R .1979. World without Trees. Wildwood House, London.

Lombardero, M.J., Ayres, M.P., Hofstetter, R.W., Moser, J.C. and Klepzig, K.D. 2003. Strong indirect interactions of *Tarsonemus* mites (Acarina: Tarsonemidae) and *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Oikos* 102: 243–252.

Loo, J.A. 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* 11: 81-96.

Madden, J.L. 1988. Sirex in Australasia. In: Dynamics of forest insect populations, (Berryman, A.A. ed.), Plenum Press, New York, pp 407-429.

Malloch, D. and Blackwell, M. 1993. Dispersal biology of the ophiostomatoid fungi. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*, (Wingfield, M.J., Seifert, K.A. and Webber, J.F. eds), APS Press, St. Paul, U.S.A., pp. 195–206.

Moran, V.C and Hoffmann, J.H. 2012. Conservation of the fynbos biome in the Cape Floral Region: the role of biological control in the management of invasive alien trees. *BioControl* 57:139–149.

Moser, J.C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. *Transactions of the British Mycological Society* 84: 750–753.

Moser, J.C., Perry, T.J., Bridges, J.R. and Yin, H.-F, 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus*, a mycangial fungus of the southern pine beetle. *Mycologia* 87: 84-86.

Paine, T.D., Raffa, K.F. and Harrington, T.C. 1997. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* 42: 179–206.

Peace, T. 1960. The status and development of elm disease in Britain. *Forestry Commission Bulletin* 33: 1–44.

Price, T.S., Doggett, C., Pye, J.L. and Holmes, T.P. 1992. A history of southern pine beetle outbreaks in the southeastern United States. Sponsored by the Souhtern Forest Insect Workshop Conference. The Georgia Forestry Commission, Macon, GA, p. 65.

Reay, S.D., Walsh, P.J., Ram, A. and Farrell, R.L. 2002. The invasion of *Pinus radiata* seedlings by sapstain fungi, following attack by the Black Pine Bark Beetle, *Hylastes ater* (Coleoptera: Scolytidae). *Forest Ecology and Management* 165: 47-56.

Roets, F., Dreyer, L.L., Crous, P.W. and Wingfield, M.J. 2009. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* 38: 143-152.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Roux, J., Dunlop, R. and Wingfield, M.J. 1999. Susceptibility of elite *Acacia mearnsii* families to Ceratocystis wilt in South Africa. *Journal of Forestry Research* 4: 187–190.

Roux, J., Heath, R.N., Labuschagne, L., Kamgan Nkuekam, G. and Wingfield, M.J. 2007. Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* 37: 292-302.

Roux, J. and Wingfield, M.J. 1997. Survey and virulence of fungi occurring on diseased *Acacia Mearnsii* in South Africa. *Forest Ecology and Management* 99: 327-336.

Seifert, K.A. 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis* and *Ophiostoma*: *taxonomy, ecology and pathogenicity*. (Wingfield, M.J., Seifert, K.A and Webber, J.F. eds.), APS Press, Minnesota, U.S.A.

Shigo, A.L. 1984. Compartmentalization: A conceptual framework for understanding how trees grow and defend themselves. *Annual Review of Phytopathology* 22: 189-214.

Six, D.L. 2003. Bark beetle-fungus symbioses. In: *Insect Symbioses*, (Bourtzis, K. and Miller, T. eds.), CRS press, Boca Raton, FL, USA., pp. 97-114.

Six, D.L. and Wingfield, M.J., 2011. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. *Annual Review Entomology* 56: 255-272.

Smiley, R.T. & Moser, J.C. 1974. New Tarsonemids associated with bark beetles (Acarina: Tarsonemidae). *Annals of the Entomological Society of America* 69: 713-715.

Webber, J.F. 1990. The relative effectiveness of *Scolytus scolytus*, *S. multistriatus* and *S. kirschii* as vectors of Dutch elm disease. *European Journal of Forest Pathology* 20: 184–192.

Wingfield, M.J. 2003. Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from *Cryphonectria* canker. *Australasian Plant Pathology* 32: 133–139.

Woolhouse, M.E.J., Haydon, D.T and Antia, R. 2005. Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology and Evolution* 20: 238-244.

Chapter 7: Conclusion

Prior to this study, very little was known about the diversity of micro-organisms such as fungi in the Afromontane forests of the CFR of South Africa. Most research on CFR fungi has focussed on the survey of plant-pathogenic and a few saprobic species (Lee *et al*. 2004; Crous *et al*. 2004; Swart 1999; Knox-Davies *et al*. 1986). This has resulted in a gap in our understanding of the fungal biodiversity and ecology in the CFR, especially in terms of fungi associated with native trees in the Afromontane forests. Numerous ophiostomatoid fungi are known as pathogens of native and introduced trees in South Africa (Kamgan 2008; Roux *et al* 2004; Roux & Wingfield 1997). Others are well-known saprophytes that are important in wood degradation (De Meyer *et al*. 2008; Marimon *et al*. 2008; 2007; Aghayeva *et al*. 2004; Zhou *et al*. 2004). Ophiostomatoid fungi therefore provided an excellent model system to study the biodiversity and ecology of tree-associated CFR fungi, and formed the central focus of this study.

Kamgan *et al*. (2008) suggested that fungal diversity in the CFR may be high, and that many of these fungal species may be associated with Afromontane forest trees. Results of this study strongly confirmed this, as many members of the Ophiostomatales were isolated from Afromontane forests trees. Fifteen Ophiostomatoid fungal species (14 members of Ophiostomatales and one member of Microascales) were isolated from native trees (mostly *Rapanea melanophloeos*), ten of which were newly described in this study. In addition three fungal species, two of which are probably new to science, were isolated exclusively from non-native *Pinus* species growing in these forests. Three fungal species were present on both native and a non-native tree species (*Acacia mearnsii* De Wild). A high diversity of arthropods, including subcortical beetles and mites, was also recorded, and many of them were associated with the fungal species isolated. Some of the fungal species collected in this study proved to be pathogenic on both native and exotic tree taxa. This is important, because if such fungi are associated with vectoring arthropod species, they can shift hosts, which could have devastating consequences to both native and alien trees.

Members of the Ophiostomatales represent some of the most common fungal associates of bark and ambrosia beetles (Klepzig & Six 2004; Jacobs & Wingfield 2001). In this study, we isolated four Ophiostomatales species (including three newly described species) that were associated with subcortical beetles on *Rapanea melanophloeos* and *Olea capensis* L. *ssp. macrocarpa* (C. H. Wright) I. Verd.. This was the first study that explored the associations

between subcortical beetles and ophiostomatoid fungi on native trees in the CFR. This also provided the first record of an ophiostomatoid fungal species associated with the host species *O. capensis* ssp. *macrocarpa*. *Sporothrix pallida* was isolated from two beetles, *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 associated with *O. capensis* ssp. *macrocarpa*. Although this fungus has been recorded from other environmental hosts, this was the first time it was found associated with subcortical beetles. *Sporothrix aemuluphilus*, another newly described species, is associated with the beetle *Xyleborinus aemuluphilu*s. It belongs to the *Sporothrix schenckii–O. stenoceras* complex, along with *S. pallida*. In addition to *S. pallida*, the newly described *Raffaelea scabbardiae* was also isolated form *Lanurgus* sp. 1. *Raffaelea rapaneae* represents yet another new species, which was isolated from a Platypodinae beetle infesting *Rapanea melanophloeos*. Although a couple of other members of the Ophiostomatales have been isolated from *R. melanophloeos* in the CFR before (Kamgan *et al*. 2008), they were only isolated from wounds and were never beetle-associated.

Wounds on trees caused by heavy storms, bark stripping and sivicultural practises (Kamgan *et al*. 2008; Morris *et al* 1993) can act as infection points for ophiostomatoid fungi (Wingfield *et al*. 1993). Although some ophiostomatoid fungi have previously been collected from wounds on *Rapanea melanophloeos* in the Afromontane forests, more fungal species were expected to be infecting this tree, especially given the signs of stress it displays in many localities. This suspicion was confirmed by results from this study. In addition to the two fungal species associated with subcortical beetles, five more wound-associated species (*Ophiostoma stenoceras*, *Sporothrix reniformis*, *S. rapaneae*, *S. lunateae* and *S. noisomeae*) were collected from *R. melanophloeos*. All but *O. stenoceras* were new to science, and were thus formally described here. All of the wound-associated species collected from *Rapanea melanophloeos* belong to the *Sporothrix schenckii–O. stenoceras* complex, except for *S. noisomeae* that has provisionally been placed in the *S. lignivora* complex. The placement of most of these fungi in the *S. schenckii–O. stenoceras* complex (including two species isolated from beetles, namely *Sporothrix pallida* and *S. aemuluphilus*) was not surprising, as many other species in this complex had been collected from southern Africa (De Beer *et al*. 2013). The *S. lignivora* complex to which *Sporothrix noisomeae* belongs is a contingent that De Beer *et al*. (2013) suggested may represent a separate genus. This needs to be confirmed by further phylogenetic work. Besides *Rapanea melanophloeos*, other tree taxa also had wounds resulting from storm damage. Three more new ophiostomatoid fungal species were collected from such wounds on other tree species. *Sporothix capensis*, also a member of the

S. schenckii – O. stenoceras complex, was collected from *O. capensis* ssp. *macrocarpa*. *Graphilbum roseus* was collected from many different, unrelated tree species. This was unusual, as most of the other new fungal species described here were more host tree specific. *Graphium ilexiense* (Microascales) was isolated from wounds on *Ilex mitis*, which represents the first isolation of an ophiostomatoid fungus from this tree species.

Several non-native trees have become invasive in the Afromontane forests of the CFR. Although associations between fungi and these exotic trees have been studied more than associations between fungi and native trees in South Africa, we still managed to collect two possibly new fungal species (*Sporothrix* sp. 1, *Ceratocystiopsis* sp. 1) as well as *Ophiostoma ips* associated with three bark beetles (*Orthotomicus erosus*, *Hylurgus ligniperda* and *Hylastes angustatus*) exclusively from *Pinus*. Several fungal species were collected from both native trees and non-native trees. These included *Sporothrix fusiforme* from *Brabejum stellatifolium* and *Acacia mearnsii*. Similarly, *O. quercus* and *O. pluriannulatum*-like fungus were collected from several native trees and from *A. mearnsii*. This suggests a possibility for host shifting of some of these fungi between native and non-native hosts or even between different native hosts. Such shifts have the potential to be detrimental if the fungus becomes pathogenic on the new host. Eight other subcortical beetle taxa, from which we did not collect any ophiostomatoid fungi, were found also to infest native trees in the Afromontane forests. Together with the beetle species associated with ophiostomatoid fungi, we collected more than 4500 beetle individuals. Given the biological diversity in the CFR, it is very likely that more beetle species await discovery in these forests.

In addition to their association with subcortical beetles, ophiostomatoid fungi were also found to be associated with other arthropods such as mites (Roets *et al*. 2007; Lombardero *et al*. 2003; Moser 1985). Some mites are phoretic on beetles and carry the same fungi as their phoront beetles, while other mites are just wound-associated. Four of the phoretic mites species collected were associated with ophiostomatoid fungi (*Dendrolaelaps quadrisetus*, *Histiogaster* sp. 3, *Elattoma* sp. 1 & 2), which was also carried by their phoront beetles. *Dendrolaelaps quadrisetus* was phoretic on both a native beetle (*Lanurgus* sp. 1) from *Olea capensis* ssp. *macrocarpa* and a non-native beetle (*Orthotomicus erosus*) from *Pinus* species. The fungal species it carried when phoretic on one beetle species was different to the species it carried on the other beetle species. Sixteen species of wound-associated mites were collected from 12 native trees. Of these, nine were associated with several ophiostomatoid (*Graphilbum roseus*, *O. pluriannulatum*-like, *O. quercus*) fungi that were isolated from

several different host trees. This suggests that they may aid in the transport of these fungi from one host species to another.

Accidental introductions of fungal species and their associated vectors into new environments or host shifts by the fungi can cause disease outbreaks with serious consequences (Brasier 2008). When we tested for possible consequences if some members of the Ophiostomatales had to move to newly encountered hosts through pathogenicity tests, some of the fungi proved to be pathogenic on several trees. Pathogenicity is most likely in fungal species associated with wounds, especially those also associated with mites, because the mites vector such fungi. When phoretic mites were tested for their specificity to their vector beetles, they proved to be highly specific. Although some of the fungi associated with these mites and their beetles were also pathogenic, it is less likely for these to be transferred to other host tree species due to the high specificity of their arthropod associates.

This is one of only a few studies that focused on ophiostomatoid fungi, subcortical beetles and mites associated with trees in the Afromontane forests of South Africa. Although a high diversity of Ophiostomatales members was collected, many more probably still await discovery. Now that we have some idea of the extent of the diversity of ophiostomatoid fungi, beetles and mites associated with Afromontane tree hosts, more detailed studies should also focus at unraveling the complex inter-organismal interactions in many of these systems.

References

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W. and Kirisits, T. 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* 96:866–878.

Brasier, C.M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57: 792–808.

Crous, P.W., Denman, S., Taylor, J.E., Swart, L. and Palm, E. 2004. Cultivation and diseases of Proteaceae: *Leucadendron*, *Leucospermum* and *Protea*. CBS Biodiversity Series 2, Centraalbureau voor Schimmelcultures, Utrecht.

De Beer, Z.W., Seifert, K.A. and Wingfield, M.J. 2013. A nomenclator for ophiostomatoid fungi genera and species in the Ophiostomatales and Microascales. In: *Ophiostomatoid fungi: expanding frontiers* (Seifert, K.A., De Beer, Z.W., Wingfield, M.J. eds.), CBS Biodiversity Series 12, pp. 245-322.

De Meyer, E.M., De Beer, Z.W., Summerbell, R.C., Moharram, A.M., De Hoog, G.S., Vismer, H.F. and Wingfield, M.J. 2008. Taxonomy and phylogeny of new wood- and soil-

inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* 100:647–661.

Jacobs, K. and Wingfield, M.J. 2001. *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. APS press, St Paul, Minnesota, U.S.A.

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. and Roux, J. 2008. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.

Klepzig, K.D and Six, D.L. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis* 37: 189-205.

Knox-Davies, P.S., van Wyk, P.S. and Marasas, W.F.O. 1986. Diseases of proteas and their control in the South-western Cape. *Acta Horticulturae* 185: 189–200.

Lee, S., Mel'nik, V., Taylor, J.E. and Crous, P.W. 2004. Diversity of saprobic hyphomycetes on Proteaceae and Restionaceae from South Africa. *Fungal Diversity* 17: 91–114.

Lombardero, M.J., Ayres, M.P., Hofstetter, R.W., Moser, J.C. and Klepzig, K.D. 2003. Strong indirect interactions of Tarsonemus mites (Acarina: Tarsonemidae) and *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Oikos* 102: 243–252.

Marimon, R., Gené, J., Cano, J. and Guarro, J. 2008. *Sporothrix luriei*: a rare fungus from clinical origin. *Medical Mycology* 46: 621–625.

Marimon, R., Cano, J., Gene, J., Sutton, D.A., Kawasaki, M. and Guarro, J. 2007. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. *Journal of Clinical Microbiology* 45:3198–3206.

Morris, M.J., Wingfield, M.J. and De Beer, C. 1993. Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* 42: 814-817.

Moser, J.C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. *Transactions of the British Mycological Society* 84: 750–753.

Roets, F., Wingfield, M.J., Crous, P.W. and Dreyer, L.L. 2007. Discovery of fungus-mite mutualism in a unique niche. *Environmental Entomology* 36: 1226–1237.

Roux, J., VanWyk, M., Hatting, H. and Wingfield, M. J. 2004. *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* 53: 414–421.

Roux, J. and Wingfield, M.J. 1997. Survey and virulence of fungi occurring on diseased *Acacia Mearnsii* in South Africa. *Forest Ecology and Management* 99: 327-336.

Swart, L. 1999. Pathogens associated with diseases of *Protea*, *Leucospermum* and *Leucadendron* spp. MSc thesis, Department of Plant Pathology, University of Stellenbosch, Stellenbosch.

Wingfield, M.J. and Kemp, G.H.J. 1993. Diseases of Pines, Eucalyptus and Wattles. In: *South African Forestry Handbook*, (Van der Sidje, H.A. ed.), The South African Association of Forestry. Pretoria, South Africa, pp. 231-248.

Zhou, X.D., De Beer, Z.W., Ahumada, R., Wingfield, B.D. and Wingfield, M.J. 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* 15: 261-274.