AN INVESTIGATION INTO THE DEATH OF NATIVE *VIRGILIA* TREES IN THE CAPE FLORISTIC REGION OF SOUTH AFRICA

NETSAI MARGARETH MACHINGAMBI

Thesis presented in the partial fulfilment of the requirements for the degree of

Master of Science

in the

Faculty of Science (Department of Botany and Zoology)

at the

University of Stellenbosch

Promoters: Dr. Francois Roets, Prof. Léanne L. Dreyer and Prof. Jolanda Roux

March 2013

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

Date: 8 February 2013

i

ABSTRACT

The Cape Floristic Region (CFR) of South Africa is well-recognised for exceptionally high plant species diversity and endemism. However, little attention has been bestowed on the pests and pathogens in this region, even though these may greatly influence plant distribution and evolution. In this study we identify various arthropods and fungi as pests and disease-causing organisms of the ecologically and economically important CFR-endemic tree taxa of *Virgilia*. We isolated, identified and determined the pathogenicity of key fungal taxa from diseased *Virgilia* trees throughout the CFR. In addition we evaluated the role of possible pest arthropod taxa, including bark beetles, phoretic mites, larvae of a cerambycid beetle and larvae of the endemic *Leto venus* (ghost moth), in the death of *Virgilia* trees. Key fungal taxa were identified by comparisons of the internal transcribed spacer rDNA regions of the isolated taxa with those available on GenBank. Pathogenicity of the most commonly encountered fungal taxa was determined both in the field and under greenhouse conditions. Five different disease symptoms were observed on *Virgilia* trees throughout the CFR. At Table Mountain, *Virgilia oroboides* subsp*. oroboides* showed symptoms of: (1) several small cankers on stems, seemingly caused by a *Fusarium acuminatum*-like fungus, (2) a root rot disease caused by *Armillaria mellea* and (3) small bracket fungi on stems associated with *Schizophyllum commune*. *Virgilia oroboides* from the Harold Porter National Botanical Garden was diagnosed with a root disease consistently associated with an un-described *Phomopsis* species. *Virgilia oroboides* subsp. *ferruginea* and *V. divaricata* from Knysna and the Tsitsikamma area often showed symptoms of rapid wilting and death. The *Virgilia* stems were damaged by the tunnelling larvae of the ghost moth and those of an unidentified cerambycid beetle. Galleries and the surrounding wood tissues often housed the ophiostomatoid fungi *Ceratocystis tsitsikammensis* and *Ophiostoma plurianulatum*. These seem to originate from nitidulid beetles found feeding on gum exudate. Pathogenicity trials confirmed the virulence of the undescribed *Phomopsis* species, the *F. acuminatum*-like fungus, *S. commune* and *C. tsitsikammensis* to *Virgilia*. All four morpho-species of bark beetles found in this study, together with phoretic mites on two of the beetle morpho-species, were only collected from dead and dying *Virgilia* hosts and were classified as secondary pests. Both beetle taxa and mites commonly carried spores of various *Geosmithia* spp. These are not pathogenic to *Virgilia* trees, but may be an important food source for the

bark beetles, as it dominated the fungal community in galleries. The phoretic mites were unable to feed on their *Geosmithia* associates, but have been observed to feed on dead bark beetle larvae within galleries. This suggests that the relationship of bark beetles, mites and their associated *Geosmithia* species in this system is complex and in need of further study. Our results show that natural populations of *Virgilia* play host to numerous destructive pathogens, some of which are non-native (e.g. *A. mellea*) and a cause for special concern. Additionally, the isolation of the undescribed *Phomopsis* species and *A. mellea* from botanical gardens, with *A. mellea* now spreading to natural areas, calls for stricter control over the movement of organic material from these areas.

OPSOMMING

Die Kaapse Floristiese Streek (KFS) van Suid-Afrika is bekend vir buitengewoon hoë plantspesie-diversiteit en endemisme. Min aandag is egter tot dusver geskenk aan die peste en patogene in hierdie streek, al mag hulle plantverspreiding en evolusie dramaties beinvloed. In hierdie studie identifiseer ons verskeie geleedpotige diere en fungi as peste en organismes wat siektes veroorsaak in die ekologies en ekonomies belangrike, KFS-endemiese boom genus *Virgilia*. Ons het die sleutel fungi vanaf *Virgilia* oor die hele KFS geisoleer, geidentifiseer en hulle patogeniteit bepaal. Addisioneel het ons ook die rol van moontlike pes geleedpotiges, insluitende baskewers, cerambycid kewerlarwes en die endemiese *Leto venus* (spookmot) in die dood van *Virgilia* bome geevalueer. Sleutel fungi taksa is geidentifiseer deur die interne getranskribeerde spasieerder rDNS streke van die geisoleerde taksa met die wat op GenBank beskikbaar was te vergelyk. Patogenisiteit van die mees algemeen geisoleerde fungi taxa is beide in die veld en onder glashuis-toestande bepaal. Vyf verskillende siekte simptome is by *Virgilia* bome regdeur die KFS waargeneem. By Tafelberg het *Virgilia oroboides* subsp. *oroboides* simptome getoon van: (1) verskeie klein kankers op stamme, blykbaar veroorsaak deur 'n *Fusarium acuminatum*-agtige fungus, (2) 'n wortelvrot siekte veroorsaak deur *Armillaria mellea* en (3) klein rakswamme op stamme geassosieer met *Schizophyllum commune*. *Virgilia oroboides* in die Harold Porter Nationale Botaniese Tuin is gediagnoseer met 'n wortelvrot siekte wat altyd met 'n onbeskryfde *Phomopsis* spesie geassosieer is. *Virgilia oroboides* subsp. *ferruginea* and *V. divaricata* van Knysna en die Tsitsikamma area het dikwels simptome getoon van vinnige verwelking en dood. Die *Virgilia* stamme is deur die tonnelende larwes van die spookmot en dié van 'n ongeidentifiseerde cerambycid kewer beskadig. Galerye en die omringende houtweefsel het dikwels die ophiostomatoid fungi *Ceratocystis tsitsikammensis* en *Ophiostoma plurianulatum* gehuisves. Dit lyk asof hierdie fungi van nitidulid kewers afkomstig is wat op die gomuitskeidings gevoed het. Patogeniteitsproewe het die kwaadaardigheid van die onbeskryfde *Phomopsis* spesie, die *F. acuminatum*-agtige fungus, *S. commune* en *C. tsitsikammensis* teenoor *Virgilia* bevestig. Al vier morfo-spesies baskewer wat in hierdie studie gevind is, sowel as die foretiese myte op twee van die kewer morfo-spesies, is slegs van dooie of sterwende *Virgilia* gashere versamel, en is as sekondêre peste geklassifiseer.

iv

Beide kewerspesies en myt taksa het algemeen spore van verskeie *Geosmithia* spesies (*Geosmithia pallida*, *G. flava*, *G. microcorthyli*, *G*. sp. 1 en *G*. sp. 2) gedra. Die *Geosmithia* spesies is nie patogenies teenoor *Virgilia* bome nie, maar mag 'n belangrike voedselbron vir die baskewers wees, aangesien dit die fungus-gemeenskap in die galarye gedomineer het. Die foretiese myte was nie instaat om op *Geosmithia*-assosiate te voed nie, maar is waargeneem om op dooie baskewer larwes te voed binne die galerye. Dit stel voor dat die verhouding van die baskewers, myte en hulle geassosieerde *Geosmithia* spesies in die sisteem kompleks is, en verdere studie benodig. Ons resultate dui aan dat natuurlike populasies van *Virgilia* gashere is vir verskeie destruktiewe patogene, sommige waarvan nie-inheems (bv. *A. mellea*) wat 'n bron van groot kommer is. Verder noodsaak die isolasie van die *Phomopsis* spesie en *A. mellea*, wat beide wortelvrot siektes in botaniese tuine veroorsaak, strenger kontrole oor die verskuiwing van organiese materiaal uit hierdie areas, veral gegewe dat *A. mellea* reeds na natuurlike areas versprei het.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance, help, financial and moral support of several individuals and institutions, listed below, that in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

- I thank my personal Lord and saviour, Jesus Christ, for bringing me this far, the divine protection and being my strength and helper up to this day.

- I would like to express my sincere gratitude to my supervisors, Dr. F. Roets, Prof. L. L. Dreyer and Prof. J. Roux for their continuous support, guidance, advice and training throughout this study. I have been extremely lucky to have supervisors who cared so much about my work, and who responded to my needs, questions and queries so promptly. I was continually amazed by their willingness to proof read and edit countless pages of my thesis chapters. To Francois Roets and Léanne Dreyer I am forever grateful for their patience and steadfast encouragement to complete this study.

- My sincere gratitude goes to Stellenbosch University and departments of Botany and Zoology and Conservation Ecology and Entomology for their facilities and equipment.

- My sincere thanks goes to The Centre of Excellence In Tree Health Biotechnology for granting me a bursary and funding the research conducted in this study.

- I thank SANParks and CAPE Nature for granting me permission to work in their forests.

- I must express my gratitude to my husband and son, Keith and Arthur Mhlanga, for being my greatest inspiration and for their continued moral support and encouragement. My gratitude also goes to my in-laws, Mr. and Mrs. Mhlanga, for taking care of my son during my period of study, I am forever grateful for their love and care.

- Last but not the least, I would like to thank my colleagues and friends (Tendai Musvuugwa, Joice Ndhlovu, Anicia Mateyisi, Mohau Mateyisi, Dewidine Van Der Collf, Dr. Kenneth Oberlander, PC Benade, Janeke Allyward, Hannibal Musarurwa, Dr. Funlola Olojede, Chota Sulleman, Oluwaceyi Aboyade, Wale Aboyade) for their assistance with laboratory and field work and the friendship we had throughout the period of my study. Dr. Kenneth Oberlander took his time to teach me much about molecular work and phylogenetic analyses. Tendai Musvuugwa accommodated me at some stage. I am indebted to these for their help.

The work was presented at the **13th Student Conference on Conservation Science** at Cambridge University, UK, in March 2012 as a poster.

DEDICATIONS

I dedicate this thesis to my beloved husband, Keith Mhlanga, I love you so much.

This is also for my late parents who would have been so proud of my achievements and for fulfilling their dreams.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 4

CHAPTER 1

GENERAL INTRODUCTION

1.   The Greater Cape Floristic Region

The Greater Cape Floristic Region (GCFR, Born *et al*. 2007) hugs the South African coastline along the southwestern and southern tip of Africa (Fig. 1). It includes two biodiversity hotspots, the Cape Floristic Region (CFR, Goldblatt and Manning 2000) and the Succulent Karoo Region (Born *et al*. 2007). The Succulent Karoo Region is situated in the semi-arid southern valleys of the GCFR, extending to the north of the CFR along the coast onto the semi-arid uplands (Born *et al*. 2007). Both regions receive the majority of their rainfall during winter. The CFR encompasses an entire floral kingdom; the smallest of the six global floral kingdoms (Goldblatt and Manning 2000). Despite this small size, it is the richest per unit area, with almost 69% of flowering plant species endemic to the area (Goldblatt and Manning 2000). Faunal diversity is not as rich as that of the flora, with the possible exception of the invertebrates that also display exceptionally high levels of endemism in some habitats (Rebelo 1992).

Linder (2003) suggested that geographical and ecological isolation of the GCFR gave rise to the exceptionally high levels of endemism recorded in the region. The GCFR is isolated by arid regions to the north and east and two oceans in the south and west. The surrounding terrestrial areas also differ climatically from the GCFR and receive most precipitation during summer. Plant speciation rates in the GCFR has been high due to divergence caused by adaptation to a mosaic of physical environments, giving rise to high levels of species diversity (Linder 2003; Van der Niet and Johnson 2009). Drivers of this species diversification include different soil types, complex topography and differential seasonality and precipitation.

1

Different and complex interactions of these physical parameters create steep environmental gradients, resulting in this proposed speciation model (Goldblatt and Manning 2000; Linder 2003; Van der Niet and Johnson 2009).
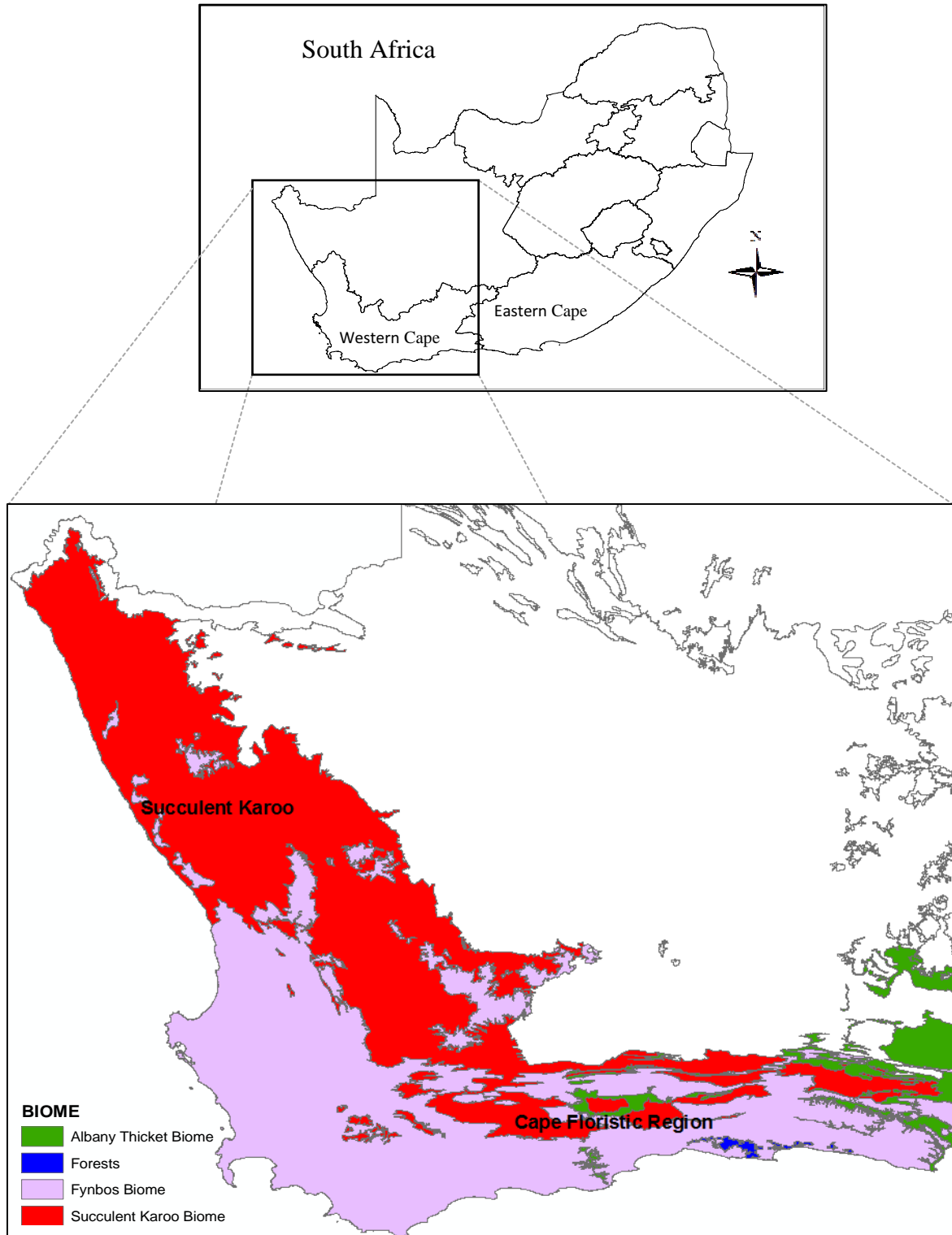
Fig.1. Map of the GCFR showing the CFR and Succulent Karoo with the four of the five included biomes (excluding Thicket biome) indicated.

2

Mucina and Rutherford (2006) recognize five biomes within the GCFR, of which the Fynbos Biome and the Succulent Karoo Biome are the most unique and species-rich (Cowling *et al*. 1997). The Fynbos Biome includes three major vegetation types, namely Fynbos, Renosterveld and Strandveld (Mucina and Rutherford 2006). Fynbos vegetation dominates the region, and is defined by the families Proteaceae, Ericaceae and Restionaceae. It is prone to fire and grows on infertile, sandy or rocky soils (Linder 2003; Goldblatt and Manning 2002). Renosterveld is the second largest vegetation type, dominated by Renosterbos (*Dicerothamnus rhinocerotis* (L. f.) Koekemoer), grasses and seasonally active geophytes and is specific to areas with richer, fine-grained soils (Goldblatt and Manning 2002). The Succulent Karoo Biome is found in arid parts and is dominated by succulent shrubs and sub-shrubs (Goldblatt and Manning 2002). The remaining biomes include thicket (semi-succulent and shrub land to low forest) and evergreen forest (Goldblatt and Manning 2002). Thickets are restricted to the eastern parts of the GCFR and mostly occur in river valleys. It is characterised by the lack of strata, with a mixture of shrubs, vines and evergreen succulent or sclerophyllous trees (Cowling 1984; Everard 1987; Vlok *et al*. 2003). The evergreen forests often have trees that are 10-30 meters high and are outliers of the tropical African high mountain Afromontane forests (Turpie *et al*. 2003).

1.1   Afromontane forests in GCFR

Afromontane forests in the GCFR are the southern remnants of a chain of the Afromontane forests of Africa (Turpie *et al*. 2003). They exist in small fragmented areas on mountains, foothills, coastal platforms, river valleys, coastal scarps and dunes and along the mountainous arc in the southwest of the Western Cape Province of South Africa (Geldenhuys 2010; Morgenthal and Cilliers 2000). According to Geldenhuys (1997), these forests are characterised by evergreen trees with closed canopies. They inhabit well-watered areas with relatively fertile, well-drained soils that are deep in valleys, but shallower on steeper slopes. Forest vegetation occur at altitudes that range from sea level to above 1 500 m. The largest Afromontane forests in South Africa are found in the Tsitsikamma and Knysna regions (Lubke and McKenzie 1996). Rainfall can exceed 2 000 mm annually (Lubke and McKenzie 1996). Dominant canopy tree species include yellowwoods (*Podocarpus* spp.), stinkwood (*Ocotea bullata* (Burch.) Baill), white pear (*Apodytes dimidiata* E. Mey. ex Arn.

subsp. *dimidiata*), Terblanz beech (*Faurea macnaughtonii* E. Phillips) and assegaai (*Curtisia dentata* C.A. Sm.). Species like *Virgilia divaricata* Adamson, *Laurophyllus capensis* Thunb. and *Wilddringtonia schwartzii* (Marloth) Mast. occupy forest margins at moist sites, while *Lachnostylis bilocularis* R.A. Dyer and *Loxostylis alata* A. Spreng. prefer the drier forest margins. The understory is multi-layered, with epiphytes and lianas common, but ground cover is almost absent because of canopy shade. These forests are fire intolerant and their expansion is limited by the frequent fires experienced by the surrounding fynbos (Mucina and Rutherford 2006).

To date, research in the GCFR mainly explored the botanical diversity and uniqueness of this region (Linder 2003; Taylor *et al*. 2001). Unfortunately, unlike in the Northern Hemisphere, the diversity of pests and pathogens associated with these forests in the GCFR have received very limited attention (Taylor *et al*. 2001), despite the critical importance of these organisms in ecosystem function (Castello *et al*. 1995). There is thus a great need for research focused on the interaction between pests and pathogens and the plant species that host them within the GCFR.

2.   PESTS AND PATHOGENS

2.1   Native insects and pathogens in natural ecosystems

Insect pests and pathogens are vital constituents of natural ecosystems and usually co-exist with their hosts, causing insignificant damage (Brasier 2008; Loo 2009). It is when there are sporadic outbreaks due to human or natural disturbances that weaken the hosts, in space and/or time, that the devastating effects of pests and pathogens on their hosts become apparent (Allard *et al*. 2003; Gilbert 2002). Global studies have indicated that pests and diseases impact species abundance and diversity, thus also forest structure and composition, diversity and succession (Anagnostakis 1987; Brasier 1990; Gilbert 2002; Haack and Byler

1993; Von Broembsen 1989). The survival, growth rate and health status of seedlings are also influenced by pests and pathogens, thereby influencing the abundance and distribution of plant species (Abdurahman 1992; Gilbert 2002; Speight and Wylie 2001). This has led to the development of the escape hypothesis (Connell 1971; Howe and Smallwood 1982), which states that seedlings far away from their parents or other conspecific species, survive attack from pests and pathogens better than seedlings in close vicinity to their mother trees and conspecific trees. This results in juvenile populations growing a distance away from their parents after some time. Survival of seedlings is believed to increase with increased dispersal distance and with a decrease in seedling density (Augspurger and Kelly 1984). Seedlings of *Platypodium elegans* Vogel in Barro moist forest, Colorado Island, Panama, exemplify the escape theory well (Augspurger and Kelly 1984). These seedlings suffered high levels of mortality from damping-off fungal pathogens when growing close to their parents and other conspecific trees and when the seedling density was high (Augspurger and Kelly 1984). In another study in the same forest by Gilbert *et al*. (1994), seedlings of *Ocotea whitei* Woodson showed characteristics of the escape theory. Seedlings growing around parent trees contracted stem canker disease, while those growing away from parental and/or conspecific trees tended to be healthy.

Arthropods such as the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann (Coleoptera, Curculionidae, Scolytinae), can also shape the structure and function of forests (Schowalter *et al*. 1981). Hosts of the SPB in south-eastern United States coniferous forests include *Pinus palustris* Mill. (longleaf pine), *P. echinata* Mill. (shortleaf pine), *P. taeda* L. (loblolly pine) and *P. elliottii* Engelm. (slash pine) (Schowalter *et al*. 1981). Longleaf and slash pines thrive in areas with high SPB activity, while at low SPB activity, shortleaf and loblolly pines have a competitive advantage, because their seedlings grow rapidly and are shade tolerant (Walker 1962). The result is that upland forest sites are dominated by longleaf pine and shortleaf pine, because the more susceptible loblolly pine is selectively eliminated by the SPB (Schowalter *et al*. 1981). Lowland forests favour growth of dense hardwood understory trees, such that pines become more susceptible to attack due to stress from overcrowding and other abiotic conditions (Walker 1962). The SPB eliminates all the stressed pines, enhancing the transformation of lowland pine and pine-hardwood forests into hardwood forests (Schowalter *et al*. 1981).

Pests and pathogens can cause significant economic losses in the forestry industry. The SPB is capable of killing healthy pine trees because it is extremely aggressive (Payne 1980). It caused an estimated $900 million loss in pine production in the southern United States from 1960 to 1990 (Price *et al.* 1992). During 2000 and 2001, Belize, Nicaragua and Honduras lost over 60 000 hectares of mature and developing pine stands in severe SPB outbreaks (Billings and Schmidtke 2002). In Mediterranean Europe and North Africa, an outbreak of larvae of the processionary moth (*Thaumetopoea pityocampa* (Denis & Schiffermüller)) severely defoliates several pine hosts, including *Pinus brutia* Ten., *P. nigra* Arnold and *P. halepensis* Miller (Hódar *et al*. 2002; Speight and Wainhouse 1989), reducing their growth and reproduction. This leads to population declines and huge economic losses (Hódar *et al*. 2002). Very little is, however, known about the impacts of pests and pathogens on native systems in South Africa, or about how they shape these systems. If the current emphasis on biodiversity conservation and sound ecosystem management with sustainable resource use is to be achieved in South Africa (Reyers and McGeoch 2007), the roles played by pests and pathogens must be carefully scrutinised.

2.2 Introduced pests and pathogens

Invasions and accidental introduction of non-native pests and pathogens pose great hazards to natural ecosystems and forest plantations (Brasier 2008; Fraedrich *et al*. 2008; Liebhold 1995; Hansen 2008). Introduced pests and pathogens, without their natural enemies and in the presence of vulnerable hosts they did not co-evolve with, can cause outbreaks with devastating consequences (Brasier 2008; Desprez-Loustau *et al*. 2007; Loo 2009). Humans greatly facilitate the dispersal of pests and pathogen into new areas through trade in international agricultural and forest products, long distance air travel, seaborne trade, exchanges of plant material and through inefficient quarantine measures (Brasier 2008; Desprez-Loustau *et al*. 2007; Jones and Baker 2007; Loo 2009; Pimentel *et al*. 2001; Skarpaas and Økland 2009; Tatem *et al*. 2006; Von Broembsen 1989). The chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, is a good example of this threat. The fungus was introduced to the United States from Asia, probably on ornamental nursery seedlings, in the late 1890s (Walker 1957; Loo 2009). It almost eliminated American chestnut trees (*Castanea dentate* (Marsh.) Borkh.) in the eastern USA, completely changing natural forest structure

and species composition of both macro- and micro-flora and fauna (Anagnostakis 1987; Brasier 2008; Loo 2009; Quimby 1982). It was also introduced to Europe, where it affected native *Castanes sativa* Mill. (Brasier 2008) trees. Another example is Dutch elm disease, caused by *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which rapidly spread on American elms throughout Europe, North America and central Asia (Brasier 2008; Loo 2009; Tainter and Baker 1996). The rapid spread was aided by both its native vector, the European elm beetle (*Scolytus multistriatus* Marsh.(Scolytinae)) and the native American elm bark beetle (*Hylurgopinus rufipes* Eichhoff (Scolytinae)) (Bingham *et al*. 1971; Loo 2009; Quimby 1982). The streets and forests of America and Europe lost their aesthetic value and most areas were left bare, while several organisms like insects, mammals, micro-organisms and birds lost their habitats and food sources (Brasier 2008; Loo 2009; Osborne 1985). In another, more recent example, the Redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Scolytinae), was introduced to the southeastern USA, along with a fungal associate, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva (Fraedrich *et al*. 2008; Harrington *et al*. 2008). This beetle and its fungal associate have been causing a severe wilt disease of Redbay trees (*Persea borbonia* (L.) Spreng.) and other members of the Lauraceae in the USA (Whitney 1982; Harrington *et al*. 2008). These Lauraceae trees and shrubs are vital habitats for *Papilio troilus* L. (spicebush swallowtail) and *Papilio palamedes* Drury (Palamedes swallowtail) and food sources for wildlife (Reed and Muzika 2010), such that their extensive death can negatively impact organisms depending on them for survival.

The invasion of Jarrah forests in Western Australia by *Phytophthora cinnamomi* Rands provides a good example of the invasion of natural ecosystems by pathogens in the Southern Hemisphere. This pathogen caused extensive death of the dominant canopy tree species, *Eucalyptus marginata* Donn ex Sm., and of other native plants in the mid- and under-storey, causing significant changes to the floral and faunal diversity (Brasier 2008; Davison and Shearer 1989; Desprez-Loustau *et al*. 2007). It also spread to other natural ecosystems such as national parks and state forests from Cape Arid in the south-west to Kalbarri National Park in Western Australia, Brisbane Ranges, Wilsons Promontory and the Grampians, Victoria and Adelaide Hills in South Australia (Podger 1972; Weste 1994, 2003), as well as other areas world-wide (Brasier, 2008; Desprez-Loustau *et al*. 2007). Human disturbance and repeated droughts accelerated the spread of this plant pathogen (Hardham 2005; Podger 1972; Weste

7

1994, 2003). Similarly, the introduction of the European wood wasp, *Sirex noctilio* Fabricius, and its associated fungus, *Amylostereum areolatum* (Fr.) Boidin, into pine plantations in Australia, Chile, New Zealand and South Africa (Gilbert and Miller 1952; Tribe 1995), signifies the devastating effect of introduced pests and pathogens on plantation forestry. *Sirex noctilio* is native to southern Europe and North Africa, and causes little or no damage to its host trees in its native range (Gilbert and Miller 1952). In non-native ranges, however, it has attacked several *Pinus* L. species in plantations, including *Pinus caribaea* Morelet*, Pinus contorta* var. *latifolia* Douglas ex Louden Engelm. ex S. Watson*, Pinus elliottii* Engelm., *Pinus muricata* D. Don*, Pinus patula* Schiede & Deppe*, P. pinaster* Aït. *, Pinus ponderosa* Laws.*, Pinus radiata D. Don, Pinus taeda* L. and *Pinus sylvestris* L. *Pinus patula, P. radiata* and *P. taeda* have been identified as particularly susceptible. *Sirex noctillio* has caused very extensive tree mortality, resulting in major financial losses (Carnegie *et al*. 2005; Haugen *et al*. 1990; Hurley *et al*. 2007; Tribe 1995; Tribe and Cillié 2004).

2.3   Successful utilisation of newly encountered hosts by forest pests and pathogens

Pests and pathogens are often capable of utilising newly encountered hosts, both indigenous and non-native, especially when the new hosts are phylogenetically related to the original/previous hosts (Brasier 2008; Desprez-Loustau *et al*. 2007; Skarpaas and Okland 2009; De Vienne *et al*. 2009). Several factors contribute to the successful utilisation of a new host. Two principal hypotheses help to summarize the processes and factors involved, namely the host relatedness and the host habitat hypothesis (Nikoh and Fukatsu 2000; Norton and Carpenter 1998; Spatafora *et al*. 2007). The host relatedness hypothesis suggests that an organism could more easily utilise newly encountered hosts that are phylogenetically related to the natural host as it may provide similar resources. In contrast, the habitat hypothesis explains how some pests and pathogens can use unrelated hosts when it encounters a new host in a microhabitat similar to its original host. When a pest or pathogen successfully utilises newly encountered hosts, it can lead to epidemics, resulting in devastating disruptions of natural ecosystems.

Examples of the utilisation of non-native trees by native organisms and of native trees by non-native organisms abound in the literature. Ohmart and Edwards (1991) described how several exotic *Eucalyptus* L'Hér species in China were severely attacked by a total of 96 indigenous insects, with leaf cutting ants and termites as the major pests. Gryzenhout *et al*. (2004) and Wingfield (2003) reported that various species of the fungal genus *Chrysoporthe* Gryzenh. & M.J. Wingf. could successfully infect both native and introduced hosts in the Myrtales in the respective areas of origin of these fungi. For example, *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf. (Cryphonectriaceae) is an economically important pathogen that causes canker disease and death of *Eucalyptus* species in southern Africa (Nakabonge *et al*. 2006; Roux *et al*. 2005; Wingfield *et al*. 1989). It is most likely native to Africa (Heath *et al*. 2006; Nakabonge *et al*. 2006 ; Van der Merwe *et al*. 2010; Vermeulen *et al*. 2011), but has adapted to related, but non-native trees in Africa, including Australian species of *Eucalyptus* and the ornamental purple glory tree (*Tibouchina granulosa* (Desr.) Cogn. (Melastomataceae)) (Myburg *et al*. 2002), resulting in disease on these trees.

As discussed above, infection of newly encountered hosts by fungal pathogens may have considerable ecological impacts on natural ecosystems. In South Africa the infection of native Proteaceae in the Fynbos vegetation by the introduced pathogens *Armillaria mellae* (Vahl. Fr.) Kummer and *A. gallica* Marxm. & Romagn. (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010), may have severe impacts on natural systems. The Proteaceae is recognised as one of the defining plant families in fynbos vegetation (Linder 2003; Goldblatt and Manning, 2002). Since its introduction into Cape Town *ca*. 360 years ago, *A. mellea* has successfully colonised and killed the species *Leucadendron argenteum* (L.) R.Br., *L. gandogeri* Schinz ex Gand., *L. grandiflorum* (Salisb.) R.Br., *Protea longifolia* Andrews, *P. eximia* (Salisb. ex Knight) Fourc. and *P. scolymocephala* (L.) Reichard, and other ornamental trees and native shrubs in Kirstenbosch National Botanical Garden (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010). *Armillaria gallica* is considered a secondary pathogen and has also been causing mortality of the same species as *A. mellea*, already infected and weakened by *Phytophthora cinnamomi* (Coetzee *et al*. 2003). Both *Armillaria* species are thus likely to severely negatively impact natural ecosystem function, as these taxa often form the structurally dominant components in fynbos communities. However, at this stage these pathogens seem to be confined to semi-natural areas and gardens.

2.4   Tree pests and pathogens in South African forestry and viticulture industries

The South African commercial forestry industry contributes significantly to economic development and employment in South Africa. In 2008/9, the industry contributed R20 376 million to the gross domestic product (GDP) of South Africa (FSA, 2010). Unfortunately both native and introduced pests and pathogens often cause significant losses to the industry (Mitchell *et al*. 2004; Crous 2005; Wingfield *et al*. 2008). For example, the pitch canker fungus *Fusarium circinatum* Nirenberg & O'Donnell, rated among the most important pathogenic threats to pines in most parts of the world (Carlucci *et al*. 2007; Coutinho *et al*. 2007; Dwinell *et al*. 2001; McCain *et al*. 1987; Santos and Tovar 1991; Viljoen *et al*. 1994; Wingfield *et al*. 2002), threatens the sustainability of South African pine forestry industry (Mitchell *et al*. 2011). It causes collar root infections of nursery stock and transplanted seedlings (Bayley and Blakeway 2002; Crous 2005; Mitchell *et al*. 2004; Viljoen *et al*. 1994; Wingfield *et al*. 2002, 2008) and the pitch canker disease that leads to serious mortalities on mature trees (Coutinho *et al*. 2007; Wingfield 1999). The most susceptible species is *Pinus patula*, with other species like *P. radiata* and *P. greggii* Engelm. ex Parl. also being attacked (Coutinho *et al*. 2007; Mitchell *et al*. 2011). *Teratosphaeria nubilosa* (Cooke) Crous & U. Braun, a serious leaf pathogen of *Eucalyptus* trees, is another example. South Africa had to abandon the commercial propagation of *Eucalyptus globulus* Labill. around the 1930s because of this pathogen (Lundquist and Purnell 1987). An alternative species, *Eucalyptus nitens* H.Deane & Maiden, had to be used, although seedlings originating from Victoria, Australia, are also susceptible to this fungus, particularly in the first two to three years of growth (Hunter *et al*. 2009). *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris is a serious wilt pathogen of *Acacia mearnsii* De Wild., causing rapid wilt and mortality in plantations (Roux and Wingfield 1997; Roux 2002; Roux *et al*. 1999). Armillaria root rot, caused by *Armillaria fuscipes* Petch, is another serious disease in pine plantations, especially in areas previously occupied by indigenous trees (Coetzee *et al*. 2000). The disease has also been recorded on *Eucalyptus* species in the Sabie area and on *Acacia mearnsii* (Wingfield and Knox-Davies 1980).

Acacia mearnsii is damaged by several indigenous insect pests in South Africa (Govender 2007). According to Govender (2007), these pests belong to different feeding guilds, including bud and flower feeders, root and bark feeders, leaf eaters and seed insects. The most damaging pests among these is the bagworm, *Chaliopsis* (*Kotochalia*) *junodi* Heylaerts (Lepidoptera: Psychidae), which is a serious defoliating pest. According to Atkinson and Laborde (1996), 12 000-20 000 ha of wattle plantations are infested annually and data from 1953-1994 showed that an average of 25% of planted areas were infested. Insecticides were used to control this pest and 800-4 000 ha were sprayed annually. Other damaging insects include larvae of white grubs (Coleoptera: Scarabaeidae: Melolonthinae, Rutelinae) and cutworms (Lepidoptera: Noctuidae), grasshoppers (Orthoptera: Acrididae, Pyrgomorphidae) and millipedes (Diplopoda: Juliformia), amongst others (Govender 2007).

Bark and ambrosia beetles are well-known to cause extensive losses in commercial forestry, and may pose an equally large threat to native trees, resulting in loss of biodiversity (FAO 2007). *Hylastes angustatus* Herbst is a bark beetle that ring-barks pine seedlings, causing significant losses in newly established plantations (Kirsten *et al*. 2000; Tribe 1992). These Scolytine beetles are often associated with pathogenic fungi such as ophiostomatoid fungi (Whitney 1982; Wingfield *et al.* 1993), which can be pathogens or sap-stainers (Zhou *et al*. 2002). Zhou *et al*. (2002) isolated *Leptographium lundbergii* Lagerb. & Melin and *L. serpens* (Goid.) Siemaszko from *H. angustatus* collected from *Pinus patula* Scheide et Deppe in Mpumalanga Province. In the same study, *Ophiostoma ips* (Rumbold) Nannf. was isolated from *Orthotomicus erosus* Wollaston that was collected from *P. patula* in Mpumalanga and *Pinus elliottii* Engelm. in Kwazulu-Natal. *Ophiostoma ips* and *L. lundbergii* are common sap-stain pathogens of pines (Gibbs 1993; Zhou *et al*. 2002).

The genus *Phomopsis* includes numerous species of economic interest. Phomopsis cane and leaf spot disease of grapevines is common in most countries where these vines are grown (Punithalingam 1979). According to Mostert *et al*. (2001b), this disease was first recorded in South Africa in 1935, and has since spread to several areas in the Western Cape Province. It is caused by *Phomopsis viticola* (Sacc.) Sacc. and can cause up to a 50% loss of normal fruit yield (Mostert *et al*. 2001b).

Similar to pathogens, numerous pest arthropods have been documented on native plants. Again most of these are from commercially important plant species. For example, in 1990, Höppner and Ferreira recorded presence of the native quince borer, *Coryphodema tristis* (Drury) (Lepidoptera: Cossidae) on grape vines (*Vitis vinifera* L.) in the southwestern Cape, South Africa. Its gregarious feeding behaviour is destructive as the larvae bores into trunks and arms of grapevines, feeding and living inside the wood, causing serious tunnelling in the wood and eventually die-back. It is reported to have a wide range of native plant hosts, including quince (*Cydonia oblonga* Mill.) and bushwillows (*Combretum* Loefl. sp.) (Picker *et al*. 2002).

2.5   Tree pests and pathogens on native South African trees

Very little documented information is available on the pests and diseases of native trees in South Africa compared to the Northern Hemisphere and Australia. In South Africa, past phytopathogenic research includes published work of Doidge (1924, 1950), Doidge and Bottomley (1931) and Doidge *et al*. (1953), which was later revised and updated by Gorter (1977, 1979, 1981, 1982). Other old documented examples include Phytophthora root rot caused by *Phytophthora cinnamomi* Rands. This non-native pathogen is soil-borne and has a wide, non-specific host range (Von Broembsen 1989; Zentmyer 1980). It was first recorded on members of the genus *Protea* L. (Proteaceae) in South Africa in 1931, both in the wild and in cultivated areas (Doidge and Bottomley 1931; Wager 1931). Other hosts of this pathogen include, among many other fynbos plants and the tree species, *Ocotea bullata* (Burch.) Baill. in southern Cape forests (Lübbe and Geldenhuys 1990; Von Broembsen 1989; Von Broembsen and Kruger 1985). It caused extensive death of *Leucospermum cordifolium* (Salisb. Ex Knight) Fourc. (common pincushion) in 1976 on a commercial farm in the southwestern Cape Province (Von Broembsen and Brits 1985).

More recent documentations include the discovery by Roux and Coetzee (2005) of the pink disease pathogen, *Erythricium salmonicolor* (Berk. & Broome) Burds, on *Dais cotonifolia* L., *Podocarpus henkelii* Stapf ex Dallim. & A.B.Jacks. and *P. latifolius* (Thunb.) R.Br. ex Mirb. in Mpumalanga Province and *Ekebergia capensis* Sparrn. and *Maesa lanceolata* Forsk. in the

Midlands of Kwazulu-Natal. In 2007, Roux *et al*. found the well-known *Acacia mearnsii* wilt and die-back pathogen, *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris, on several native trees. Host tree species include *Acacia caffra* (Thunb.) Wild., *Burkea africana* Hook, *Combretum molle* R.Br. ex G.Don, *Ochna pulchra* Hook, *Protea gaguedi* J.F.Gmel. and *Terminalia sericea* Burch. ex DC, amongst other hosts. This fungus was shown to most likely be native to Africa and has not been found to cause mortality of native hosts in South Africa (Roux *et al*. 2007). A recently described fungal pathogen, *Graphium adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux, was found on wounds on *Adansonia digitata* L. made by elephants in the Kruger National Park and a motor vehicle on the N1 highway near Mesina (Cruywagen *et al*. 2010). In another example, Mehl *et al*. (2010) attributed the die-back and death of *Pterocarpus angolensis* DC. (kiaat) to the interaction between drought, fire stress and fungal pathogens. Several fungal species isolated from dead and dying kiaat trees included *Candida*, *Humicola*, *Penicillium*, with *Cytospora* spp., *Lasiodiplodia theobromae* and *Fusarium* spp. suggested as the potential pathogens (Mehl *et al*. 2010). A follow up study on the kiaat die-back and death suggested Botryospaeriaceae species and environmental stresses to be responsible for mortalities (Mehl *et al*. 2011). Among the isolated species, a newly described fungus *Diplodia alatafructa* J.W.M. Mehl & B. Slippers and *Lasiodiplodia pseudotheobromea* A.J.L. Phillips, A. Alves & Crous proved to be pathogenic to kiaat trees, with the latter fungus suggested as the species responsible for die-back and death of kiaat due to its high virulence and common occurrence (Mehl *et al*. 2011).

Some of the most recent examples include the recently described virulent pathogen, *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf., & Jol. Roux, causing serious cankers on stems and branches and mortality of *Rapanea melanophloeos* (L.) Mez. trees in Harold Porter National Botanical Garden in Betty's Bay (Chen *et al*. 2012). During pathogenicity tests, the fungus killed the inoculated stems and branches (Chen *et al*. 2012). Van der Linde *et al*. (2012) isolated *Gondwanamyces serotectus* van der Linde, Jol. Roux and *Gondwanamyces ubusi* van der Linde, Jol. Roux from diseased *Euphorbia ingens* E. Meyer: Boissier trees and from insects (and inside their tunnels) associated with dying *E. ingens* trees. Both fungal species proved to be pathogenic to *E. ingens* trees during pathogenicity trials and hence suggested both fungi as the cause of the decline of *E. ingens* (Van der Linder *et al*. 2012).

Similar to pathogens, few records of pest arthropods have been documented on native plants. Examples of such records include the native quince borer, *Coryphodema tristis* (Drury) (Lepidoptera: Cossidae), which was reported to have a wide range of native plant hosts, including quince (*Cydonia oblonga* Mill.) and bushwillows (*Combretum* Loefl. sp.) (Picker *et al*. 2002). Orwa *et al.* (2009) identified pests of *Accacia karoo* Haines as the psychid wattle bagworm (*Kotochalia junodi*), a defoliator and several species of Bruchids that are seed parasites.

In the CFR, most studies have included pathogens from native Proteaceae, as they are often grown commercially and numerous records of fungal pathogens to this family have been published. In a study by Coetzee *et al.* (2003), *Armillaria gallica* and *A. mellea* were isolated from dead and diseased *Leucadendron argenteum*, *L. gandogeri*, *L. grandiflorum*, *Protea longifolia*, *P. eximia* and *P. scolymocephala* in the Kirstenbosch National Botanical Garden. In another study by Mostert *et al*. (2001a), *Phomopsis saccharata* J.-C. Kang, L. Mostert & P. W. Crous was identified as the cause of a canker and die-back disease of *Protea repens* (L.) L. on Jonkershoek Mountains in Stellenbosch. Rooibos tea is obtained from an indigenous leguminous shrub, *Aspalathus linearis* (N.L.Burm.) R.Dahlgr., and is susceptible to the die-back disease caused by a complex of *Phomopsis* Sacc. (teleomporh: *Diaporthe*) species. Outbreaks of this disease in 1989 led to large economic losses, as 89% of 3 year old plants in cultivation were affected in the Clanwilliam area alone (Van Rensburg *et al*. 2006). The order of pathogenicity of the five pathogens isolated from *A. linearis* in that study was as follows: *Diaporthe aspalathi* E. Jansen, Castl. & Crous (formerly identified as *D. phaseolorum* (Cke. & Ell.) Sacc. or *D. phaseolorum* var. *meridionalis* F.A.. Fernández) was the most virulent pathogen, followed by by *D. ambigua* Nitschke, *Phomopsis theicola* Curzi, a *Libertella* Desm. species and a *Phomopsis* species (Van Rensburg *et al*. 2006).

2.6   Bark and ambrosia (scolytine) beetles

Bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) rate among the most economically important forestry and forest pests globally (Avtzis *et al*. 2012; Harrington 2005; Linnakoski *et al*. 2012; Paine *et al*. 1997; Wood 1982b). Worldwide there are more

than 6 000 species of these beetles from 225 genera (Avtzis *et al*. 2012; Six 2012). Scolytine beetles are distributed across a wide range of host tree taxa and can be categorized based on the status of host plant substrate (Six 2012). These are recognised as "nearly obligate parasitic" (primary), "facultative parasitic" (secondary) or "saprophytic" (Linnakoski *et al*. 2012; Raffa *et al*. 1993) pests.

Primary scolytine beetles are very aggressive and can kill host trees due to mass colonization (Linnakoski *et al*. 2012; Paine *et al*. 1984; Raffa *et al*. 1993; Six and Wingfield 2011; Wood 1982a). Healthy trees defend themselves from attack by producing chemicals like phenolics, monoterpenes and resins that immobilize, flush out, kill, or suffocate the insects (Christiansen *et al*. 1987; Franceschi *et al*. 2005; Hudgins *et al*. 2004; Raffa and Smalley 1995; Six and Wingfield 2011). Despite these defence mechanisms, a very large outbreak of these insects may overcome the natural defence of trees, causing mortality (Berryman 1982; Franceschi *et al*. 2005; Six and Wingfield 2011; Thatcher *et al*. 1980). The mountain pine beetle (*Dendroctonus ponderosae* (Hopkins)) is an example of a primary scolytine beetle and according to Lewis and Hartley (2006), it killed pine trees in an area spanning more than 7 million ha during an outbreak from 1993 to 2006 in North America. The most affected trees were lodge-pole pines (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm), while ponderosa pine (*Pinus ponderosa* P. Laws. ex C. Laws.), western white pine (*Pinus monticola* Dougl. ex D. Don) and white bark pine (*Pinus albicaulis* Engelm.) were also attacked, but to a lesser extent (Lewis and Hartley 2006).

Secondary scolytine beetles are known to attack stressed, weakened and recently dead trees (Avtzis *et al*. 2012; Paine *et al*. 1997; Raffa *et al.* 1993; Six and Wingfield 2011). They also feed on logging residue or fallen trees (Paine *et al*. 1997). Examples of this group include *Ips confusus* LeConte, which destroyed an estimated 40-80% of drought stressed *Pinus edulis* Engelmann in the southwestern United States between 2002 and 2003 (Breshears *et al*. 2005). These trees are the dominant species throughout this region (Breshears *et al*. 2005).

The saprophytic scolytine beetles constitute the largest group and exclusively colonize dead hosts (Paine *et al*. 1997; Raffa *et al*. 1993). Their saprophytic feeding behaviour makes them important initiators of wood decomposition (Jordal and Cognato 2012). They help in nutrient recycling through digestion of pectins and cellulose (Levieux *et al*. 1989) when they introduce saprophytic fungi that decompose wood (Halloin 2003).

2.7   Bark and ambrosia beetles and their microbial associates

Scolytine beetles have complex associations with micro- and macro-organisms, including bacteria (Bridges 1984), fungi (Linnakoski *et al*. 2012; Six and Paine 1998; Six and Wingfield 2011; Whitney 1982), mites (Cardoza *et al*. 2008; Klepzig *et al*. 2001; Moser *et al*. 1995, 2005; Roets *et al*. 2009) and nematodes (Cardoza *et al*. 2008; Moser *et al*. 2005). Some associations are mutualistic, whereby, for example, some fungi provide nutritional benefits to the beetles (Harrington and Zambino 1990; Hofstetter *et al*. 2006; Klepzig *et al*. 2001) and bacteria help to enhance digestion of host tissues (Brand *et al*. 1976). The scolytine beetles transport the fungi and bacteria to new hosts for feeding and survival purposes. Bacterial associates may also benefit the bark beetles by inhibiting pathogenic fungi (Cardoza *et al*. 2006). However, some fungal associates of these bark and ambrosia beetles are pathogenic to both the beetles and the host trees, such as the blue-staining ascomycete *Ophiostoma minus* (Hedgecock) H. & P. Sydow on the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmerman (Barras 1970; Harrington and Zambino 1990; Hofstetter *et al*. 2006; Klepzig *et al*. 2001; Six and Wingfield 2011).

Scolytine beetles that can colonize host trees in huge numbers, with their fungal associates, are of particular interest to forestry and natural ecosystems (Kirkendall 1983; Klepzig *et al*. 2001; Moser *et al.* 1995; Wood 1982). These beetles can be grouped into two ecological groups, those that exercise fungal farming, the ambrosia beetles, and those that do not exercise fungal farming, the bark beetles (Jordal and Cognato 2012). Scolytine ambrosia beetles bore into xylem and sapwood of host trees, inoculating specialized fungi during colonization and excavation of new tunnels, which will grow on tunnel walls and serve as principal food for their larvae (Beaver 1989; Cognato and Grimaldi 2009; Jordal and Cognato

2012; Rabaglia *et al*. 2006; Six 2012; Wood 1982b). Scolytine bark beetles and their larvae primarily feed on subcortical tissue, phloem beneath the bark, although their fungal associates may have nutritional value to the beetles and may enhance palatability of their food (Cognato and Grimaldi 2009; Jordal and Cognato 2012; Rabaglia *et al*. 2006; Six 2012; Wood 1982b).

Several studies have shown mutualistic and/or antagonistic relationships between scolytine beetles and fungi. Spores and/or mycelia of the fungal associates can either be carried on the exoskeletons of these beetles or inside cuticular structures called mycangia (Francke-Grosmann 1967; Paine *et al*. 1997; Six 2003; Six and Wingfield 2011). The fungi are inoculated into trees when the trees are attacked by the beetles. Klepzig *et al*. (2001) suggested that the manner in which fungal associates are carried determines their role in the life cycles of bark and ambrosia beetles. Fungi carried inside mycangia provide a source of nutrition to larvae of the beetles, while those transported externally may be pathogenic to host trees and antagonistic to beetle larvae. Fungi benefit by being vectored to new plant hosts (Paine *et al*. 1997; Six 2003). The SPB, for example, vectors *Ceratocystiopsis ranaculosa* J.R. Bridges & T.J. Perry and *Entomocorticium sp.* A (an undescribed Basidiomycete) in their mycangia. The SPB is thought to feed on these fungi (Harrington and Zambino 1990; Klepzig *et al*. 2001). The SPB beetle is also known to carry the blue-staining ascomycete *Ophiostoma minus* externally on their exoskeleton. This fungus is known to be a particularly virulent pathogen to host trees and also antagonistic to this beetle (Barras 1970; Harrington and Zambino 1990; Rumbold 1936; Klepzig *et al*. 2001). The ambrosia beetle, *Microcorthylus* Ferrari sp., was found gardening *Geosmithia microcirthyli* M. Kolařík in its tunnels on *Cassia grandis* L.f. host trees in Costa Rica (Kolařík and Kirkendall 2010). According to Kolařík and Kirkendall (2010), *G. microcirthyli* dominated the ambrosial layers of these beetles and had nutritional value to both larvae and adults.

2.8   Scolytine beetles, mites and fungi

The relationship between mites phoretic on bark and ambrosia beetles and their fungal associates has not received enough scientific research attention in the Southern Hemisphere

(Roets *et al.* 2009). It is known that scolytine beetles carry several species of mites, which are parasitic, predatory, fungivorous, nematophagous and/or omnivorous (Cardoza *et al.* 2008; Hofstetter *et al.* 2006; Kinn 1984; Klepzig *et al.* 2001; Moser 1975, 1995). Phoretic fungivorous mites are particularly important to forestry. They are transported from tree to tree on the bodies of these beetles and neither undergo morphogenesis nor feed during the transportation period (Moser and Roton 1971; Smiley and Moser 1974). Some have specialised flap-like structures called sporothecae, in which ascospores of fungi are transported, and they are capable of feeding on the fungi they transport in their sporothecae (Klepzig *et al.* 2001; Roets *et al.* 2009).

The relationship between bark and ambrosia beetles, phoretic mites and fungi is well-studied in the SPB system. Several studies have shown that the SPB is associated with 57 mite species (Moser and Roton 1971), with only a few species truly phoretic (Moser and Roton 1971; Smiley and Moser 1974). Species of the genus *Tarsonemus* (Acari: Tarsonemidae) are truly phoretic and the common associates of SPB. This genus includes *Tarsonemus ips* Lindquist, *Tarsonemus krantzii* Smiley and Moser, and *Tarsonemus fusarii* Cooreman (Klepzig *et al.* 2001). *Tarsonemus ips* and *T. krantzii* carry spores of *Ophiostoma minus* and/or *Ceratocystiopsis ranaculosa* in their sporothecae (Klepzig *et al.* 2001; Moser *et al.* 1995). During a study by Klepzig *et al.* (2001), all three mite species successfully reproduced and showed positive growth rates when fed on new hyphal growth of their fungal associates. This suggests a mutual relationship between the mites and their fungal associates. The fungal associates of the mites can also be pathogenic to host trees (Hofstetter *et al.* 2006; Klepzig *et al.* 2001; Levieux *et al.* 1989; Six and Wingfield 2011).

Fungal taxa, other than ophiostomatoid fungi, are also common associates of scolytine beetles and mites. *Trichosporium symbioticum* Wright is an associate of *Scolytus ventralis* LeConte (Livingston and Berryman 1972 in Paine *at al.* 1997), and the genus *Geosmithia* Pitt (Hypocreales: Boinectriaceae and Eurotiales: Trichocomaceae) recently emerged as common associates of scolytine beetles (Cizkova *et al.* 2005; Jiri and Dunn 2011; Kolařík *et al.* 2004, 2005, 2007; Ogawa *et al.* 1997). It is distributed worldwide and has more than 20 bark beetle associates in central Europe alone and five ambrosia beetles in the tropics of Asia, America

and Australia (Kirschner 2001; Kolařík *et al*. 2007, 2008; Kubátová *et al*. 2004). They usually replace ophiostomatoid fungi as main associates of a number of scolytine beetles that infest deciduous trees (Kirschner 2001; Kolařík *et al*. 2004, 2005, Kubátová *et al*. 2004). *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley & Tisserat was found associated with the walnut twig beetle (*Pityophthorus juglandis* Blackman) (Tisserat *et al*. 2009). It is recognised as a serious threat to black walnut trees (*Juglans nigra* L.), causing the disease known as thousand cankers disease (Tisserat *et al*. 2009). *Geosmithia* and other fungal associates of scolytine beetles that are not particularly phytopathogenic are usually ignored in most studies and very little is known about what their roles are in these associations (Kolařík *et al*. 2004; Six and Wingfield 2011).

Inter-organismal associations are understudied in natural systems in general and even more so in South Africa. Studies by Zhou *et al*. (2002) highlighted the importance of bark beetles and their associated fungi in natural ecosystems and plantations of non-native tree species, respectively. These beetles and their fungal associates may be a threat to South African native ecosystems, especially when such ecosystems are severely impacted by anthropogenic or climatic threats.

---

3.   PROBLEM IDENTIFICATION

Recently, personal observations revealed extensive decline and death of *Virgilia* trees in populations at Betty's Bay, Table Mountain and Silver Mine Nature Reserve in the Western Cape Province of South Africa. Pilot studies suggested that these deaths may be attributed to attacks by a range of unidentified fungal species and/or bark beetles species. These pests and pathogens seem to be non-specific in terms of the age-class of the trees they attack, as many seedlings, saplings and mature trees were found to be affected. It is unknown whether these bark beetles and fungi are native or were introduced to South Africa, since such incidences have never been recorded before.

3.1   The genus *Virgilia* Pior.

The genus *Virgilia* (Fabaceae) is endemic to the Western Cape Province of South Africa and comprises of two species, *Virgilia oroboides* (P. J. Bergius) Salter and *Virgilia divaricata* Adamson. Both species were formerly known as *Virgilia capensis* Brown (Phillips 1926, 1928). *Virgilia oroboides* is further divided into subspecies *oroboides* and subspecies *ferruginea* B–E. van Wyk (Palgrave 2002). *Virgilia* trees are known to be fast growing and short-lived; the average lifespan ranges from 12 to 20 years (Mbambezeli and Notten 2003). Despite their short life expectancy, both species produce long-lived seed banks (up to 150 years) (Goldblatt and Manning 2000). *Virgilia* species are found in riparian vegetation, thickets, on hillsides and along forest margins (Palmer and Pitman 1972) throughout the western and southern regions of the CFR.

Virgilia oroboides has compound leaves with 13 to 25 impari-pinnate leaflets that have a broadly tapering tip with a hair-like apex (Palgrave 1983; Palgrave 2002; Palmer and Pitman 1972; Van Wyk and Van Wyk 1997). Leaflets are glossy green above and covered with dense hairs below (Van Wyk and Van Wyk 1997). Its flowers can be pale or dark pink and are carried in short racemes. Pods are velvety brown, flat and dehiscent (Palgrave 1983; Palmer and Pitman 1972). The leaflets of *V. oroboides* subspecies *oroboides* have whitish hairs abaxially and its flowers are white or pink, while subspecies *ferruginea* has rusty hairs on the abaxial side of its leaflets and the flowers are violet-purple or rose-violet (Van Wyk and Van Wyk 1997). This species flowers from January to April (Palmer and Pitman 1972) and can reach up to 15 m in height (Mbambezeli and Notten 2003; Palgrave 2002). *Virgilia oroboides* is found along the coastal regions of the CFR, in a range that extends from George in the east to the Cape Peninsula in the west (Palmer and Pitman 1961, 1972).

Virgilia divaricata has compound leaves with usually 11 to 19 impari-pinnate leaflets (Palgrave 2002). The leaflets appear green, because they are almost hairless. It has pinkish mauve to violet-pink flowers with dark-tipped keels in short racemes (Palmer and Pitman 1972; Van Wyk and Van Wyk 1997). According to Palgrave (2002), this species hardly exceeds 10 m in height. It flowers from August to December and pods are dehiscent, flat and

velvety brown (Palgrave 1983; Palmer and Pitman 1972). It is commonly found in the southern and eastern parts of the CFR and has been recorded in the Klein Swartberg Mountains, and then eastward form George to Port Elizabeth. It reaches inland to as far north as Grahamstown, and up to an altitude of 1200 m (Palgrave 1983; Palgrave 2002; Palmer and Pitman 1961, 1972). It is also common along the Keurbooms River, which was named after this tree (Smith 1966).

Keurboom trees are today known from Australia, USA and England, where they have been in cultivation as ornamental trees for many years. They were introduced to England around 1767 (Palmer and Pitman 1961, 1972; Smith 1966). In the USA they are known as Cape Lilac or Tree-in-a-hurry.

3.2   Importance of *Virgilia* trees

Virgilia trees are important, both ecologically and for subsistence use. Their elimination from the ecosystem could detrimentally impact other organisms that depend on them for feeding, breeding or as habitats. Such organisms include the rare ghost moth (*Leto venus* Cramer), which lays its eggs only on Keurboom bark, just above the ground-level. The stems of *Virgilia* trees are the only source of food (monophagy) for the caterpillars of this moth (Nielsen *et al*. 2000). The caterpillars bore into the trunk and stems of the tree, where they feed and live (Palmer and Pitman 1972; Van Wyk and Van Wyk 1997). Loss of *Virgilia* trees can thus directly lead to the extinction of this moth species. The moth has adaptively evolved to utilize *Virgilia* trees as breeding sites and as its larval food source, and is thus unlikely to find an alternative tree species to fulfil these functions. Another species, the Lucerne Blue butterfly (*Lampides boeticus* Linnaeus) also breeds on *Virgilia*, as well as on the crop lucerne (Palmer and Pitman 1972). *Virgilia* trees are also important habitats for nesting white-eyes and doves and their flowers are very rich in nectar for honey bees, ants, sunbirds and carpenter bees (Palmer and Pitman 1961).

Virgilia trees are important pioneer trees in forest plant succession, because they grow well and fast in the open, thereby creating essential shade for slower-growing, more permanent tree species (Geldenhuys 1994; Phillips 1926; Van Daalen 1981). Due to their dense foliage, *Virgilia* trees are wind tolerant and often act as wind screens for the other young forest tree species. Without the nursing role of the *Virgilia* species, such areas will never fully develop into a stable and diverse forest (Geldenhuys 1994; Van Daalen 1981). The subsistence uses of *Virgilia* trees include the making of yokes, rafters and furniture from its soft and light wood (Palmer and Pitman 1961; Smith 1966). Traditionally, the transparent gum has also been used as a starch substitute (Palmer and Pitman 1961, 1972).

3.3   Aims and objectives of study

We hypothesise that the death of *Virgilia* trees is attributed to fungal diseases and bark beetle attacks and that both species of *Virgilia* at all stages of development are equally susceptible to attack by fungi and bark beetles.

The main aim of this study was to identify the bark beetles and fungal species that are attacking native *Virgilia* trees across their natural range, and to determine the sequence of attack by these beetles and pathogens. The specific objectives were to:

1. Establish the extent of fungal and bark beetle attack on *Virgilia* species throughout their natural range.

2. Establish which stage of development of *Virgilia* trees is more susceptible to attack.

3. Determine whether both *Virgilia* species and their subspecies are equally susceptible to bark beetle and fungal attack.

4. Isolate and identify the fungal species infecting *Virgilia* trees in the Cape Floristic Region.

5. Determine the pathogenicity of key fungal species isolated from *Virgilia* species.

6. Isolate and identify the bark beetle species attacking *Virgilia* trees in the Cape Floristic Region.

7. Isolate and identify fungal associates of bark beetles attacking *Virgilia* species.

8. Determine the pathogenicity of key fungal species isolated from the bark beetles (and the mites they may vector) collected from *Virgilia* species.

REFERENCES

Abdurahman A, 1992. Research need in forest entomology. In: *Proceedings of the National Workshop on Setting Forestry Research Priorities in Ethiopia*, April 27-30 1992. Forestry Research Centre, Addis Ababa, Ethiopia, pp. 113-121.

Atkinson PR, Laborde de RM, 1996. Estimates of the extent of infestation and potential losses caused by common forestry pests in South Africa. In: Nair KSS, Sharma JK, Varma RV (eds.), *Impact of Diseases and Insect Pests in Tropical Forests*. Proceedings of the IUFRO symposium, 23-26 November 1993, Peechi, India, pp. 274-288.

Allard GB, Fortuna S, See LS, Novotny J, Baldini A, Courtinho T, 2003. Global information on outbreaks and impact of major forest insect pests and diseases. XII World Forestry Congress, Canada.

Anagnostakis SL, 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* **79**: 23-37.

Augspurger CK, Kelly CK, 1984. Pathogen mortality of tropical tree seedlings: experimental studies of the effects of dispersal distance, seedling density, and light conditions. *Oecologia* **61**: 211-217.

Avtzis DN, Bertheau C, Stauffer C, 2012. What is next in bark beetle phylogeography? *Insects* **3:** 453-472.

Barras SJ, 1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. *Annals of the Entomological Society of America* **63**: 1187-90.

Bayley AD, Blakeway F, 2002. Deployment strategies to maximise value recovery from tree improvement: the experience of two South African companies. *Southern African Forestry Journal* **195**: 11-22.

Beaver RA, 1989. Insect - fungus relationships in the bark and ambrosia beetles. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds.), *Insect - fungus interactions*. London: Academic, pp. 121-143.

Berryman AA, 1982. Population dynamics of bark beetles. In: Mitton JB, Austin SKB (eds.), *Bark Beetles in North American Conifers*. University Texas, pp. 264-314.

Billings RF, Schmidtke PJ, 2002. Central American southern pine beetle/fire management assessment. College Station, Texas, USA, USAID. (Unpublished report).

Bingham RT, Hoff RT, McDonald GI, 1971. Disease resistance in forest trees. *Annual Review Phytopathology* **2**: 433-452.

Born J, Linder HP, Desmet P, 2007. The Greater Cape Floristic Region. *Journal of Biogeography* **34**: 147-162.

Brand JM, Bracke JW, Britton LN, Markovetz AJ, Barras SJ, 1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis. Journal of Chemical Ecology* **2**: 195-199.

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

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

Breshears DD, Cobb NS, Rich PM, Price KP, Allen CD, Balice RG, Romme WH, Kastens JH, Floyd ML, Belnap J, Anderson JJ, Myers OB, Meyer CW, 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 JR, 1984**.** A quantitative study of the yeasts and bacteria associated with laboratory-reared *Dendroctonusfrontalis* Zimm (Coleopteran: Scolytidae). *Journal of Applied Entomology* **97**: 261.

Cardoza YJ, Klepzig KD, Raffa KF, 2006. Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology* **31**: 636-645.

Cardoza YJ, Moser JC, Klepzig KD, Raffa KF, 2008. Multipartite symbioses among fungi, mites, nematodes and the spruce beetle, *Dendroctonus rufipennis. Environmental Entomology* **37**(4): 956-963.

Carlucci A, Colatruglio L, Frisullo S, 2007. First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Disease* **91**: 1683.

Carnegie AJ, Eldridge RH, Waterson DG, 2005. The history and management of sirex wood wasp, *Sirex noctilio* (Hymenoptera: Siricidae), in pine plantations in New South Wales, Australia. *New Zealand Journal of Forestry Science* **35**: 3-24.

Castello JD, Leopold DJ, Smallidge PJ, 1995. Pathogens, patterns and processes in Forest Ecosystems. *BioScience* **45**(1): 16-24.

Chen SF, Wingfield MJ, Roets F, Roux J, 2012. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea melanophloeos* in South Africa. *Plant Pathology* (In press).

Christiansen E, Waring RH, Berryman AA, 1987. Resistance of conifers to bark beetle attack: searching for general relationships. *Forest Ecology and Management* **22**: 89-106.

Čizkova D, Šrutka P, Kolarik M, Kubatova A, Pazoutova S, 2005. Assessing the pathogenic effect of *Fusarium*, *Geosmithia* and *Ophiostoma* Fungi from broad-leaved Trees. *Folia Microbiologica* **50**(1): 59-62.

Coetzee MPA, Wingfield BD, Coutinho TA, Wingfield MJ, 2000. Identification of the causal agent of Armillaria root rot in pine plantations in South Africa. *Mycologia* **92**: 777-785.

Coetzee MPA, Wingfield BD, on Harring TC, Steimel J, Coutinho TA, Wingfield MJ, 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* **10**: 387-396.

Coetzee MPA, Wingfield BD, Roux J, Crous PW, Denman S, Wingfield MJ, 2003. Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa. *Plant Pathology* **52**: 604-612.

Cognato AI, Grimaldi D, 2009. 100 million years of morphological conservation in bark

beetles (Coleoptera: Curculionidae: Scolytinae). *Systematic Entomology* **34**: 93-100.

Connell JH, 1971. On the role of natural enemies in preventing competitive exclusion in some marine animals and in rainforest trees. In: den Boer PJ, Gradwell R (eds.), *Dynamics of Populations*. Proceedings of the Advanced Study Institute on Dynamics of numbers in Populations. Centre for Agricultural Publishing and Documentation, Wageningen, pp. 298-310.

Cowling RM, 1984. A syntaxonomic and synecological study in the Humansdorp region of the Fynbos Biome. *Bothalia* **15**(1): 177-227.

Cowling RM, Richardson DM, Mustart PJ, 1997. Fynbos. In: Cowling RM, Richardson DM, Piece SM (eds.), *Vegetation of South Africa*. Cape Town: Cambridge University Press, pp. 99-123.

Coutinho TA, Steenkamp ET, Mongwaketsi K, Wilmot M, Wingfield MJ, 2007. First outbreak of pitch canker in a South African pine plantation. *Australasian Plant Pathology* **36**: 256-261.

Crous JW, 2005. Post establishment survival of *Pinus patula* in Mpumalanga, one year after planting. *Southern African Forestry Journal* **205**: 3-8.

Cruywagen EM, De Beer ZW, Roux J, Wingfield MJ, 2010. Three new *Graphium* species from baobab trees in South Africa and Madagascar. *Persoonia* **25**: 61-71.

Davison EM, Shearer BL, 1989. *Phytophthora* spp. in indigenous forests in Australia. *New Zealand Journal of Forestry Science* **19**: 277-289.

De Vienne DM, Hood ME, Giraud T, 2009. Phylogenetic determinants of potential host shifts in fungal pathogens. *Journal of Evolutionary Biology* **22**: 2532-2541.

Desprez-Loustau ML, Robin C, Buée M, Courtecuisse R, Garbaye J, Suffert F, Sache I, Rizzo DM, 2007. The fungal dimension of biological invasions. *TRENDS in Ecology and Evolution* **22**(9): 472-480.

Doidge EM, 1924. A preliminary checklist of plant diseases occurring in South Africa. *Botanical Survey of South Africa, Memoir No*. **6**: 1-56.

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

Doidge EM, Bottomley AM, 1931. A revised list of diseases occurring in South Africa. *Botanical Survey of South Africa, Memoir No*. **11**: 1-78.

Doidge EM, Bottomley AM, Plank JE van der, Pauer GD, 1953. A revised list of plant diseases in South Africa. *Union of South Africa, Department of Agriculture, Science Bulletin* **346**: 1-122.

Dwinell LD, Fraedrich SW, Adams D, 2001. Diseases of pines caused by the pitch canker fungus. In: Summerell BA, Leslie JF, Backhouse D, Bryden WL, Burgess LW (eds.), '*Fusarium*: *Paul E. Nelson Memorial Symposium'*. The American Phytopathological Society: St Paul, MN, pp. 225-232.

Everard DA, 1987. A classification of the subtropical transitional thicket in the eastern Cape, based on syntaxonomic and structural attributes. *South African Journal of Botany* **53**(5): 329-340.

FAO, 2007. Over view of forest pests South Africa. Forestry Department. Working Paper FBS/30E. FAO, Rome, Italy.

Forestry South Africa, 2010. Forestry and Forest Products Industry Facts 1980-2009. *Forestry South Africa*: (Online). Available at: http://www.forestry.co.za. (2012, October 28).

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR, 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. *Plant Diseases* **92**:215-224.

Franceschi VR, Krokene P, Christiansen E, Krekling T, 2005**.** Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist* **167**: 353-376.

Francke-Grosmann H, 1967. Ectosymbiosis in wood inhibiting insects. In: Henry SM (ed.), *Symbiosis*. New York: Academic **2**: 141-205.

Geldenhuys CJ, 1994. Bergwind fires and the location pattern of forest patches in the southern Cape landscape, South Africa. *Journal of Biogeography* **21**: 49-62.

Geldenhuys CJ, 1997. Composition and biogeography of forest patches on the inland mountains of the southern Cape. *Bothalia* **27**(1): 57-74.

Geldenhuys CJ, 2010. Distribution, size and owenrship of forests in the southern Cape. *South African Forestry Journal* **158**: 51-66.

Gibbs JN, 1993. The biology of Ophiostomatoid fungi causing sap-stain in trees and freshly cut logs. In: Wingfield MJ, Seifert KA, Webber JF (eds.), *Ceratocystis and Ophiostoma*. *Taxonomy, Ecology and Pathogenicity*. American Phytopathological Press, St. Paul, Minnesota, pp. 153-160.

Gilbert GS, 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology* **40**: 13–43.

Gilbert GS, Hubbell SR, Foster RB, 1994. Density and distance-to-adult effects of a canker disease of trees in a moist tropical forest. *Oecologia* **98**: 100-108.

Gilbert JM, Miller LW, 1952. An outbreak of *Sirex noctilio* (F.) in Tasmanis. *Australian Forestry* **16**: 63-69.

Goldblatt P, Manning J, 2000. Cape plants: A conspectus of the Cape flora of southern Africa. National Botanical Intitute, Cape Town & Missouri Botanical Garden, St Louis.

Goldblatt P, Manning J, 2002. Plant diversity of the Cape region of southern Africa. *Annals of the Missouri Botanical Garden* **89**(2): 281-302.

Govender P, 2007. Status of seedling establishment pests of *Acacia mearnsii* De Wild. (Mimosaceae) in South Africa. *South African Journal of Science* **103:** 141–147.

Gorter GJMA, 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. *Republic of South Africa Department of Agricultural Technical Services Science Bulletin* **392**: 1–177.

Gorter GJMA, 1979. An annotated check list and selected bibliography of South African fungi for the period 1946-1977. *Republic of South Africa Department of Agricultural Technical Services Science Bulletin* **163**: 1-34.

Gorter GJMA, 1981. Index of plant pathogens II and the diseases they cause in wild growing plants in South Africa. *Republic of South Africa Department of Agricultural Technical Services Science Bulletin* **398**: 1-84.

Gorter GJMA, 1982. Supplement to Index of plant pathogens (I). *Republic of South Africa Department of Agricultural Technical Services Science Bulletin* **392**: 1–14.

Gryzenhout M, Myburg H, van der Merwe NA, Wingfield BD, Wingfield MJ, 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. *Studies in Mycology* **50**: 119-142.

Haack RA, Byler JW, 1993. Insects and pathogens: Regulators of forest eco-systems. *Journal of Forestry* **91**: 32-37.

Halloin L, 2003. Major bark beetles of the intermountain west. Washington Department of Natural Resources.

Hansen EM, 2008. Alien forest pathogens: *Phytophthora* species are changing world forests. *Boreal Environmental Research* **13**: 33–41.

Hardham AR, 2005. Pathogen profile: *Phytophthora cinnamomi*. *Molecular Plant Pathology* **6**(6): 589-604.

Harrington TC, 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE, Blackwell M (eds.), *Ecological and Evolutionary Advances in Insect-Fungal Associations*. Oxford University Press, New York, pp. 257-291.

Harrington TC, Fraedrich SW Aghayeva DN, 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxonomy* **104**: 399-404.

Harrington TC, Zambino PJ, 1990. *Ceratocystiopsis ranaculosis* not *Ceratocystis minor* var. *barrasii* is the mycangial fungus of the southern pine beetle. *Mycotaxonomy* **38**: 103-115.

Haugen DA, Bedding RA, Underdown MG, Neumann FG, 1990. National strategy for control of *Sirex noctilio* in Australia. *Australian Forest Grower* **13**: special liftout section No. 13: 8.

Heath RN, Gryzenhout M, Roux J, Wingfield MJ, 2006. Discovery of *Chrysoporthe cubensis* on native *Syzygium* species in South Africa. *Plant Disease* **90**: 443-438.

Hódar JA, Zamora R, Castro J, 2002. Host utilisation by moth and larval survival of pine processionary caterpillar *Thaumetopoea pityocampa* in relation to food quality in three *Pinus* species. *Ecological Entomology* **27**: 292-301.

Hofstetter RW, Cronin J, Klepzig KD, Moser JC, Ayres MP, 2006. Antagonisms, mutualisms and commensalisms affect outbreak dynamics of the southern pine beetle. *Oecologia* **147**: 679-91.

Höppner GFJ, Ferreira JHS, 1990. Fungi Associated with the Quince Borer, *Coryhodema tristis* (Drury) (Lepidoptera: Cossidae), in Grapevines. *South African Journal for Enology and Viticulture* **11**(2): 67-69.

Howe HF, Smallwood J, 1982. Ecology of seed dispersal. *Annual Review of Ecology and Systematics* **13**: 201-228.

Hudgins JW, Christiansen E, Franceschi VR, 2004. Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. *Tree Physiology* **24:** 251-264

Hunter GC, Crous PW, Carnegie AJ, Wingfield MJ, 2009. *Teratosphaeria nubilosa*, a serious leaf disease pathogen of *Eucalyptus* spp. in native and introduced areas. *Molecular Plant Pathology* **10**: 1–14.

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

Jiri H, Dunn RR, 2011. The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. Proceedings of the Royal Society Biological Siences, p. 1130-1138.

Jones DR, Baker RHA, 2007. Introductions of non-native plant pathogens into Great Britain, 1970–2004. *Plant Pathology* **56**: 891–910.

Jordal BH, Cognato AI, 2012. Molecular phylogeny of bark and ambrosia beetles reveals multiple origins of fungus farming during periods of global warming. *BMC Evolutionary Biology* **12**: 133-151.

Kinn DN, 1984. Life cycle of *Dendrolaelaps neodisetus* (Mesostigmata: Digamasellidae), a nematophagous mite associated with pine bark beetles (Coleoptera: Scolytidae). *Environmental Entomology* **13**: 1141-1144.

Kirkendall LR, 1983. The evolution of mating systems in bark and ambrosia beetles (Coleoptera: Scolytidae). *Zoological Journal of the Linnean Society* **77**: 293-352.

Kirsten JF, Tribe GD, van Rensburg NJ, Atkinson PR, 2000. Insect pests in South African forest plantations. In: Owen DL (ed.), *South African Forestry Handbook*, South African Institute of Forestry, pp. 221-239.

Kirschner R, 2001. Diversity of filamentous fungi in bark beetle galleries in Central Europe. In: Misra JK, Horn BW (eds), Trichomycetes and other Fungal Groups: Professor Robert W. Lichtwardt Commemoration Volume. Science Publishers, Enfield, pp. 175-196.

Klepzig KD, Moser JC, Lombardero MJ, Hofstetter RW, Ayres MP, 2001. Symbiosis and competition: complex interactions among beetles, fungi and mites. *Symbiosis* **30**: 83-96.

Kolařík M, Kirkendall LR, 2010. Evidence for a new lineage of primary ambrosia fungi in Geosmithia Pitt (Ascomycota: Hypocreales). *Fungal Biology* **114**: 676-689.

Kolařík M, Kubátová A, Cepicka I, Pazoutova S, Srutka P, 2005. A complex of three new white-spored, sympatric, and host range limited *Geosmithia* species. *Mycological Research* **109**(12): 1323-1336.

Kolařík M, Kubátová A, Hulcr J, Pažoutová S, 2008. *Geosmithia* fungi are highly diverse and consistent bark beetle associates: Evidence from their community structure in temperate Europe. *Microbial Ecology* **55**(1): 65-80.

Kolařík M, Kubátová A, Pažoutová S, Srutka P, 2004. Morphological and molecular characterisation of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. *Mycological Research* **108**(9): 1053-1069.

Kolařík M, Kostovčík M, Pažoutová S, 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. *Mycological Research* **111**(11): 1298-1310.

Kubátová A, Kolařík M, Prasil K, Novotny D, 2004. Bark beetles and their galleries: well-known niche for little known fungi, case of *Geosmithia*. *Czech Mycology* **55**: 1-18.

Levieux J, Lieutier F, Moser JC, Perry TJ, 1989. Transportation of phytopathogenic fungi by the bark beetle *Ips sexdentatus* Boerner and associated mites. *Journal of Applied Entomoloy* **108**: 1-11.

Lewis KJ, Hartley ID, 2006. Rate of deterioration, degrade, and fall of trees killed by mountain pine beetle. *BC Journal of Ecosystems and Management* **7**(2):11-19.

Liebhold AM, Macdonald WL, Bergdahl D, Maestro VC, 1995. Invasion by exotic forest pests – a threat to forest ecosystems. *Forest Science Monographs* **30**: 1-49.

Linder HP, 2003. The radiation of the Cape flora, southern Africa. *Biological Reviews* **78:** 597-638.

Linnakoski R, de Beer ZW, Niemelä P, Wingfield MJ, 2012. Associations of Conifer-infesting bark beetles and fungi in Fennoscandia. *Insects* **3**: 200-227.

Livingston RL, Berryman AA, 1972. Fungal transport structures in the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae). *Canadian Entomologist* **104**: 1793-800.

Lundquist JE, Purnell RC, 1987. Effects of Mycosphaerella leaf spot on growth of *Eucalyptus nitens*. *Plant Disease* **71**: 1025-1029.

Loo JA, 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* **11**: 81-96.

Lübbe WA, Geldenhuys CJ, 1990. Decline and mortality of *Ocotea bullata* trees in the southern Cape forests. *South African Forestry Journal* **154**: 7-14.

Lubke RA, Mackenzie B, 1996. Afromontane Forest. In: Low AB, Rebelo GA (eds.), *Vegetation of South Africa, Lesotho and Swaziland*. Pretoria: Department of Environmental Affairs and Tourism, p. 12.

Mbabezeli G, Notten A, 2003. *Virgilia divaricata* Adamson and *Virgilia oroboides* (Berg.) Salter. South African National Biodiversity Institute, South Africa.

Mehl JWM, Geldenhuys CJ, Roux J, Wingfield MJ, 2010. Die-back of kiaat (*Pterocarpus angolensis*) in southern Africa: a cause for concern? *Southern Forests: a Journal of Forest Scien*ce **72**: 3-4, 121-132.

Mehl JWM, Slippers B, Roux J, Wingfield MJ, 2011. Botryosphaeriaceae associated with *Pterocarpus angolensis* (kiaat) in South Africa. *Mycologia* **103**: 534–553.

McCain AH, Koehler CS, Tjosvold SA, 1987. Pitch canker threatens California pines. *California Agriculture* **41**: 22-23.

Mitchell RG, Steenkamp ET, Coutinho TA, Wingfield MJ, 2011. The pitch canker fungus, *Fusarium circinatum*: implications for South African forestry. *Southern Forests: a Journal of Forest Science* **73**(1): 1-13

Mitchell RG, Zwolinski J, Jones NB, Coutinho TA, 2004. The effect of applying prophylactic measures on the post-planting survival of *Pinus patula* in South Africa. *Southern African Forestry Journal* **200**: 51-58.

Morgenthal TL, Cilliers SS, 2000. Species composition and phytogeographical significance of an afromontane forest fragment in the Mpumalanga province, South Africa. *The Southern African Forestry Journal* **189** (1): 81-102.

Moser JC, 1975. Mite predators of the southern pine beetle. *Annals of the Entomological Society of America* **68**: 1113-1116.

Moser JC, Roton LM, 1971. Mites associated with southern pine bark beetles in Allen Parish, Lousiana. *Canadian Entomologist* **103**: 1775-1798.

Moser JC, Perry TJ, Bridges JR, Yin H.-F, 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus*, a mycangial fungus of the southern pine beetle. *Mycologia* **87**: 84-86.

Moser JC, Konrad H, Kiristis T, Carta LT, 2005. Phoretic mites and nematodes associates of *Scolytus multistriatus pygmaeus* (Coleoptera: Scolytidae) in Austria. *Journal of Agricultural and Urban Entomology* **7**: 169-177.

Mostert L, Crous PW, Kang JC, Phillips AJL, 2001b. Species of *Phomopsis* and a *Libertella* sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterization. *Mycologia* **93**(1): 146-167.

Mostert L, Kang J.-C, Crous PW, Denman S, 2001a. *Phomopsis saccharata* sp. nov., causing a canker and die-back disease of *Protea repens* in South Africa. *Sydowia* **53**(2): 227-235.

Mucina L, Rutherford MC (eds.), 2006. Vegetation of South Africa, Lesotho and Swaziland. Streletzia 19. Pretoria: *South African National Biodiversity Institute*: 77: 93-94: 181-182.

Myburg H, Gryzenhout M, Heath R, Roux J, Wingfield BD, Wingfield MJ, 2002. Cryphonectria canker on *Tibouchina* in South Africa. *Mycological Research* **106**: 1299–1306.

Nakabonge G, Gryzenhout M, Roux J, Wingfield BD, Wingfield MJ, 2006. *Celoporthe dispersa* gen. et sp. nov. from native Myrtales in South Africa. *Studies in Mycology* **55**: 255-267.

Nielsen ES, Robinson GS, Wagner DL, 2000. Ghost-moths of the world; a global inventory and bibliography of the Exoporia (Mnesarchaeoidea and Hepialoidea). (Lepidoptera). *Journal of Natural History* **34**(6): 823-837.

Nikoh N, Fukatsu T, 2000. Interkingdom host jumping underground: Phylogenetic analysis of entomoparasitic fungi of the genus Cordyceps. *Molecular Biology and Evolution* **17**: 629-638.

Norton DA, Carpenter MA, 1998. Mistletoes as parasites: host specificity and speciation. *Trends in Ecology and Evolution* **13**: 101-105.

Ogawa H, Yoshimura A, Sugiyama J, 1997. Polyphyletic origin of species of the anamorphic genus *Geosmithia* and the relationships of the cleistothecial genera: evidence from 18S, 5S, and 28S rDNA sequence analysis. *Mycologia* **89**: 756-771.

Ohmart CP, Edwards PB, 1991. Insect herbivory on eucalypts. *Annual Review of Entomology* **36**: 637-657.

Orwa C, Mutua A, Kindt R, Jamnadass R, Simons A, 2009. Agroforestree Database: a tree reference and selection guide version 4.0 (http://www.worldagroforestry.org/af/treedb/).

Osborne P, 1985. Some effects of the Dutch Elm Disease on the birds of a dorset dairy farm. *Journal of Applied Ecology* **22**: 681-691.

Paine TD, Raffa KF, Harrington TC, 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42***: 179-206.

Paine TD, Stephen FM, Taha HA, 1984. Conceptual model of infestation probability based on bark beetle abundance and host tree susceptibility. *Environmental Entomology* **13**: 619-24.

Palgrave KC, 1983. Keith Coated Palgrave Trees of Southern Africa. Edition 2. Struik, Cape Town.

Palgrave CM, 2002. Keith Coates Palgrave Trees of southern Africa. Edition 3. Struik, Cape Town.

Palmer E, Pitman N, 1961. Trees of South Africa. Balkema. Cape Town.

Palmer E, Pitman N, 1972. Trees of South Africa Vol. 2. Balkema. Cape Town.

Payne TL, 1980. Life history and habits. In: Thatcher RC, Searcy JL, Coster JE, Hertel GD (eds.), *The southern Pine Beetle. USDA Forest Service Technical Bulletins* **1631**: 7-23.

Phillips JFV, 1926. *Virgilia capensis* Lam. ('Keurboom'): A contribution to its ecology and silviculture. *South African Journal of Science* **23**: 435-454.

Phillips EP, 1928. *Virgilia capensis*. *The flowering plants of South Africa* 8: 305.

Picker M, Griffiths C, Weaving A, 2002. Field guide to Insects of South Africa. Struik, pp. 440.

Pimentel D, McNair S, Janecka J, Wightman J, Simmonds C, O'Connell C, Wong E, Russel L, Zern J, Aquino T, Tsomondo T, 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture, Ecosystems and Environment* **84**: 1-20.

Price TS, Doggett C, Pye JL, Holmes TP (eds.), 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.

Podger FD, 1972. *Phytophthora cinnamomi*, a cause of lethal disease in indigenous plant communities in West Australia. *Phytopathology* **62**: 972-981.

Punithalingam E, 1979. *Phomopsis viticola*. CMI Descr no 635. Kew, Surrey, England: CMI.

Quimby jr. PC, 1982. Impact of diseases on plant populations. In: Charudattan R, Walker HL (eds.), *Biological Control of Weeds with Plant Pathogens*, John Wiley & Sons, New York, pp. 47-60.

Rabaglia RJ, Dole SA, Cognato AI, 2006. Review of American Xyleborina (Coleoptera: Curculionidae: Scolytinae) occurring North of Mexico, with an illustrated key. *Annals of the Entomological Society of America* **99**: 1034-1056.

Raffa KF, Phillips TW, Salom SM, 1993. Strategies and mechanisms of host colonization by bark beetles. In: Schowalter T, Filip G (eds.), *Beetle-Pathogen Interactions in Conifer Forests.* San Diego: Academic, pp. 102-28.

Raffa KF, Smalley EB, 1995. Interaction of pre-attack and induced monoterpene concentrations in host conifer defence against bark beetle-fungal complexes. *Oecologia* **102**(3): 285-295.

Rebelo AG, 1992. Preservation of biotic diversity. In: Cowling RM (ed.), *The ecology of Fynbos, nutrients, fire and diversity*. Oxford University Press, Cape Town.

Reed SE, Muzika RM, 2010. The influence of forest stand and site characteristics on the composition of exotic dominated ambrosia beetle communities (Coleoptera: Curculionidae: Scolytinae). *Environmental Entomology* **39**(5): 1482-1491.

Reyers B, McGeoch MA, 2007. A biodiversity monitoring framework for South Africa: progress and directions. *South African Journal of Science* **103**: 295-300.

Roets F, Crous PW, Wingfield MJ, Dreyer LL, 2009. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* **38**(1):143-152.

Roux J, Coetzee MPA, 2005. First report of pink disease on native trees in South Africa and phylogenetic placement of *Erythricium salmonicolor* in the Homobasidiomycetes. *Plant Disease* **89**: 1158-1163.

Roux J, Dunlop R, Wingfield MJ, 1999. Susceptibility of elite *Acacia mearnsii* families to Ceratocystis wilt in South Africa. *Journal of Forestry Research* **4**: 187-90.

Roux J, Heath RN, Labuschagne L, Nkuekam GK, Wingfield MJ, 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 GC, Nakabonge G, Heath RN, Wingfield MJ, 2005. Diseases of plantation forestry tree species in eastern and southern Africa. *South African Journal of Science* **101**: 409-413.

Roux J, 2002. Threats to Wattle plantations III: Diseases. In: Dunlop RW, MacLennan LA (eds.) *Black wattle: The South African research experience*Institute for Commercial Forestry Research, Pietermaritzburg, South Africa, pp. 121-126.

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

Rumbold CT, 1936. Three blue-stain fungi, including two new species, associated with bark beetles. *Journal of Agricultural Research* **52**: 419-37.

Santos JJG, Tovar DB, 1991. Algunos aspectos sobre el cancro resinoso de los pinos. In 'VI Simposio Nacional Sobre Parasitologia Forestal, October 1991'. p. 31. (Unidad de Xcongresos del Colegio de Postgraduados Montecillos: Edo, Mexico).

Schowalter TD, Coulson RN, Crossley Jr. DA, 1981. Role of southern Pine beetle and fire in maintenance of structure and function of the southern coniferous forest. *Environmental Entomology* **10**: 821-825.

Six DL, 2003**.** Bark beetle-fungus symbioses. In: Bourtzis K,Miller T (eds.), *Insect symbioses*. CRC, Boca Raton, FL, pp. 96-116.

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

Six DL, De Beer ZW, Beaver RA, Visser L, Wingfield MJ, 2005. Exotic invasive elm bark beetle, *Scolytus kirschii*, detected in South Africa: research in action. *South African Journal of Science* **101**(5/6): 229-232.

Six DL, Paine TD, 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomol*ogy **27**: 1393-1401.

Six DL, Wingfield MJ, 2011. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. *Annual Review Entomology* **56**: 255-72.

Skarpaas O, Økland B, 2009. Timber import and the risk of forest pest introductions. *Journal of Applied Ecology* **46**: 55-63.

Smiley RT, Moser JC, 1974. New Tarsonemids associated with bark beetles (Acarina:Tarsonemidae). *Annals of the Entomological Society of America* **67**: 713-715.

Smith CA, 1966. Common names of South African plants. Memories of the Botanical Survey of South Africa 35.

Spatafora JW, Sung G -H, Sung J -M, Hywel-Jones NL, White JR, 2007. Phylogenetic evidence for an animal pathogen origin for ergot and the grass endophytes. *Molecular Ecology* **16**: 1701-1711.

Speight MR, Wainhouse D, 1989. *Ecology and management of forest insects.* Clarendon Press, Oxford, UK, pp. 374.

Speight MR, Wylie FR, 2001. Insect Pests in Tropical Forestry. CABI Publishing, pp. 307.

Tatem AJ, Hay SI, Rogers DJ, 2006. Global traffic and disease vector dispersal. *PNAS* **103**(16): 6242-6247.

Taylor JE, LeeS, Crous PW, 2001. Biodiversity in the Cape Floral Kingdom: fungi occurring on *Proteaceae. Mycological Research* **105** (12): 1480-1484.

Tainter FH, Baker FA, 1996. Principles of Forest Pathology. New York: John Wiley & Sons, Inc, p. 805.

Thatcher RC, Searcy JL, Coster JE, Hertel GD (eds.), 1980. The Southern Pine Beetle. USDA, Expanded southern pine beetle research and application program, Forest Service, Science and Education Administration, Pineville, LA. Technical Bulletin 1631, pp. 265.

Tisserat N, Cranshaw W, Leatherman D, Utley C, Alexander K, 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Online. Plant Health Progress doi:10.1094/PHP-2009-0811-01-RS.

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

Tribe GD, 1995. The woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), a pest of *Pinus* species, now established in South Africa. *African Entomology* **3**: 215-217.

Tribe GD, Cillié JJ, 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.

Turpie JK, Heydenrych BJ, Lamberth SJ, 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.

Van Daalen JC, 1981. The dynamics of the indigenous forest-fynbos ecotone in the southern Cape. *South African Forestry Journal* **119**: 14-23.

Van der Linde JA, Six DL, Wingfield MJ, Roux J, 2012. New species of *Gondwanamyces* from dying *Euphorbia* trees in South Africa. *Mycologia* **104**(2): 574-584.

Van der Merwe NA, Gryzenhout M, Steenkamp ET, Wingfield BD, Wingfield MJ, 2010. Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. *Fungal Biology* **114**:966-979.

Van Der Niet T, Johnson SD, 2009. Patterns of plant speciation in the Cape floristic region. *Molecular Phylogenetics and Evolution* **51**:85-93.

Van Rensburg JCJ, Lamprecht SC, Groenewald JZ, Castlebury LA, Crous PW, 2006. Characterisation of *Phomopsis* spp. associated with die-back of rooibos (*Aspalathus linearis*) in South Africa. *Studies in Mycology* **55**: 65-74.

Van Wyk B, Van Wyk P, 1997. Field guide to trees of Southern Africa. Struik. Cape Town.

Vermeulen M, Gryzenhout M, Wingfield MJ, Roux J, 2011. New records of the Cryphonectriaceae from southern Africa including *Latruncellus aurorae* gen. sp. nov. Mycologia **103**(3): 554-569.

Viljoen A, Wingfield MJ, Marasas WFO, 1994. First report of *Fusarium subglutinans* f. sp. *pini* on pines seedlings in South Africa. *Plant Disease* **78**: 309-312.

Vlok JHJ, Euston-Brown DIW, Cowling RM, 2003. Acock's Valley Bushveld 50 years on: new perspectives on the delimitation, characterisation and origin of subtropical thicket vegetation. *South African Journal of Botany* **67**(1): 27-51.

Von Broembsen SL, 1989. Invasions of natural ecosystems by plant pathogens. In: Drake JA, Mooney HA, di Castri F, Groves RH, Kruger FJ, Rejmanek M, Williamson M (eds.), *Biological Invasions: A Global Perspective* **37**: 77-83.

Von Broembsen SL, Brits GJ, 1985. Phytophthora root rot of commercially cultivated proteas in South Africa. *Plant Diseases* **69**: 211-213.

Von Broembsen SL, Kruger FJ, 1985. *Phytophthora cinnamomi* associated with mortality of native vegetation in South Africa. *Plant Diseases* **67**: 715-717.

Wager VA, 1931. Diseases of plants in South Africa due to members of the Pythiaceae. Union South Africa. Department of Agricultural Science. *Bullet* 103.

Walker JC, 1957. *Plant Pathology*. Mc-Graw-Hill Book Company, New York.

Walker LC, 1962. The coastal plain southern pine region. In: Barret JW (ed.) *Regional silviculture of the Unites States*. The Ronald Press Co., Bew York, p. 246-295.

Weste G, 1994. Impact of *Phytophthora* species on native vegetation of Australia and Papua New Guinea. *Australian Plant Pathology* **23**: 190-109.

Weste G, 2003. Disease caused by *Phytophthora* in Australia and its impact on native forests, woodlands and heathlands. Sudden Oak Death Online Symposium. www.apsnet.org/online/SOD (website of The American Phytopathological Society).

Whitney HS, 1982. Relationships between bark beetles and symbiotic organisms. In: Mitton JB, Sturgeon KB (eds.)*, Bark beetles in North American Conifers.* Austin: University of Texas Press, pp. 183-211.

Wingfield MJ, 1999. Pathogens in exotic plantation forestry. *International Forestry Review* **1**: 163-168.

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

Wingfield MJ, Coetzee MPA, Crous PW, Six D, Wingfield BD, 2010. Fungal phoenix rising from the ashes? *International Mycological Association Fungus* **1**(2): 149-153.

Wingfield MJ, Coutinho TA, Roux J, Wingfield BD, 2002. The future of exotic plantation forestry in the Southern Hemisphere: lessons learnt from pitch canker. *Southern African Forestry Journal* **195**: 79-82.

Wingfield MJ, Hammerbacher A, Ganley RJ, Steenkamp ET, Gordon TR, Wingfield BD, Coutinho TA, 2008. Pitch canker caused by *Fusarium circinatum* – a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology* **37**: 319-334.

Wingfield MJ, Knox-Davies PS, 1980. Observations on diseases in pine and *Eucalyptus* plantations in South Africa. *Phytophylactica* **12**: 57-63.

Wingfield MJ, Seifert KA, Webber JF, 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Press, St. Paul, Minnesota.

Wingfield MJ, Swart WJ, Abear BJ, 1989. First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**: 311-313.

Wood DL, 1982a. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annual Review Entomology.* **27**: 411–46.

Wood SL, 1982b. The bark and ambrosia beetles of North and Central America Coleoptera: Scolytidae), a taxonomic monograph. Great Basin Naturalist Memoirs, No. 6. Brigham Young University, Provo, UT.

Zentmyer GA, 1980. *Phytophthora cinnamomi* and the diseases it causes. The American Phytopathological Society, St. Paul, N. M.

Zhou XD, De Beer W, Wingfield BD, Wingfield MJ, 2002. Infection sequence and pathogenicity of *Ophiostoma ips, Leptographium serpens* and *L. lundbergii* to pines in South Africa. In: Hyde KD, Lones EBG (Eds.), *Fungal Succession*. *Fungal Diversity* **10**: 229-240.

CHAPTER 2

DEATH OF *VIRGILIA* TREES IN THE HAROLD PORTER NATIONAL BOTANICAL GARDEN OF SOUTH AFRICA

ABSTRACT

The South African endemic tree genus *Virgilia* contains two ecologically and economically important species, *V. oroboides* and *V. divaricata*. Individuals of *V. oroboides* in the Harold Porter National Botanical Garden (HPNBG) were recently observed to show symptoms of disease, that varied from leaf-yellowing and shoot die-back to ultimate death. Bark beetles (Curculionidae; Scolytinae) and root-rot symptoms were commonly observed on dead and dying trees. In this study we identified the main fungal and bark beetle taxa involved in the death of *V. oroboides* in the HPNBG, and evaluated their importance in causing tree mortality. We monitored tree health decline in the HPNBG over a year. Bark, roots and branches with bark beetles were collected randomly from diseased individuals. Fungi were isolated from surface sterilised bark and root samples and from the surfaces of bark-beetles that emerged from collected branches. Key fungal isolates were identified by comparisons of the internal transcribed spacer regions (ITS1, ITS2), including the 5.8S gene, of the rDNA to taxa available on GenBank. Pathogenicity tests of the most commonly found fungal taxa were conducted in the field and under greenhouse conditions. The fungus most consistently isolated from declining *V. oroboides* roots and bark was an un-described *Phomopsis* species. Four morpho-species of scolytine beetles, associated with various *Geosmithia* species, were collected from *Virgilia* stems and branches. Pathogenicity tests revealed that the *Phomopsis* sp. is a virulent pathogen of *V. oroboides*, while the *Geosmithia* spp. are non-pathogenic. Trees of all ages were susceptible to root-rot disease caused by the *Phomopsis* species, with subsequent bark beetle attack. The new species is here described as *Phomopsis virgiliansis* sp. nov. It has caused a significant decline in the health of the *Virgilia oroboides* population

in the HPNBG over a short period of time and may pose a significant threat to *V. oroboides* trees in general.

---

1.   INTRODUCTION

The Cape Floristic Region (CFR) of South Africa is home to the genus *Virgilia* Pior. (Fabaceae). Their showy flowers and rapid growth make them very popular garden trees, and they are widely planted in Australia, England, South Africa and the United States of America (Palmer and Pitman 1961, 1972; Smith 1966). *Virgilia* is endemic to the southwestern and southern coastal regions of the CFR (Van der Bank *et al*. 1995) and includes two species; *Virgilia divaricata* Adamson and *Virgilia oroboides* (P. J. Bergius) Salter. *V. divaricata* is distributed eastward from George to Port Elizabeth, while *V. oroboides* occurs from just east of George to the Cape Peninsula in the west (Palgrave 1983, 2002; Palmer and Pitman 1961, 1972; Smith 1966). Natural habitats include riparian vegetation, thickets, hillsides and forest margins (Palmer and Pitman 1972). *Virgilia* trees are important pioneer species in forest plant succession, because they grow well in the open (Phillips 1926). Their dense foliage act as wind breaks and they provide shelter for young forest trees to establish (Geldenhuys 1994; Phillips 1926; Van Daalen 1981). They are also important breeding sites for numerous animals and the only known food source of the rare *Leto venus* moth (Nielsen *et al*. 2000; Palmer and Pitman 1972; Van Wyk and Van Wyk 1997).

Limited information is available on the health of native trees in South Africa in general. However, reports of diseases and death of native trees have been emerging over the years. Examples include Phytophthora root rot disease and mortalities of *Protea* L. species and *Leucospermum cordifolium* (Salisb. Ex Knight) Fourc. (common pincushion) both in the wild and in cultivated areas due to the non-native soil-borne pathogen *Phytophthora cinnamomi* Rands (Doidge and Bottomley 1931; Von Broembsen 1989; Von Broembsen and Brits 1985; Wager 1931; Zentmyer 1980). Other hosts of this pathogen include the tree species *Ocotea*

bullata (Burch.) Baill. in southern Cape forests and many other fynbos plants (Lübbe and Geldenhuys 1990; Von Broembsen 1989; Von Broembsen and Kruger 1985). A more recent example, among many others, is the discovery of the fungal pathogen *Graphium adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux on wounds on *Adansonia digitata* L. made by elephants in the Kruger National Park and a motor vehicle on the N1 highway near Mesina (Cruywagen *et al*. 2010). For a number of years the causes of die-back of *Pterocarpus angolensis* DC. (kiaat) were unknown, but recently Mehl *et al*. (2010) suggested the cause to be the interaction of drought, fire stress and fungal pathogens. A follow up study on the kiaat die-back and death suggested Botryospaeriaceae species and environmental stresses to be responsible for mortalities (Mehl *et al*. 2011). In this study, pathogenicity trials showed that a newly described fungus *Diplodia alatafructa* J.W.M. Mehl & B. Slippers and *Lasiodiplodia pseudotheobromea* A.J.L. Phillips, A. Alves & Crous are virulent to kiaat trees, with the latter fungus suggested as the species responsible for die-back and death of kiaat due to its high virulence and common occurrence (Mehl *et al*. 2011).

Botanical gardens are often sources of various pests and pathogens due to active nurseries that outsource planting material from other places (Coetzee *et al*. 2001; Von Broemsen 1989). It has been shown that non-native pathogens such as *Armillaria mellae* (Vahl. Fr.) Kummer, *A. gallica* Marxm. & Romagn and *Phytophthora cinnamomi* in Kirstenbosch National Botanical Garden were accidentally introduced into the country on rooted infected material (Coetzee *et al*. 2001, 2003; Doidge and Bottomley 1931; Wager 1931; Wingfield *et al*. 2010). *Armillaria mellea* has successfully colonised and killed the species *Leucadendron argenteum* (L.) R.Br., *L. gandogeri* Schinz ex Gand., *L. grandiflorum* (Salisb.) R.Br., *Protea longifolia* Andrews, *P. eximia* (Salisb. ex Knight) Fourc. and *P. scolymocephala* (L.) Reichard, and other ornamental trees and native shrubs in Kirstenbosch National Botanical Garden since its introduction (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010). *Armillaria gallica* has also caused mortality of these same hosts, although it is considered a secondary pathogen infecting hosts already infected and weakened by *Phytophthora cinnamomi* (Coetzee *et al*. 2003).

Bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) are phytophagous insects that can pose a primary, secondary or saprophytic threat to trees (Linnakoski *et al*. 2012; Raffa *et al*. 1993). Primary scolytine beetles are very aggressive and can kill host trees due to mass colonization (Paine *et al*. 1997; Six and Wingfield 2011). Healthy trees defend themselves from attack by producing insecticides and/or other chemicals like resins, phenolics or monoterpenes that immobilize or kill the insects (Hudgins *et al*. 2004; Raffa and Smalley 1995; Six and Wingfield 2011). Despite these defence mechanisms, a very large outbreak of these insects may overcome the natural defence of trees and ultimately lead to tree death (Franceschi *et al*. 2005; Lee *et al*. 2011; Six and Wingfield 2011). Secondary scolytine beetles attack stressed, weakened and dying trees (Avtzis *et al*. 2012; Paine *et al*. 1997; Raffa *et al.* 1993; Six and Wingfield 2011), where stress symptoms may be ascribed to drought, disease or physical damage. They may also feed on logging residue or fallen trees (Langstrom and Hellqvist 1993; Paine *et al*. 1997). The largest group of scolytine beetles prefer saprophytic habitats, as they exclusively colonize dead hosts (Paine *et al*. 1997).

Several studies have shown various specialised associations between bark beetles and fungi. Depending on the ecological roles of fungi, these can be obligatory or incidental, mutualistic and/or antagonistic (Harrington 2005; Six 2003; Six and Wingfield 2011). Spores and/or mycelia of the fungal associates can be carried on the exoskeletons of these beetles or inside specialised structures called mycangia (Six 2003; Six and Wingfield 2011). The fungi are inoculated into trees as the beetles bore into the bark and/or wood, sometimes causing tree mortality. The first documented association between bark beetles and fungi were between conifer bark beetles and *Ophiostoma minus* (Hedgcock) H. & P. Sydow, a bluestain ascomycetous fungus (Hartig 1878; Münch 1907; in Harrington 2005). Ophiostomatoid fungi (Wingfield *et al*. 1993) are common associates of bark and ambrosia beetles and many members are economically important as serious tree pathogens (Roux and Wingfield 2009). They have evolved and adapted to an entomochoric lifestyle by producing sticky conidia or ascospores that can adhere to the exoskeleton and mycangia of bark beetles (Kolařík *et al*. 2008; Six 2003). The Dutch elm pandemics caused by *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier killed millions of Elm trees throughout America and Europe (Brasier 2008; Loo 2009; Tainter and Baker 1996). These fungi are disseminated by both their native vector and an American elm bark beetle (Bingham *et al*. 1971; Loo 2009; Quimby 1982).

The genus *Geosmithia* Pitt (Ascomycota: Hypocreales*:* Bionectriaceae and Eurotiales*;* Trichocomaceae) (Čizkova *et al*. 2005; Kolařík *et al*. 2005) is an understudied, common associate of various bark and ambrosia beetles with a worldwide distribution (Kolařík *et al*. 2008; Kolařík and Kirkendall 2010). Their association with many bark beetles is consistent (Kolařík *et al*. 2007), with more than 20 bark beetle species associated with *Geosmithia* species in central Europe alone (Kirschner 2001; Kolařík *et al*. 2008; Kubátová *et al*. 2004). Unlike ophiostomatoid fungi that have entomochoric adaptations, *Geosmithia* produce dry conidia (Kolařík *et al*. 2007; 2008). However, they usually replace ophiostomatoid fungi as main associates of a large group of bark beetles that infest deciduous trees (Kirschner 2001; Kolařík *et al*. 2004, 2005; Kubátová *et al*. 2004). Phytopathogenic species include *Geosmithia morbida* M. Kolarík, E. Freeland, C. Utley & Tisserat that is a serious threat to black walnut trees (*Juglans nigra* L.) in the USA (Tisserat *et al*. 2009). A symptom unique to this disease is the formation of several cankers, hence the common name "thousand cankers disease". The walnut twig beetle (*Pityophthorus juglandis* Blackman) is responsible for the dispersal of *G. morbida* between hosts (Tisserat *et al*. 2009).

A dramatic decline in the health of *Virgilia oroboides* trees in the Harold Porter National Botanical (HPNBG) garden in the Western Cape Province of South Africa was recently observed. Diseased individuals exhibited root-rot symptoms and their trunks were heavily colonised by bark beetles. No pests or diseases on *Virgilia* have previously been reported. As botanical gardens are often introductory points for various diseases (Coetzee *et al*. 2001; Von Broemsen 1989), these tree deaths raised concerns about the origins of the causal organisms considering Chen *et al*. (2012) recently discovered a new serious stem canker pathogen of *Rapanea melanophloeos*, *Immersiporthe knoxdaviesiana*, causing mortality in the same garden. Therefore, the aim of this study was to identify the causal organisms of *Virgilia* tree death in the Harold Porter National Botanical Garden. The specific objectives were to isolate, identify and determine the pathogenicity of key fungal species associated with diseased *Virgilia* trees, to identify the bark beetles (and their associated fungi) infesting these *Virgilia* trees and to establish which life stages of *Virgilia* trees are susceptible to attack. We also monitored *Virgilia* individuals in the HPNBG over a year to establish whether there was a significant decline in population health over this short time period.

2   MATERIALS AND METHODS

2.1   Disease progression

Field surveys were conducted in the HPNBG (S 34' 20.893 E 18' 55.519) in Betty's Bay, South Africa during March 2011. Three 50 m x 10 m transects were randomly plotted and the health status of all *V. oroboides* individuals encountered therein were assessed. A scoring system was developed to categorise trees into observed health status and these scores were recorded. Healthy trees were given a score of six, those at the leaf yellowing stage a score of five, those dropping leaves a score of four, trees with less than 50% shoot die-back a score of three, trees with more than 50% shoot die-back a score of two and dead trees a score of one. The presence or absence of bark beetles on stems and trunks was also recorded. The health status of the same individual trees initially assessed was again assessed in June 2012. To determine if population health had stabilised or deteriorated after a year, mean health status of *V. oroboides* individuals from each of the three transects were compared between 2011 and 2012 using repeated measures ANOVA in Statistica 10 (Statsoft Corporation, USA). Significant differences are reported when $P \leq 0.05$.

2.2   Effect of tree age on health status

Trunk diameters, used as surrogate for plant age, were measured for all individuals encountered within the three transects during 2011. Diameters were taken at breast height for mature trees and at 10 cm above the ground for individuals less than 1 m tall. We tested for the effect of tree age on health status by comparing stem diameters among the different health status categories. Normality of the stem diameter data was tested using a Shapiro-Wilk test (Shapiro and Wilk 1965) and was subsequently analysed using Kruskal-Wallis ANOVA and Median test procedures in Statistica 10 (Statsoft Corporation, USA). Significant differences are reported when $P \leq 0.05$.

2.3   Sample collection and fungal isolations

Root samples were collected from both diseased and healthy individuals growing within transects. Bark samples and branches containing bark beetles were collected from diseased individuals and placed in labelled sample bags. Branches containing bark beetles were placed in insect emergence cages. These consisted of cardboard boxes (ca. 49 x 49 x 32.6 cm) fitted with two clear plastic bottles (5.7 cm diameters) on the side (opening into boxes). These bottles allowed light to enter and attracted beetles that emerged from branches, from where they could easily be collected. Additional bark beetle individuals were collected directly from their galleries from collected bark samples.

Bark and root samples were washed under flowing tap water and air-dried prior to surface sterilisation. These were surface sterilised by soaking them in 70% ethanol for five minutes and dried in a laminar flow cabinet. The outer bark was removed with a sterile scalpel to expose any lesions. Small pieces (*ca*. 2 mm²) of inner bark and root tissue from the edges of fresh necrotic lesions were plated onto malt extract agar (MEA: 20gL-1 malt extract and 20gL-1 agar, Biolab, South Africa) in petri dishes. A subset of root tissue samples were also plated on PARP and PARPH media that are selective for *Pythium* and *Phytophtora*, respectively (Jeffers and Martin 1986). Plates were incubated at room temperature (20 to 25°C) in the dark and examined daily for fungal growth. Single hyphal tips of developing mycelium were transferred to fresh MEA plates.

Pieces of bark containing bark beetle galleries were placed into moisture chambers to stimulate fungal sporulation. After 7 – 12 days, spores from sporulating structures that formed within galleries were transferred to MEA. Pure cultures from all isolations were stored at 4°C on MEA until further use. The most consistent fungal morpho-types isolated from bark and root samples were scrutinized and their morphological characters recorded following Santos and Phillips (2009).

Collected bark beetles were studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan) and grouped into morpho-species. Fifty individuals (where available) of each morpho-species were washed and/or crushed separately in eppendorf tubes containing 0.1 ml ddH$_2$O and the water and/or solution spread onto MEA in petri dishes. Plates were again incubated in the dark at room temperature (20 to 25°C) until fungi that grew on MEA could be purified. Reference bark beetles were stored in 100% ethanol and are housed in the Stellenbosch University Insect Collection, Stellenbosch, South Africa. Representative isolates of all fungal taxa used in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

2.4   DNA extraction, amplification and sequencing

Ten pure representative isolates of each morphologically similar isolates of fungi consistently isolated from root samples and bark beetles were randomly chosen for sequencing (NM1-NM20 for fungi from roots and NM70 to NM109 for fungi from scolytine beetles). Fungal mycelium from representative isolates was harvested from colonies of pure cultures on MEA with a sterile scalpel. DNA was extracted using a Sigma-Aldrich™ plant PCR kit (USA) following the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer region (ITS1, ITS2), including the 5.8S gene of the rDNA, was amplified using primers ITS1-f (Gardes and Bruns 1993) and ITS4 (White *et al*. 1990). The 20 µL PCR reaction volumes used consisted of 10 µL ddH$_2$O, 5 µL REDExtract-N-Amp PCR ready mix (Sigma-Aldrich™, USA), 4 µL extracted fungal DNA and 0.5 µL (10mM) of each primer. A Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.) was used for the DNA amplification. PCR reaction conditions followed instructions from Sigma and were as follows: 2 minutes of initial denaturing at 95°C, followed by 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 1 min 30 seconds elongation at 72°C and a final elongation step at 72°C for 8 minutes. PCR products were separated by agarose gel electrophoresis (1.5 % agarose gel containing ethidium bromide) and visualised under ultraviolet light. Amplified PCR products were purified and sequenced at the Stellenbosch University Central Analytical Facility, Stellenbosch, South Africa.

2.5   Phylogenetic analyses

Sequences generated in this study from the suspected pathogenic fungal taxa were compared to published sequences using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al*. 1990) in GenBank (http://www.ncbi.nlm.nih.gov/genbank). Closest matching taxa collected from diseased root samples based on ITS included species of *Phomopsis* and we did a phlogenetic analysis for this genus including our isolates (NM11-NM19)*.* Closest matching taxa from bark beetles using ITS were *Geosmithia* spp. (see Chapter 3 for phylogeny tee). Sequences of all taxa closely related to the *Phomopsis* sp. isolated in this study were downloaded and aligned using Clustal W (Thompson *et al*. 1994) and manually adjusted in BioEdit v7. 0. 5 (Hall 2005). Supplementary sequences and the out-group taxa used (*Valsa mali* Miyabe & Yamada, *V. japonica* Miyabe & Hemmi*, Leucostoma niveum* (Hoffm.) Höhn. and *Leucostoma persoonii* (Nitschke) Höh) are based on previous phylogenetic studies on the genus *Phomopsis* (Mostert *et al*. 2001; Udayanga *et al*. 2011) and are presented in Appendix 1. We also included all known *Phomopsis* taxa from South Africa identified in previous studies for which sequence data were available (Mostert *et al*. 2005; Van Niekerk *et al*. 2005; Van Rensburg *et al.* 2006). Bayesian and Maximum Parsimony (MP) analyses were conducted to reconstruct molecular phylogenetic trees for the taxa represented in the data set using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003) and PAUP (Phylogenetic Analysis Using Parsimony PAUP*4.0b10 (Swofford 2002), respectively. Bayesian analyses were performed based on a Markov Chain Monte Carlo (MCMC) approach, using the GTR+I+K (most parameter rich) model as selected in jModelTest 0.1.1 (Posada 2008) using Akaike information criteria (Akaike 1974). The analysis implemented runs of 10 million generations with a sample frequency of 1 000. Burn-in trees, the first 2 million generations, were discarded and the remaining trees were pooled into a 95% majority rule consensus tree. Maximum Parsimony (MP) analysis used the heuristic search option with random addition of sequences (1 000 replications), tree bisection-reconnection (TBR) and MULTREES options ON. Bootstrap support values with 1 000 replications were calculated to assess the confidence of resultant nodes in the MP trees using MULTREES option OFF and 10 random sequence additions in each of 1 000 pseudo-replications. Newly generated sequence data from this study will be deposited in GenBank and alignments and phylogenetic trees will be deposited in TreeBASE (http://www.treebase.org/treebase/index.html).

2.6   Pathogenicity tests

Ten representative isolates of the *Phomopsis* sp. isolated from roots and 3 isolates of each *Geosmithia* morphospecies from bark beetles were used in pathogenicity tests at the Harold Porter National Botanical Garden. Phomopsis inoculations were done in January 2012 and Geosmithis inoculations in September 2012. A 7 mm cork borer was used to wound the bark and expose the cambium of *Virgilia oroboides* branches (*ca*. 1.5 cm diameter). Similar sized disks from actively growing fungal colonies on MEA were inserted into these wounds with mycelium facing the xylem. These wounds were covered with masking tape to prevent desiccation and contamination by other organisms. One branch was inoculated with one isolate of each fungal strain. This procedure was repeated eight times on eight different trees for each isolate, with each tree being inoculated with all strains of all species to minimise the effect of host individual immunity. Control stems were inoculated with sterile MEA plugs. After 6 weeks, resultant lesion lengths were measured and re-isolations of fungi were made to confirm that the inoculated fungi were responsible for lesion development. For re-isolations, wounds were surface sterilized with 70% ethanol and the outermost wood tissue on the wounds removed. Small pieces of inner wood (2 mm²) were plated onto MEA in petri dishes and incubated at room temperature (20 - 25°C) in the dark. Fungal cultures growing in the plates were purified and identified based on morphological characteristics. All inoculated branches were removed from the trees and destroyed after completion of these experiments to avoid accidental spreading of diseases in natural populations. To assess pathogenicity, lesion length data from fungal isolates were compared to those of the control using one-way analysis of variance (ANOVA) procedures in Statistica 10 (Statsoft Corporation, USA) after confirming normality of the data (Shapiro and Wilk 1965). A Tukey HSD post-hoc test, as implemented in Statistica, was used to test differences in mean group lesion length.

Inoculations were also conducted on the roots of potted *Virgilia oroboides* seedlings under greenhouse conditions. Four isolates of fungi that produced the longest lesions from stem inoculations were chosen for root inoculations (NM2 (P2), NM3 (P3), NM5 (P5), NM6 (P6)). The roots of *Virgilia* seedlings were exposed and a sterile scalpel was used to make small wounds on feeder roots (ca. 2mm²). Similar sized plugs of fungal colonies were inserted into

these wounds and sealed with parafilm to prevent contamination. Sterile plugs of MEA were used as control. The exposed roots were covered with potting soil. The experiment was replicated 8 times under the same conditions.The plants were monitored for as long as it took for them to die, recording visible disease symptoms and deaths over time.

3.   RESULTS

3.1   Disease progression

A total of 91 *Virgilia oroboides* trees were assayed for health status in the three transects in HPNBG. During the first field visit, 46 of these seemed healthy, 28 were diseased and 17 were dead. A reassessment of the same individuals only a year later revealed that only 33 seemed healthy, 14 were diseased and 44 were dead. The overall deterioration in health status of this *Virgilia* population over a year was found to be significant (Fig. 1).
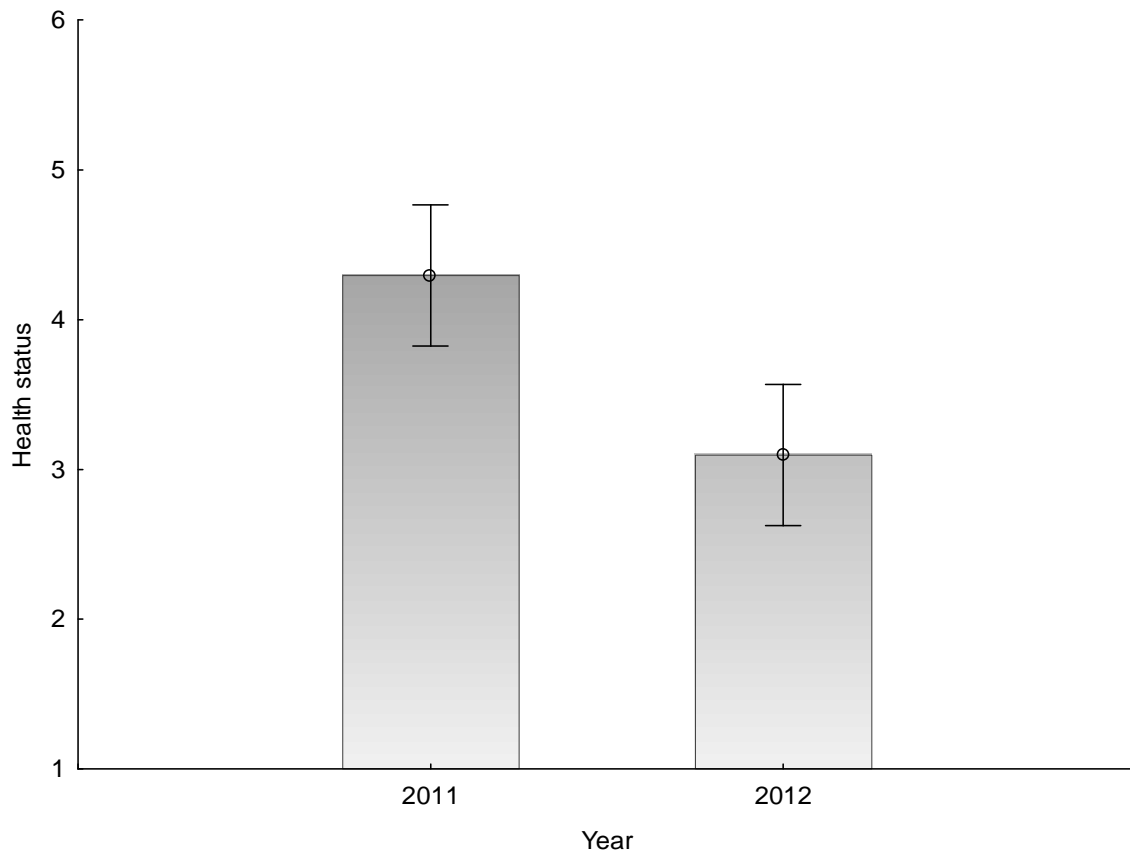
Fig. 1: Comparison of mean population health status (bars = Standard error) of *Virgilia* trees at HPNBG showing a significant decline from 2011 to 2012 (df = 90; F = 43.5; p < 0.000001). Vertical bars denote 0.95 confidence intervals.

3.2   Effect of tree age on health status

Trees of all ages and developmental stages are susceptible to disease, as there were no significant differences in size distributions between the different disease status categories (Fig. 2). All diseased individuals showed above-ground symptoms of leaf yellowing, leaf drop, die-back of main shoots and ultimate death (Fig 3 and 4). Below ground symptoms were girdled roots typical of fungal root disease. Infected roots had a brick-red colour with necrosis and lesions extending into the base of the stems/trunks (Fig. 4). Affected roots lacked young feeder roots that are present in healthy individuals (Fig. 4).
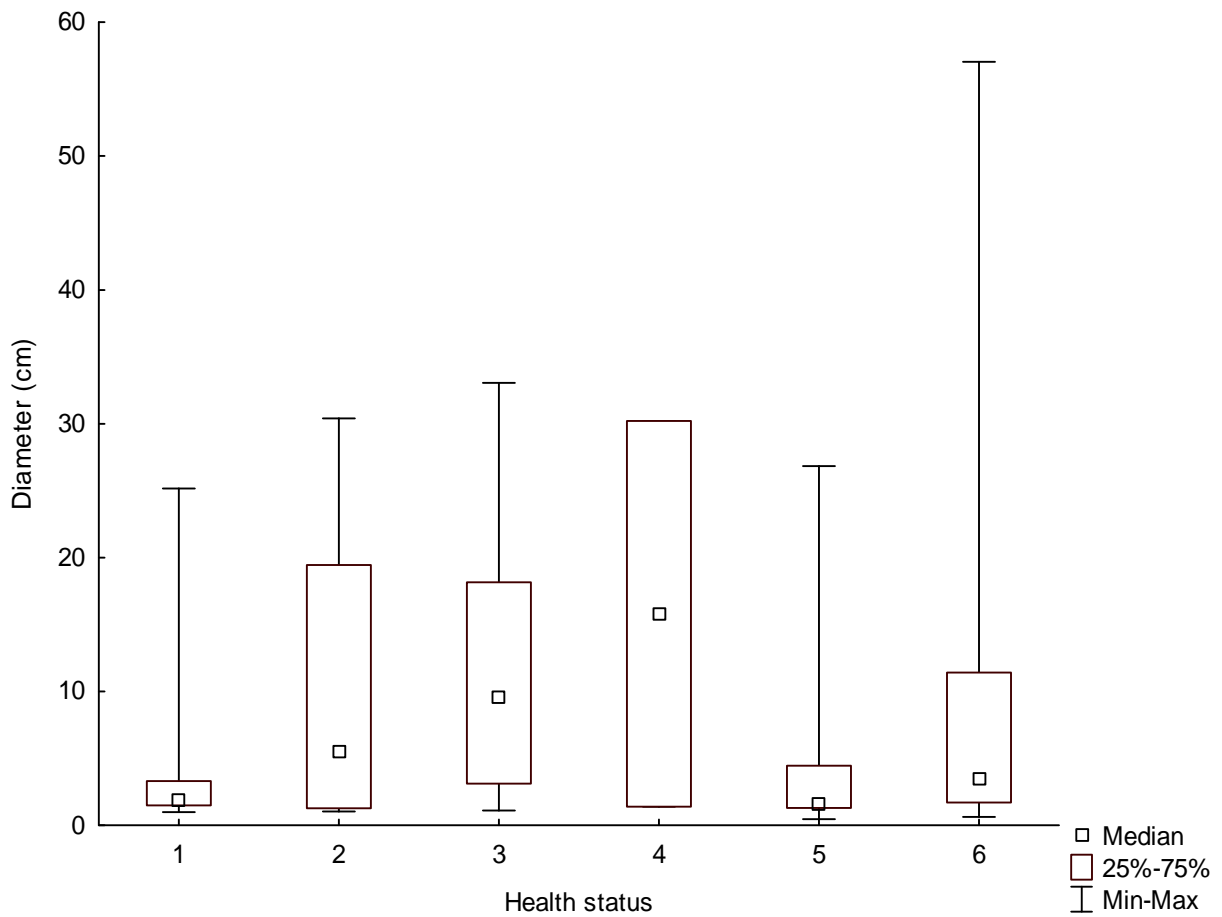
Fig. 2: Box and Whisker plot showing variation in diameter of trees at different levels of disease infection. A Kruskal-Wallis ANOVA on the diameter showed no difference between tree health status H (df=5, N= 91) =5.931235; p=0.3130) and *Virgilia* tree age (as stem diameter).

Fig. 3: Dead and dying *Virgilia* seedlings at HPNBG.
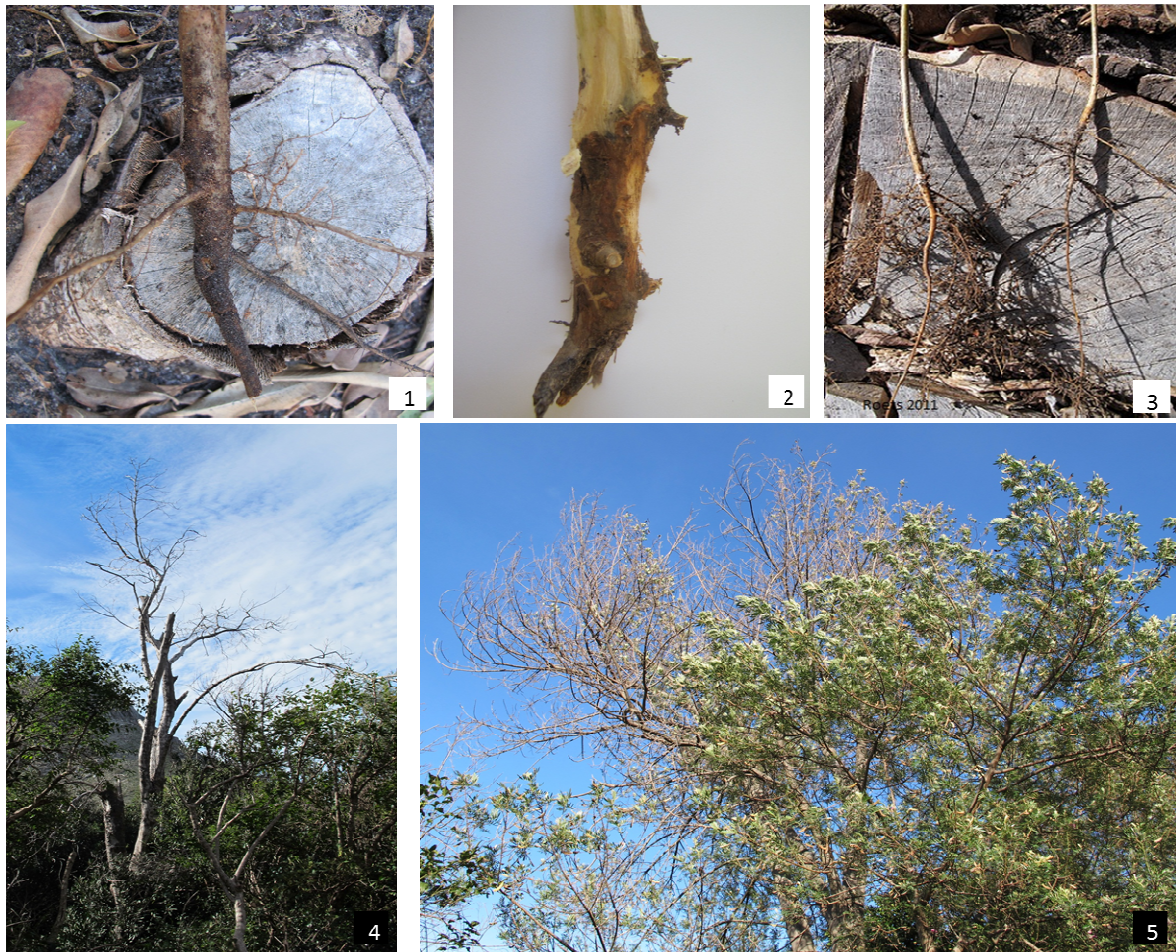
Fig. 4: Symptoms of disease of *Virgilia* trees from the HPNBG. 1- diseased root lacking young feeder roots; 2- diseased root with outer bark removed, exposing brown necrosis and lesions; 3- healthy seedling with abundant fine feeder roots; 4- dead *Virgilia* tree; 5- healthy tree (foreground) and recently killed trees (background).

3.3   Sample collection and fungal isolations

Root samples from 23 diseased individuals and 14 healthy individuals, of various ages, were collected. A total of 19 diseased individuals were sampled for bark and ambrosia beetles. These beetles were only present in dead and dying older trees that had a minimum stem diameter of 7 cm. They were first seen to colonise individuals that were at the leaf yellowing stage. Infested trees were easily identified by the presence of tiny holes in the bark (Fig. 5) and either brown or cream coloured powder on the ground immediately below the tree

trunk in severely attacked hosts. After bark removal, bark beetle galleries containing eggs, larvae and adults were often present (Fig. 5). Later in the development of these galleries, the wood became stained in a characteristic way, apparently caused by the fungal associates of the beetles (Fig. 5). Three morpho-species of bark beetles and one morpho-species of ambrosia beetle were collected from dead and dying trees (see Chapter 3).
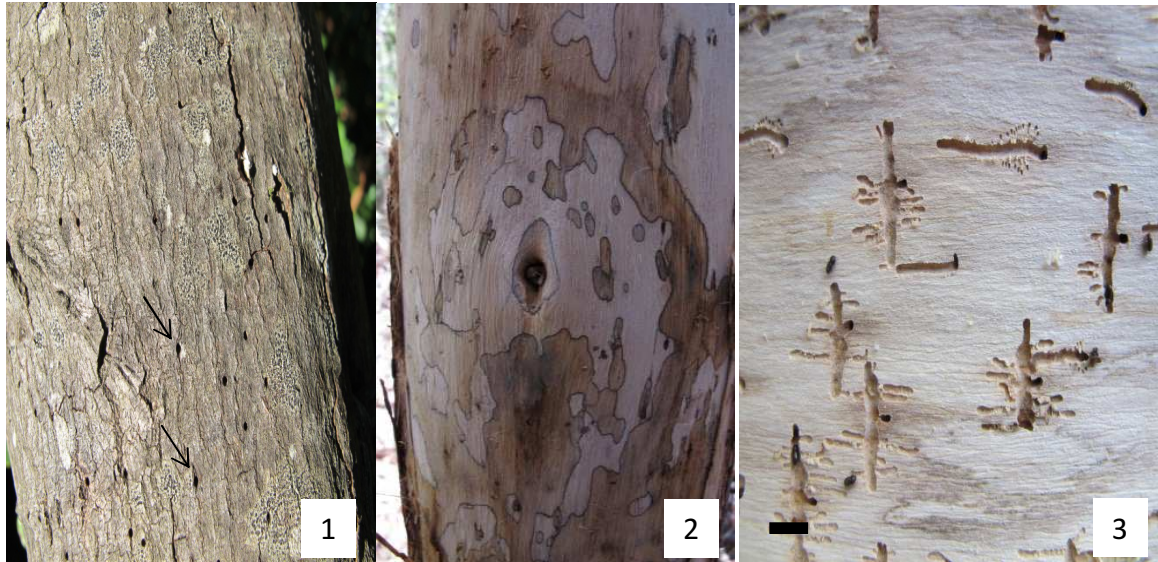
Fig. 5: Bark beetle colonisation of *Virgilia* trees in the HPNBG. 1- bark beetle holes in outer bark of diseased tree; 2- wood staining by fungal associates of bark beetles; 3- bark beetle galleries containing eggs, larvae and adults. Scale bar: 5 mm = 2 mm.

The most persistent fungal morpho-species isolated from the roots and bark of diseased individuals was never isolated from any healthy individuals. It was identified as a species of *Phomopsis* (ITS sequence data). Samples placed on PARP and PARPH media did not reveal the presence of *Phythium* or *Phytophtora* spp. Other fungal taxa were isolated from diseased roots, but were not as consistant as *Phomopsis* and they are not known to be potentially pathogenic. One fungal taxon was consistently isolated from all four morpho-species of bark beetle individuals that were crushed and washed. Morphologically these isolates resembled *Geosmithia pallida.* This was confirmed by ITS sequence data comprisons. An additional four morpho-species of *Geosmithia* were also regularly isolated from the different bark beetle morpho-species. These were identified (using ITS sequence data) as *Geosmithia flava* Kolařík, Kubátová & Pažoutová, *Geosmithia microcorthyli M. Kolarík*, a *G. pallida*-like fungus and *Geosmithia* sp.

3.4 Phylogenetic analyses

Most ITS sequences of our isolates from diseased *Virgilia* trees in HPNBG (NM11-NM17) where identical, except for NM 18 and NM 19, which differed by seven base pairs from the rest, but were identical to each other. These sequences were aligned with other ITS sequences of *Phomopsis* obtained from GeneBank. Sequences included 549 characters, of which 143 were parsimony informative, 39 variable and parsimony uninformative and 367 characters were constant. Parsimony analyses through heusteric search retrieved 661 best trees (Tree Length = 556, Consistency Index = 0.446, Retention Index = 0.776). Both parsimony analysis and Bayesian inference of the ITS marker in MrBayes resolved 9 major clades (Fig. 6). One of these major clades included all isolates obtained in this study (NM11 to NM 19) (Fig. 6). Our isolates grouped in a strongly supported clade (Bayesian posterior probability of 0.95, MP 93%) sister to two isolates only identified as fungal endophytes from China (GenBank DQ485961 and MJ025281) (Fig. 6). Our isolates did not group with any described species available on GenBank and we here describe this new taxon as *Phomopsis virgiliansis* sp. nov.

3.5 Pathogenicity tests

Many of the stems inoculated with *Phomopsis virgiliansis* produced excessive gum exuding from the wounds, a character that could also be seen at the base of a few diseased individuals at the HPNBG, while this was absent from stems inoculated with MEA only (Fig. 7). All ten isolates from the bark and roots of diseased *Virgilia oroboides* individuals caused distinct lesions (Fig. 7) on stems of *Virgilia* trees (mean from 17.5 to 35.25 mm), while control inoculations caused very small or no lesions (mean of 1.75 mm). Lesions caused by all *P. virgiliansis* isolates were significantly larger than those of the control (f = 8.8, df = 10, p < 0.00001) (Fig. 8). Isolate P2 (NM12) caused the most severe lesions, which were significantly longer than those formed by isolates P1 (NM1), P4 (NM4), P7 (NM7), P8 (NM8), P9 (NM9) and P10 (NM10) (Fig .8). None of the *Geosmithia* species were pathogenic to
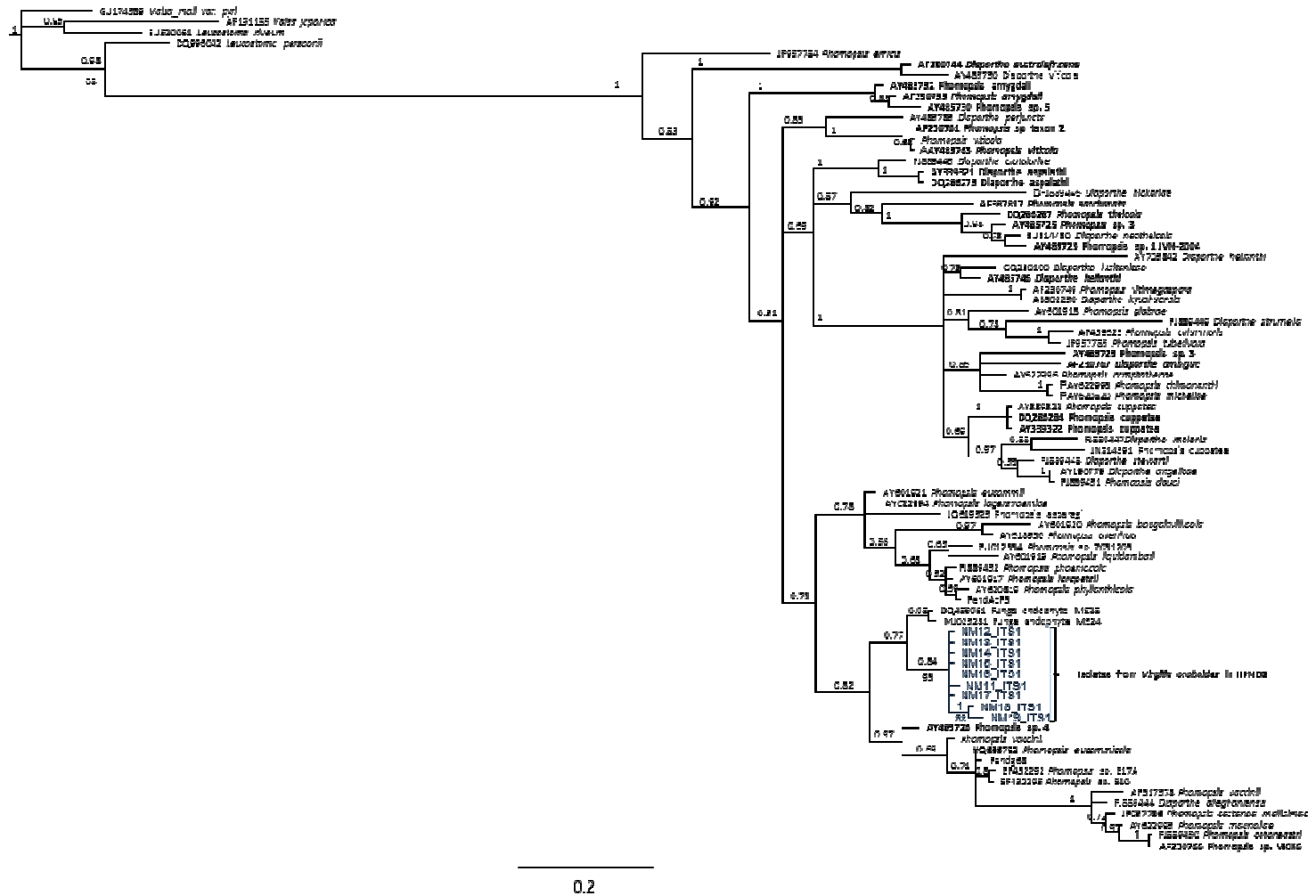
Fig. 6: Bayesian strict consensus tree (ITS sequence data) using data available from GenBank and isolates from this study. Bayesian probabilities are shown above branches and Parsimony bootstrap support values are shown below branches (only above 50%). South African isolates are in bold.

Virgilia trees, as the lesions they caused did not differ significantly from lesions of control (p > 0.05) (Fig. 9). All test isolates were successfully re-isolated from the lesions after six weeks.

Fig. 7: Field inoculations using *Phomopsis virgiliansis* isolates. 1- stem inoculated with *Phomopsis* mycelia that exudes gum after 6 weeks; 2- lesions resulting from inoculations with *Phomopsis virgiliansis* after 6 weeks (control on left hand side). Scale bars: 10.4 mm = 9 mm
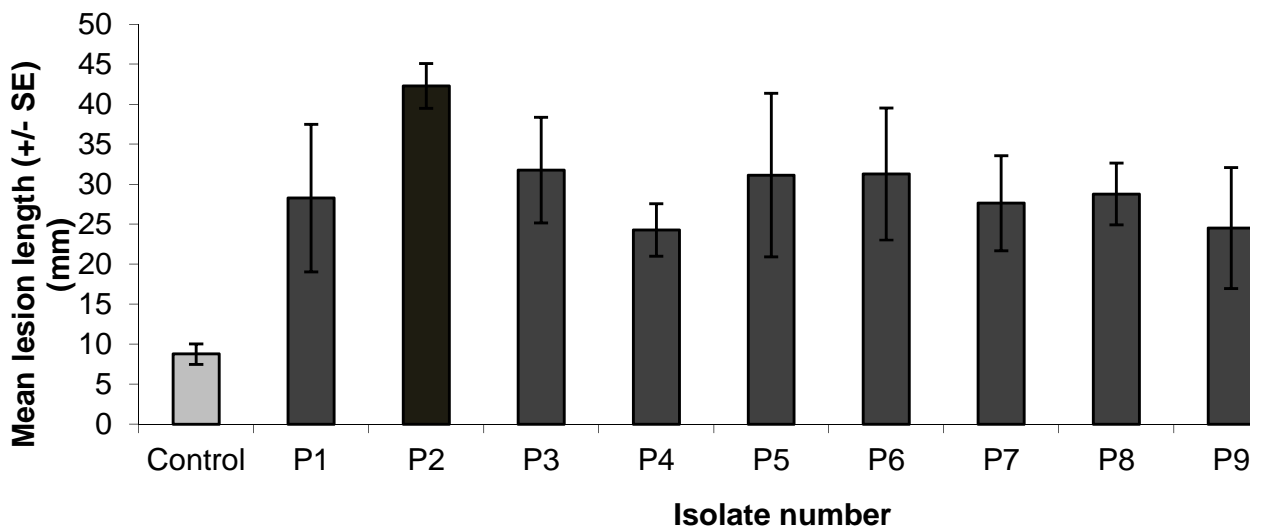
Fig. 8: Mean lesion length caused by *Phomopsis virgiliansis* isolates (P1-P10). Different shades of grey indicate differences in significance levels (One-way ANOVA (f = 8.8, df = 10, p < 0.00001)) of lesion lengths caused by *Phomopsis virgiliansis* isolates to those of control.
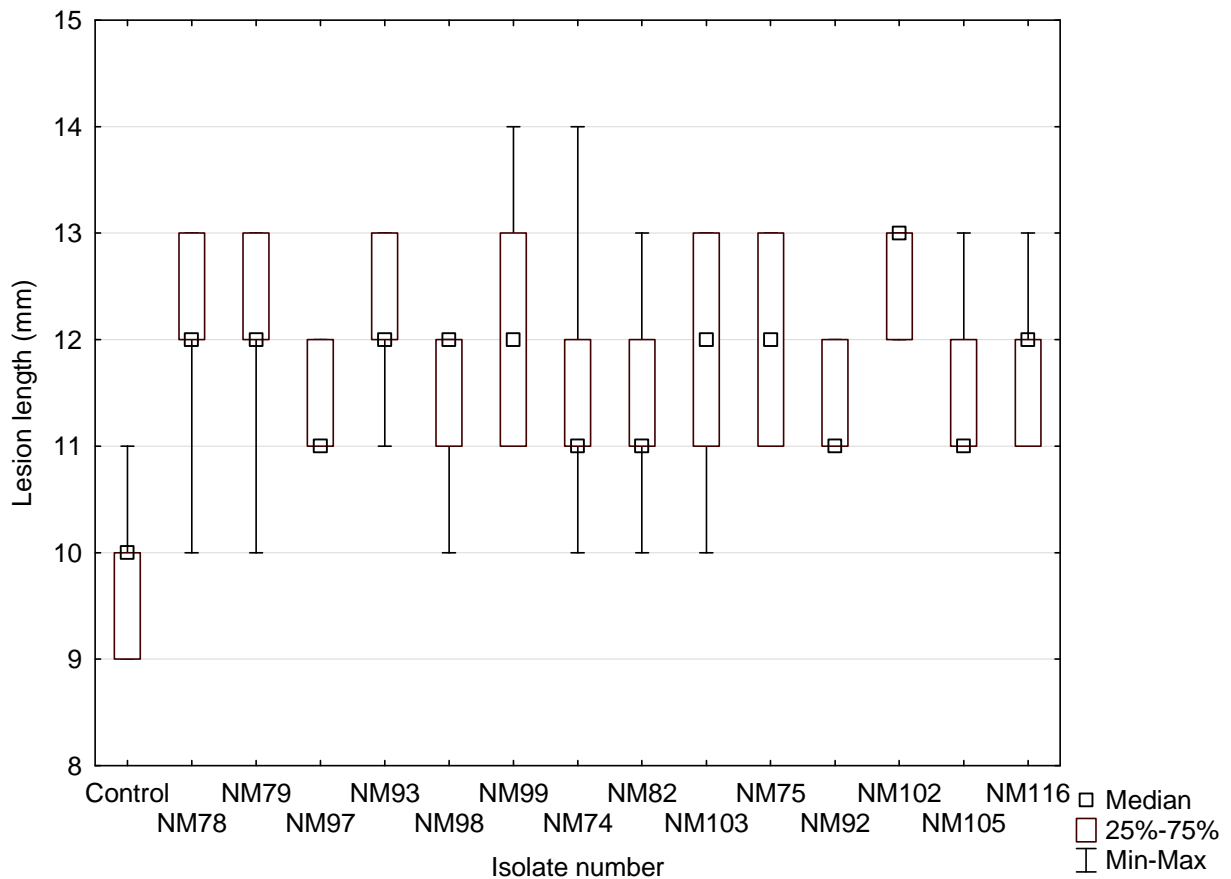
Fig. 9: Box and Whisker plot showing variation in lesion lengths caused by *Geosmithia flava* (NM78; NM79; NM97), *G. microcorthlyi* (NM93; NM98; NM99), *G. pallida* (NM74; NM82; NM103), *G.* sp. 1 (NM75; NM92; NM102) and *G.* sp. 2 (NM105 and NM116). A Kruskal-Wallis ANOVA on the lesion lengths showed no significant difference of the lesion lengths H (df=14, N= 75) =20.1853; p=0.1244) on *Virgilia oroboides* trees

After 12 weeks, 26 of the 32 *Virgilia* individuals (> 80%) that were inoculated with *P. virgiliansis* were dead. Three of the control plants (37%) were also dead. *Phomopsis virgiliansis* could not be isolated from the inoculated roots, as by the time the seedlings showed symptoms of leaf drop, the inoculated roots were long dead and invaded by contamints.

4.   DISCUSSION

Results from this study indicate that the death of *Virgilia oroboides* in the Harold Porter National Botanical Garden is caused primarily by a root disease causing fungus, here described as *Phomopsis virgiliansis* sp. nov. This fungus was consistently isolated from root and bark samples of diseased *V. oroboides* trees, but never from healthy specimens. This is supported by stem pathogenicity tests that showed significantly longer lesions than those of the control, and by root inoculations that showed that the fungi were able to kill a large proportion of plants within only 12 weeks. Diseased trees lacked fine feeder roots or, when present, they were dead and could easily be broken off. Necrotic areas and lesions extended from these fine roots into larger roots and on into the main tap root. This suggests that *P. virgiliansis* initially infect the fine, young feeder roots, from where it spreads to larger roots, and ultimately to the root crown and base of stems.

Phomopsis (Sacc.) Bubák (teleomorph: *Diaporthe* Nitschke) is a genus of ascomycota fungi (Diaporthales, Diaporthaceae) including species that exist as endophytes, saprobes or pathogens on a wide host range (Uecker 1988; Crous 2005; Udayanga *et al*. 2011). Many phytopathogenic *Phomopsis* species cause rots, spots, cankers, blights and wilts to numerous plants globally, including some that are of economic importance (Uecker 1988; Van Rensburg 2006; Udayanga *et al*. 2011). Other studies of *Phomopsis* as pathogens have shown that *Phomopsis* species can either cause disease as single species or as species complexes. For example, Mostert *et al*. (2001) discovered *Phomopsis saccharata* to be responsible for the canker and die-back disease of *Protea repens*, while a complex of *Phomopsis* spp. were found to be associated with die-back of rooibos (*Aspalathus linearis* (N.L.Burm.) R.Dahlgr.) in South Africa (Van Rensburg *et al*. 2006). In the latter study, six species were isolated with the most virulent pathogen identified as *Diaporthe aspalathi* E. Jansen, Castl. & Crous, followed by *D. ambigua* Nitschke, *Phomopsis theicola* Curzi, *Libertella* Desm., *Phomopsis* sp. and *Phomopsis cuppatea* E. Jansen, Lampr. & Crous. *Phomopsis* cane and leaf spot disease on grapevines is caused by a complex of fifteen species of *Phomopsis*. Included among them are *P. viticola* (Sacc.) Sacc. and *P. vitimegaspora* Kuo & Leu, both of which are known to be pathogenic to grapevines, while *Diaporthe viticola* Nitschke and six species of *Phomopsis* are minor pathogens of grapevines (Van Niekerk *et al*. 2005). They

cause serious economic losses in the South African viticulture industry (Pearson and Goheen 1994; Van Niekerk *et al*. 2005), as infested grapevine shoots break off at the base, leading to loss in growth vigour and die-back. This ultimately results in reduced bunch set and fruit rot (Pearson and Goheen 1994; Van Niekerk *et al*. 2005). Diogo *et al*. (2010) identified *Phomopsis amygadali* (Delacr.) Tuset & Portilla as a common twig canker and blight disease pathogen of almonds (*Prunus dulcis* (Mill.) D.A. Webb) and peach (*Prunus persica* (L.) Batsch).

Based on personal observations, we hypothesise that *P. virgiliansis* either blocks water and nutrient conducting cells or kills them (Berger *et al*. 2007; Rosskopf *et al*. 2000; Udayanga *et al*. 2011; van Kan 2006). Necrosis observed at the base of lesions is thought to directly affect the ability of the roots to absorb water and nutrients and physically block vascular tissue, preventing movement of water and nutrients up the trunk (Berger *et al*. 2007; Rosskopf *et al*. 2000; van Kan 2006). Lack of essential nutrients and water supply to the leaves compromises the growth and health of trees. This leads to subsequent symptoms like leaf yellowing and wilting over time. Continued shortage in the supply of water and nutrients to the leaves leads to leaf drop. With death of the root system, shoot die-back and plant death followed fairly rapidly thereafter. Trees attacked by *P. virgiliansis* seldom recover, as seen by the significant increase in number of dead trees in 2012 as compared to 2011. Thus, most trees that were diseased in 2011 were dead in 2012.

This study showed that trees of all ages are susceptible to this pathogen. Bark beetles were only found on dead and dying older trees (see Chapter 3). These beetles thus seem to require fairly mature trees for larval development. The presence of bark beetles at the leaf yellowing stage to severe stages of disease development suggests that the health of *V. oroboides* trees would have been severely compromised by *P. virgiliansis* by the time the beetles arrive. Healthy trees defend themselves from attack by insects by producing insecticides and/or other chemicals that immobilize or kill the insects (Hudgins *et al*. 2004; Raffa and Smalley 1995). It is assumed that *V. oroboides* trees will either not synthesise adequate chemicals to counter bark beetle attack in the presence of *P. virgiliansis* or produce stress hormones that attract the bark beetles in reaction to pathogen colonisation.

This needs to be tested in future studies. All four scolytine beetle species that were found to infest *V. oroboides* are thus considered secondary pests (see Chapter 3). However, as substantial numbers of bark beetles are attracted to dying trees, probably in response to conspecific pheromones (Six and Wingfield 2011), health of such trees probably deteriorate faster than trees that are only infected with the *Phomopsis* root disease fungus. A combination of lack of nutrients and water plus feeding behaviour of bark beetles probably accelerate the death of *Virgilia* trees. This strengthens the hypotheses that the *Phomopsis* species isolated here is primarily responsible for tree death in the HPNBG.

Fungal associates of the bark beetles in this study were dominated by various species of *Geosmithia*, including *Geosmithia pallida, G. flava, G. microcorthyli* and a *Geosmithia* sp. These associates cause a characteristic marble-like staining of *Virgilia* wood, but were not found to be pathogenic. To date, only a single species that are associated with bark beetles (*G. morbida*) was shown to be phytopathogenic (Tisserat *et al*. 2009). Their ecological roles largely remain unresolved, but may include beetle nutrition (Kolařík and Kirkendall 2010). Identifying the ecological role of the taxa isolated in this study will be an interesting field for future study.

This study is the first to record *Phomopsis virgiliansis* to cause a root rot disease in South Africa. It is currently unknown whether this fungus is native to South Africa and a common pathogen on *Virgilia*. However, in preliminary assessments no root disease symptoms have been reported or observed from *Virgilia* trees from other areas in the CFR. As introductions of foreign organisms are common via diseased nursery material or timber (Coetzee *et al*. 2001; Von Broemsen 1989) and this disease is currently only known from a botanical garden, it is quite possible that the fungus reported here is not native to this area. The high incidence of tree death in this population adds credence to this notion. Being a botanical garden with an active nursery, it is possible that this pathogen could spread to other areas via rooted plants. Until further studies are conducted, it is thus recommended that no *Virgilia* plants are translocated from this area.

5.   TAXONOMY

Phomopsis virgiliansis Machingambi N. M, Dreyer L. L & Roets F., **sp. nov.** (Fig. 10).

Teleomorph: Unknown.

Etymology: Named after *Virgilia*, the host plant.

Pycnidia black, eustromatic, subglobose to conical, aggregated or scattered. In culture up to 400 μm wide. Pycnidial wall consists of brown, thick-walled cells. Conidia exuding from pycnidia in cream to peach-coloured droplets. Conidiophores cylindrical, noticeably flexuous and tall, well-developed, simple or mostly branched, 1-2 celled, (12.254-)16.579(21.305)x (0.682)1.154(1.468) μm. Conidiogenous cells straight to curved, tapering slightly towards the apex, minute periclinal thickening and funnel shaped collarette present. Alpha-conidia fusoid-ellipsoidal, apex bluntly rounded, base obtuse to subtruncate, biguttulate, (5.19-)6.9(-7.9) x (1.1-)2.3(3.5) μm; beta-conidia curved and needle like (17.059 – 25.348 μm long and 1.038 – 1.793 μm wide at widest).

Cultural characteristics on MEA: reaching a diameter of 63 mm after 5 days at 25°C in the dark. Colonies spreading with sparse, dirty white aerial mycelium with smoke-grey to pale brown surfaces; margins becoming pale brown; reverse smoke-grey at centre becoming darker as it radiates to the edges.

Specimens examined: Holotype, Isolate NM 12, South Africa, Western Cape Province, Stellenbosch University. Paratypes: isolates NM 11 – NM 20 same data as for holotype.
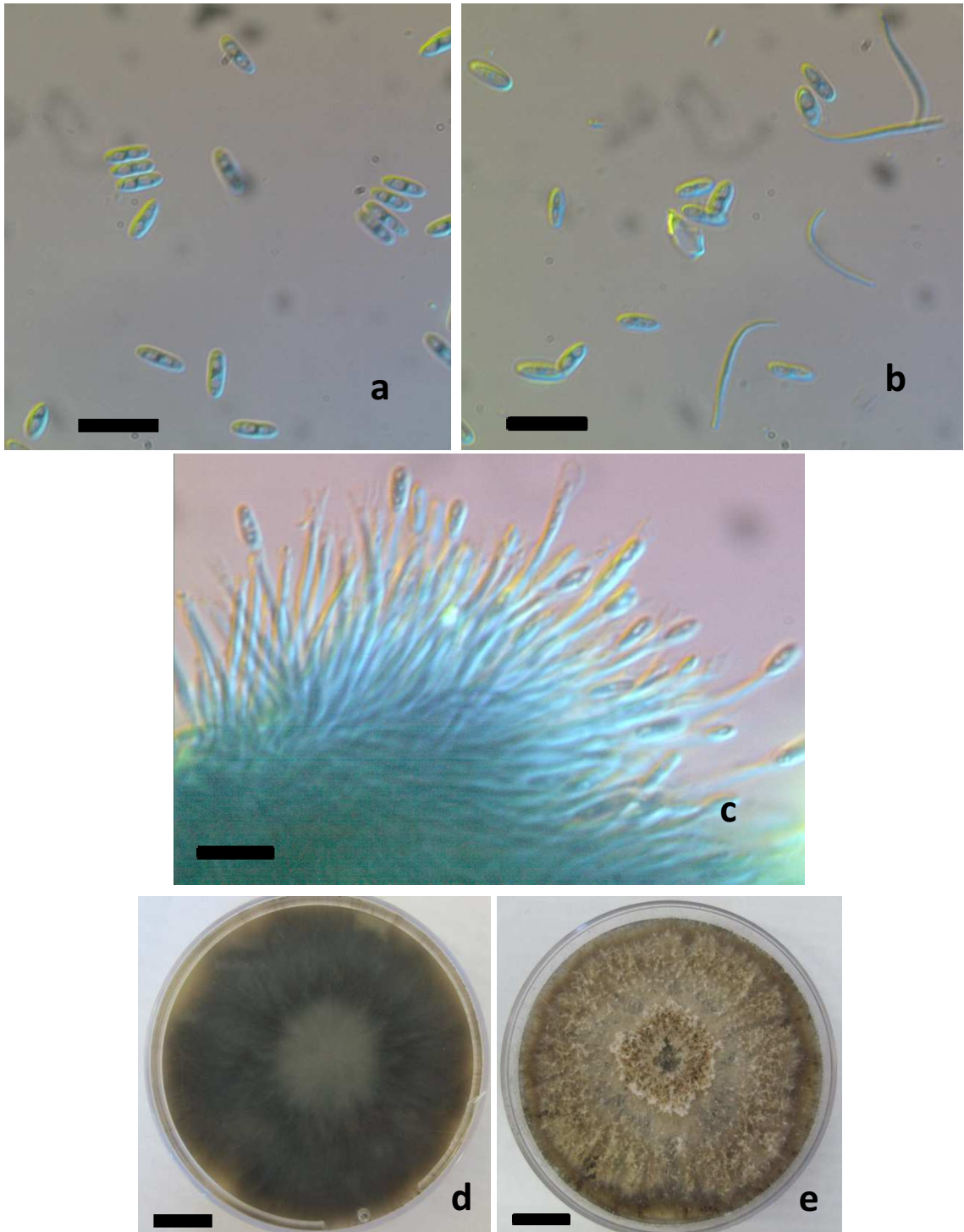
Fig. 10: *Phomopsis virgiliansis* sp. nov. NM12. (a) alpha-conidia; (b) alpha- and beta-conidia; (c) conidiophores; (d) and (e) 16 days old culture on MEA. Scale bars: a, b, c = 10 µm; d, e = 1 cm.

REFERENCES

Akaike H, 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**:716-723.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Avtzis DN, Bertheau C, Stauffer C, 2012. What is next in bark beetle phylogeography? *Insects* **3:** 453-472.

Berger S, Sinha AK, Roitsch T, 2007. Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. *Journal of Experimental Botany* **58**:4019-4026.

Bingham RT, Hoff RT, McDonald GI, 1971. Disease resistance in forest trees. *Annual Review Phytopathology* **2**: 433-452.

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

Čizkova D, Šrutka P, Kolarik M, Kubatova A, Pazoutova S, 2005. Assessing the pathogenic effect of *Fusarium*, *Geosmithia* and *Ophiostoma* fungi from broad-leaved trees. *Folia Microbiologica* **50**(1): 59-62.

Coetzee MPA, Wingfield BD, on Harring TC, Steimel J, Coutinho TA, Wingfield MJ, 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* **10**: 387-396.

Coetzee MPA, Wingfield BD, Roux J, Crous PW, Denman S, Wingfield MJ, 2003. Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa. *Plant Pathology* **52**: 604-612.

Crous PW, 2005. Impact of molecular phylogenetics on the taxonomy and diagnostics of fungi. Bull OEPP/EPPO **35**: 47-51.

Cruywagen EM, De Beer ZW, Roux J, Wingfield MJ, 2010. Three new *Graphium* species from baobab trees in South Africa and Madagascar. *Persoonia* **25**: 61-71.

Doidge EM, Bottomley AM, 1931. A revised list of diseases occurring in South Africa. *Botanical Survey of South Africa, Memoir No*. **11**: 1-78.

Diogo ELF, Santos JM, Phillips AJL, 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. *Fungal Diversity* **44**: 107-155.

Franceschi VR, Krokene P, Christiansen E, Krekling T, 2005**.** Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist* **167**: 353-376.

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

Geldenhuys CJ, 1994. Bergwind fires and the location pattern of forest patches in the southern Cape landscape, South Africa. *Journal of Biogeography* **21**: 49-62.

Hall T, 2005. BioEdit, biological sequence alignment editor for Win95/98/NT/2K/XP. Carlsbad, California: Ibis therapeutic.

Harrington TC, 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE, Blackwell M (eds.), *Ecological and Evolutionary Advances in Insect-Fungal Associations*, Oxford University Press New York, pp. 257-291.

Harrington TC, Fraedrich SW, Aghayeva DN, 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* **104**: 399-404.

Hartig R, 1878. *Die Zersetzungserscheinungen des Holzes der Nadelbäume und der Eiche forstlicher, botanischer und chemischer Richtung*. Berlin: Julius Springer.

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

Jeffers SN, Martin SB, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* **70**: 1038-1043.

Kirschner R, 2001. Diversity of filamentous fungi in bark beetle galleries in Central Europe. In: Misra JK, Horn BW (eds.), *Trichomycetes and other fungal groups*. Professor Robert W Lichtwardt Commemoration Volume Science Publishers, Enfield, (NH), USA, pp. 175-196.

Kolařík M, Kirkendall LR, 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). *Fungal Biology* **114**: 676-689.

Kolařík M, Kubátová A, Hulcr J, Pažoutová S, 2008. *Geosmithia* Fungi Are Highly Diverse and Consistent Bark Beetle Associates: evidence from their community structure in temperate Europe. *Microbial Ecology* **55**(1): 65-80.

Kolařík M, Kostovčík M, Pažoutová S, 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. *Mycological Research* **111**(11): 1298-1310.

Kolařík M, Kubátová A, Cepicka I, Pažoutová S, Srutka P, 2005. A complex of three new white-spored, sympatric and host range limited *Geosmithia* species. *Mycology Research* **109**: 1323-1336.

Kolařík M, Kubátová A, Pažoutová S, Srutka P, 2004. Morphological and molecular characterisation of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. *Mycology Research* **108** (9): 1053-1069.

Kubátová A, Kolařík M, Prasil K, Novotny D, 2004. Bark beetles and their galleries: well-known niche for little known fungi, case of *Geosmithia*. *Czech Mycology* **55**: 1-18.

Langstrom B, Hellqvist C, 1993. Induced and spontaneous attacks by pine shoot beetles on young Scots pine trees: tree mortality and beetle performance. *Journal of Applied Entomology* **115**: 25-26.

Lee J-S, Haack RA, Choi WI, 2011. Attack pattern of *Platypus koryoensis* (Coleoptera: Curculionidae: Platypodinae) in relation to crown dieback of Mongolian oak in Korea. *Environmental Entomology* **40**(6): 1363-1369.

Linnakoski R, de Beer ZW, Niemelä P, Wingfield MJ, 2012. Associations of Conifer-infesting bark beetles and fungi in Fennoscandia. *Insects* **3**: 200-227.

Loo JA, 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* **11**: 81-96.

Lübbe WA, Geldenhuys CJ, 1990. Decline and mortality of *Ocotea bullata* trees in the southern Cape forests. *South African Forestry Journal* **154**: 7-14.

Mehl JWM, Geldenhuys CJ, Roux J, Wingfield MJ, 2010. Die-back of kiaat (*Pterocarpus angolensis*) in southern Africa: a cause for concern? *Southern Forests: a Journal of Forest Scien*ce **72**: 3-4, 121-132.

Mehl JWM, Slippers B, Roux J, Wingfield MJ, 2011. Botryosphaeriaceae associated with *Pterocarpus angolensis* (kiaat) in South Africa. *Mycologia* **103**: 534-553.

Mostert L, Kang JC, Crous PW, Denman 2001. *Phomopsis saccharata* sp. nov., causing a canker and die-back disease of *Protea repens* in South Africa. *Sydowia* **53**(2): 227-235.

Moser JC, Konrad H, Kiristis T, Carta LT, 2005. Phoretic mites and nematodes associates of *Scolytus multistriatus pygmaeus* (Coleoptera: Scolytidae) in Austria. *Journal of Agricultural and Urban Entomology* **7**: 169-177.

Münch E, 1907. Die Blaufaule des Nadelhozes II. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **62**: 297-323.

Nielsen ES, Robinson GS, Wagner, DL 2000. Ghost-moths of the world; a global inventory and bibliography of the Exoporia (Mnesarchaeoidae and Hepialoidea). (Lepidoptera). *Journal of Natural History* **34**(6): 823-837.

Paine TD, Raffa KF, Harrington TC, 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42***:* 179-206.

Palgrave KC, 1983. Keith Coated Palgrave Trees of Southern Africa. Edition 2. Struik, Cape Town.

Palgrave CM, 2002. Keith Coates Palgrave Trees of Southern Africa. Edition 3. Struik, Cape Town.

Palmer E, Pitman N, 1961. Trees of South Africa Vol. 1. Balkema Cape Town.

Palmer E, Pitman N, 1972. Trees of South Africa Vol. 2. Balkema. Cape Town.

Pearson RC, Goheen C, 1994. *Phomopsis* cane and leaf spot. In: Hewitt WB, Pearson RC (eds.) *Compendium of grape diseases*. APS Press: St Paul, MI, pp. 17-18.

Phillips JFV, 1926. *Virgilia capensis* Lam. ('Keurboom'): A contribution to its ecology and silviculture. *South African Journal of Science* **23**: 435-454.

Posada D, 2008. Selection of models of DNA evolution with jModelTest. In: Posada D (ed.), *Bioinformatics for DNA sequence analysis*. Humana Press, Totowa, pp. 93-112.

Quimby Jr. PC, 1982. Impact of diseases on plant populations. In: Charudattan R, Walker HL (eds.), *Biological Control of Weeds with Plant Pathogens*. John Wiley & Sons, New York, pp. 47-60.

Raffa KF, Phillips TW, Salom SM, 1993. Strategies and mechanisms of host colonization by bark beetles. In: Schowalter T, Filip G (eds.), *Beetle-Pathogen Interactions in Conifer Forests.* San Diego: Academic, pp. 102–28.

Raffa FR, Smalley EB, 1995. Interaction of pre-attack and induced monoterpene interactions in host conifer defense against bark beetle-fungal complexes. *Oecologia* **102**: 285-295.

Ronquist FR, Huelsenbeck JP, 2003. MrBayes: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

Rosskopf EN, Charudattan R, DeValerio JT, Stall WM, 2000. Field evaluation of *Phomopsis amaranthicola*, a biological control agent of *Amaranthus* spp. *Plant Disiseases* **84**:1225-1230.

Roux J, Wingfield MJ, 2009. *Ceratocystis* species: Emerging pathogens of non-native plantation *Eucalyptus* and *Acacia* species. *Southern Forests* **71**: 115-120.

Santos JM, Phillips AJL, 2009. Resolving the complex of Diaporthe (*Phomopsis*) species occurring on *Foeniculum vulgare* in Portugal. *Fungal Diversity* **34**: 111-125.

Shapiro SS, Wilk MB, 1965. An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.

Six DL, 2003. Bark beetle-fungus symbioses. In: Bourtzis K, Miller TA (Eds.) *Insect Symbiosis Contemporary Topics in Entomology*. CRC Press, Boca Raton, London, New York, Washington D. C, pp. 97-114.

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

Smith CA, 1966. Common names of South African plants. *Memories of the Botanical Survey of South Africa* **35**.

Swofford DL, 2002. PAUP*: Phylogenetic Analysis Using Parsimony. (* and Other Methods), Version 4.0b10. Sinauer Associates, MA(2002).

Tainter FH, Baker FA, 1996. Principles of Forest Pathology. New York: John Wiley & Sons, Inc. p. 805.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Tisserat N, Cranshaw W, Leatherman D, Utley C, Alexander K, 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Online. Plant Health Progress doi:10.1094/PHP-2009-0811-01-RS.

Udayanga D, Liu X, McKenzie EHC, Chukeatirote E, Bahkali AHA, Hyde KD, 2011. The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. *Fungal Diversity* **50**:189-225.

Uecker FA, 1988. A world list of *Phomopsis* names with notes on nomenclature, morphology and biology. *Mycology Mem* **13**:1-231.

Van Daalen JC, 1981. The dynamics of the indigenous forest-fynbos ecotone in the southern Cape. *South African Forestry Journal* **119**: 14-23.

Van der Bank M, Van der Bank FH, Van Wyk B-E, 1995. Speciation in *Virgilia* (Fabacee): allopatric divergence followed by introgression? *Plant Systematics and Evolution* **201**: 57-73.

Van Kan JAL, 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci* **11**:247-253.

Van Niekerk JM, Groenewald JZ, Farr DF, Fourie PH, Halleen F, Crous PW, 2005. Reassessment of *Phomopsis* species on grapevines. *Australian Plant Pathology* **34**: 27-39.

Van Rensburg JCJ, Lamprecht SC, Groenewald J Z, Castlebury LA, Crous PW, 2006. Characterisation of *Phomopsis* spp. associated with die-back of rooibos (*Aspalathus linearis*) in South Africa. *Studies in Mycology* **55**: 65-74.

Van Wyk B, Van Wyk P, 1997. Field guide to trees of Southern Africa. Struik. Cape Town.

Von Broembsen SL, 1989. Invasions of Natural Ecosystems by Plant Pathogens. In: Drake JA, Mooney HA, di Castri F, Groves RH, Kruger FJ, Rejmanek M, Williamson M (eds.), *Biological Invasions: A Global Perspective* **37**: 77-83.

Von Broembsen SL, Brits GJ, 1985. Phytophthora root rot of commercially cultivated proteas in South Africa. *Plant Diseases* **69**: 211-213.

Von Broembsen SL, Kruger FJ, 1985. *Phytophthora cinnamomi* associated with mortality of native vegetation in South Africa. *Plant Diseases* **67**: 715-717.

Wager VA, 1931. Diseases of plants in South Africa due to members of the Pythiaceae. Union South Africa. Department of Agricultural Science. *Bullet* **103**.

White TJ, Bruns T, Lee S, Taylor, JW 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., New York, pp. 315-322.

Wingfield MJ, Coetzee MPA, Crous PW, Six D, Wingfield BD, 2010. Fungal phoenix rising from the ashes? *International Mycological Association Fungus* **1**(2): 149-153.

Zentmyer GA, 1980. *Phytophthora cinnamomi* and the diseases it causes. The American Phytopathological Society, St. Paul, N. M.

APPENDICES

Appendix 1: Species of *Phomopsis*/*Diaporthe* obtained from GenBank that were used in this study.

Species	Strain number	Host tree	Origins	GenBank accession number ITS
P. amygdali	STE-U 2632	*Vitis vinifera*	South Africa	AF230755
P. amygdali	STE-U 5151	*Vitis vinifera*	South Africa	AY485752
P. averrhoea	SCHM 3605	*Averrhoa carambola*	China	AY618930
P. bougainvilleicola	SCHM 3006	*Bougainvillea glabra*	China	AY601920
P. camptothecae	SCHM 3611	*Camptotheca acuminata*	China	AY622996
P. chimoanthi	SCHM 3614	*Chimonanthus praecox*	China	AY622993
P. castaneae mollissimae	n.e.	*Castanea mollissima*	China	JF957786
P. cuppatea	CBS117499		South Africa	AY339322

P. cuppatea	R164AN	*Aspalathus linearis*	South Africa	DQ286284
P. cuppatea	R433R			AY339322
P. cuppatea	Pho32	*Vitis* sp.		JN214591
P. columnaris	CBS109873	*Vaccinium vitis-idaea*	USA	AF439625
P. cotoneastri	CBS 439.82			FJ889350
P. dauci	CBS 315.49			FJ889451
P. emicis	BRIP 45089 a	*Emex australis*	China	JF957784
P. eucommicola	SCHM 3607	*Eucommia ulmoides*	China	AY578071
P. eucommii	SCHM 0020	*Eucommia ulmoides*	China	AY601921
P. glabrae	SCHM 3622	*Bougainvillea glabra*	China	AY601918
P. lagerstromiae	SCHM 3608	*Lagerstroemia indica*	China	AY622994
P. liquidambari	SCHM 3621	*Liquidambar formosana*	China	AY601919
P. loropetali	SCHM 3615	*Loropetalum chinense var. rubrum*	China	AY601917
P. magnoliae	SCHM 3609	*Magnolia officinalis*	China	AY622995
P. micheliae	SCHM 3603	*Michelia alba*	China	AY620820
P. phoenicicola	CBS 161.64	*Areca catechu*	India	FJ889452
P. phyllanthicola	SCHM 3680	*Phyllanthus emblicae*	China	AY620819
P. saccharata	u.n.	*Protea repens*	South Africa	AF387817
P. tuberivora	CBS 268.32	*Solanum tuberosum*	China	JF957785

P. vaccinii	CBS 160.32	*Vaccinium macrocarpon*	USA: Massachusetts	AF317578
P. viticola	STE-U 5639	*V. vinifera*	South Africa	AY485763
P. vitimegaspora	STE-U 2675	*V. vinifera*	South Africa	AF230749
Phomopsis sp. 1	STE-U 5567	*V. vinifera*	South Africa	AY485723
Phomopsis sp. 1	STE-U 2654	*V. vinifera*	South Africa	AF230757
Phomopsis sp. 3	STE-U 4407	*V. vinifera*	South Africa	AY485725
Phomopsis sp. 4	STE-U 5464	*V. vinifera*	South Africa	AY485726
Phomopsis sp. 5	STE-U 5465	*V. vinifera*	South Africa	AY485730
Phomopsis sp. MCK6	STE-U2680	*V. vinifera*	South Africa	AF230766
D. alleghaniensis	ATCC 24097, CBS 495.72	*Betula alleghaniensis*	Canada	FJ889444
D. ambigua	CBS 114015	*V. vinifera*	South Africa	AF230767
D. angelicae	CBS 111592		Australia	AY196779
D. aspalathi	CBS 117169/STE-U 5428	*Aspalathus linearis*	South Africa	DQ286275
D. aspalathi	R338E	*A. linearis*	South Africa	AY339321
D. australafricana	CBS 113487	*V. vinifera*	South Africa	AF230744
D. crotalariae	CBS 162.33	*Crotalaria spectabilis*	Portugal	FJ889445
D. helianthi	STE-U 5353[B]	*V. vinifera*	South Africa	AY485746
D. hickoriae	CBS 145.26	*Carya glabra*	Portugal	FJ889446

77

Diaporthe kyushuensis	u.n.	Grape vine	Japan	AB302250
D. lusitanicae	CBS 123213, CBS 123212	*Foeniculum vulgare*	Portugal	GQ250190
D. melonis	CBS 507.78	*Cucumis melo*	USA	FJ889447
D. neotheicola	CBS 123209, CBS 12308	*F. vulgare*	Portugal	EU814480
D. perjuncta	CBS109745	*V. vinifera*	South Africa	AY485785
D. stewarti	CBS 193.36	*Cosmos bipinnatus*	Portugal	FJ889448
D. strumella var. longispora	CBS 194.36	*Ribes* sp.	Portugal	FJ889449
D. viticola	STE-U 5683, CBS 113201	*V. vinifera*	South Africa	AY485750
Leucostoma niveum	NW341		China	EU520061
L. persoonii	ATCC 42549	*Prunus persica*	USA	DQ996042
Valsa mali var. *pyri*	GSZY113	*Malus pumila*	China	GU174589
V. japonica	CBS375.29	*Prunus persicae*	Japan	AF191185
P. virgiliansis	NM11	*Virgilia oroboides*	South Africa	
P. virgiliansis	NM12	*V. oroboides*	South Africa	
P. virgiliansis	NM13	*V. oroboides*	South Africa	
P. virgiliansis	NM14	*V. oroboides*	South Africa	

P. virgiliansis	NM15	V. oroboides	South Africa	
P. virgiliansis	NM16	V. oroboides	South Africa	
P. virgiliansis	NM17	V. oroboides	South Africa	
P. virgiliansis	NM18	V. oroboides	South Africa	
P. virgiliansis	NM19	V. oroboides	South Africa	
P. virgiliansis	NM20	V. oroboides	South Africa	

CHAPTER 3

FIRST REPORT OF SCOLYTINE BEETLE-MITE-FUNGUS ASSOCIATIONS ON NATIVE *VIRGILIA* TREES IN SOUTH AFRICA

ABSTRACT

Bark and ambrosia (scolytine) beetles are ecologically and economically important phytophagous insects that have successfully adapted to subcortical environments. They can pose primary, secondary or saprophytic threats to their hosts. Scolytine beetles often have complex relationships with fungi and mites, which may be mutualistic, commensal, incidental, obligatory and/or antagonistic. Recent reports suggested widespread scolytine beetle activity on native *Virgilia* trees in the Cape Floristic Region (CFR) of South Africa. In this study, we identified these scolytine beetles, their main fungal associates, and their phoretic mites. We also tested the ability of the phoretic mites to feed on the associated fungi. Four morpho-species of scolytine beetles, each with a unique tunnelling system, were collected from various *Virgilia* hosts. All except one inhabited the inner bark and cambium of the hosts, while one was an ambrosial type. Morpho-species A and B were associated with a single species of phoretic mite. Both the beetles and these mites were commonly associated with various species of *Geosmithia*. Morpho-species C did not carry phoretic mites, but was also associated with various *Geosmithia* spp. Morpho-species D was associated with a *Geosmithia pallida*-like fungus and had phoretic mites only when infestation was very high. *Geosmithia* associates of each morpho-species were maintained over extended geographic distances and co-existing bark beetles had similar *Geosmithia* communities. Phoretic mite numbers were determined over a seven week period as beetles appeared from emergence boxes. Mean number of phoretic mites increased significantly with time, but after *ca*. seven weeks only dead mites were present on beetles. Phoretic mites were unable to feed on their *Geosmithia* associates, but were observed to feed on dead larvae within tunnels. Our results

reveal that the relationship of bark beetles, mites and *Geosmithia* is complex, ranging from commensal to mutual. The effect of *Geosmithia* on bark beetles still remains unknown.

---

1.   INTRODUCTION

Bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) are among the most economically important forest and forestry pests globally (Avtzis *et al*. 2012; Harrington 2005; Linnakoski *et al*. 2012). Currently about 225 genera, including more than 6 000 species, of scolytine beetles have been described worldwide (Avtzis *et al*. 2012; Linnakoski *et al*. 2012). They are phytophagous insects inhabiting subcortical environments with a wide host range and can pose primary, secondary or saprophytic threats (Linnakoski *et al*. 2012; Raffa *et al*. 1993). The most aggressive group consists of primary bark and ambrosia beetles (Paine *et al*. 1984; Six and Wingfield 2011). Healthy trees produce insecticides and/or other chemicals like monoterpenes, resins or phenolics in response to insect attack, killing or immobilizing the insects (Hudgins *et al*. 2004; Raffa and Smalley 1995; Six and Wingfield 2011). However, a very large outbreak of primary scolytine beetles may overcome the natural defence of trees, ultimately leading to tree death (Franceschi *et al*. 2005; Lee *et al*. 2011; Six and Wingfield 2011). The southern pine bark beetle (SPB), *Dendroctonus frontalis* Zimmermann, is an example of a primary bark beetle that is capable of killing healthy trees. It caused an estimated $900 million loss in pine production in the southern United States between 1960 and 1990 (Price *et al*. 1992). Another example is the Redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Scolytinae), which was introduced to the southeastern USA, together with a fungal associate, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva (Fraedrich *et al*. 2008; Harrington *et al*. 2008). This ambrosia beetle and its fungal associate are responsible for the severe wilt disease of Redbay trees (*Persea borbonia* (L.) Spreng.) and other members of the Lauraceae in the USA (Whitney 1982; Harrington *et al*. 2008). The extensive death of Lauraceae species can negatively impact on organisms depending on them for survival, like spicebush swallowtail (*Papilio troilus* L.) and the Palamedes swallowtail (*Papilio palamedes* Drury) among other wildlife (Reed and Muzika 2010).

Secondary beetles attack trees weakened and/or stressed by drought, disease or physical damage (Avtzis *et al*. 2012; Paine *et al*. 1997; Raffa *et al.* 1993; Six and Wingfield 2011). They may also feed on logging residue or fallen trees (Langstrom and Hellqvist 1993; Paine *et al*. 1997). *Ips typographus* L. is an example of a secondary beetle that killed 3700 hectares of spruce trees between 1992 and 2000 in the 'Bavarian Forest' German National Park (Wermelinger 2003). In this case, host trees were thought to be weakened by the interaction of above-average temperatures, inadequate water supply and wind-throws (Wermelinger 2003). The largest group of scolytine beetles have saprophytic habitats (Raffa *et al*. 1993) and solely colonize and reproduce on dead hosts (Paine *et al*. 1997; Raffa *et al*. 1993). Despite their ecological importance in, for example, initiating nutrient cycling (Christiansen *et al*. 1987; Stark 1982), they have not attracted much research interest, as they seldom cause economic losses, except for a few that vector detrimental fungi (Lieutier *et al*. 2009).

The occurrence of bark and ambrosia beetles in South Africa has received some attention in the forestry industry, but very little in natural ecosystems. Recorded species include the introduced species *Hylastes angustatus* (Kirsten *et al*. 2000; Tribe 1990, 1992; Zhou *et al*. 2002), *Hylurgus ligniperda* Fabr. (Tribe 1992), *Orthotomicus erosus* Wollaston (Tribe 1990, 1992) and *Scolytus kirschii* Skalitzky (Six *et al*. 2005). The latter species was first detected in South Africa in February 2005, initiating galleries in the phloem of English elms (*Ulmus procera* Salisb) growing on a farm in Stellenbosch (Six *et al*. 2005). More recently, the ambrosia beetle *Xylosandrus crasiusculus* (Motschulsky) was reported from *Tabernaemontana* L. trees in the KwaZulu-Natal Province (Roux *et al*. 2011).

Bark and ambrosia beetles may have complex associations with various organisms, including fungi (Linnakoski *et al*. 2012; Six and Paine 1998; Six and Wingfield 2011; Whitney 1982), bacteria (Bridges 1984), mites (Cardoza *et al*. 2008; Klepzig *et al*. 2001; Moser *et al*. 1995, 2005) and nematodes (Cardoza *et al*. 2008; Moser *et al*. 2005). Scolytine beetles associated with ophiostomatoid fungi (for example species of *Ceratocystis*, *Ophiostoma* and *Raffaelea*) are of particular interest, as these fungi may have considerable economic importance (Kirkendall 1983; Klepzig *et al*. 2001; Moser *et al.* 1995; Wood 1982). Several studies have shown that such relationships may be incidental or obligatory, mutualistic, commensal or

antagonistic (Kolařík *et al*. 2008; Six 2003; Six and Wingfield 2011). Mycelia and/or spores of the fungal associates can either be carried on exoskeletons of these beetles or inside specialised structures called mycangia (Linnakoski *et al*. 2012; Paine *et al*. 1997; Six and Paine 1998; Six 2003; Six and Wingfield 2011; Whitney 1982). The fungi are inoculated into host trees as the beetles colonise their hosts (Linnakoski *et al*. 2012; Six and Wingfield 2011). The fungi benefits by being vectored to new plant hosts (Paine *et al*. 1997; Six 2003; Six and Wingfield 2011). Some studies have shown the ability of bark and ambrosia beetles to feed on some of their fungal associates (Six 2003; Harrington 2005), while other fungi are antagonistic to the beetles (Barras 1970; Harrington and Zambino 1990; Hofstetter *et al*. 2006; Klepzig *et al*. 2001; Six and Wingfield 2011). The SPB for example, vectors *Ceratocystiopsis ranaculosa* and *Entomocorticium sp.* A (an undescribed Basidiomycete) in their mycangia, which it is thought to feed on (Harrington and Zambino 1990; Hofstetter *et al*. 2006; Klepzig *et al*. 2001). It also carries the blue-staining ascomycete *Ophiostoma minus* externally on its exoskeleton, which is known to be particularly antagonistic to its larvae and a sap-staining pathogen of the host trees (Barras 1970; Harrington and Zambino 1990; Hofstetter *et al*. 2006; Klepzig *et al*. 2001; Six and Wingfield 2011).

In addition to fungi, mites are commonly associated with bark beetles. These are usually phoretic on the beetles and can be parasitic, predatory, fungivorous and/or omnivorous (Klepzig *et al*. 2001). The fungivorous group is of particular importance to forestry, as they can transport pathogenic or sapstain fungi (Moser *et al*. 2005). Some mites possess specialised flap-like structures called sporothecae, in which ascospores of their associated fungi are transported (Klepzig *et al*. 2001; Moser *et al.* 1995; Six and Wingfield 2011). Some studies have proved the ability of these mites to feed on the fungi they transport in their sporothecae (Cardoza *et al*. 2008; Klepzig *et al*. 2001). The relationship of scolytine beetles, phoretic mites and fungi is well-researched and understood in the SPB system. True phoretic mite species found on the SPB are *Tarsonemus ips*, *Tarsonemus krantzii* and *Tarsonemus fusarii* (Klepzig *et al*. 2001). *Tarsonemus ips* and *T. krantzii* carry spores of *O. minus* and/or *Ceratocystiopsis ranaculosa* in their sporothecae (Klepzig *et al*. 2001; Moser *et al.* 1995). In a study by Klepzig *et al*. (2001), these mite species successfully reproduced and showed positive growth rates when fed on new hyphal growth of their fungal associates. This suggests a mutual relationship between the mites and their associated fungi.

Most studies on the interactions between bark beetles and other organisms have focussed on the ophiostomatoid fungi. However, numerous other fungal taxa may be consistently associated with these beetles. For example, the genus *Geosmithia* Pitt, is a polyphyletic mitosporic ascomycete genus of fungi belonging to hypocrealean (Hypocreales: Boinectriaceae) and eurotialeans fungi (Eurotiales: Trichocomaceae) (Čizkova *et al*. 2005; Kolařík *et al*. 2004, 2005, 2007; Ogawa *et al*. 1997). They have a worldwide distribution (Čizkova *et al*. 2005; Kolařík *et al*. 2004, 2005, 2007; Ogawa *et al*. 1997). Until recently, the genus was understudied, but after the hypocrealean group was found to be commonly associated with several scolytine beetle species, there has been a growing body of literature on these fungi (Jiri and Dunn 2011; Kolařík and Kirkendall 2010; Kolařík *et al*. 2007, 2008). Entomochoric adaptations are absent in *Geosmithia* species (Kolařík and Kirkendall 2010; Kolařík *et al*. 2008). Instead, they produce hydrophobic and dry conidia that are typically air borne (Kolařík *et al*. 2007, 2008). In central Europe, more than 20 species of bark beetles have been associated with *Geosmithia* spp., while five species of ambrosia beetles have been found associated with *Geosmithia* spp. in tropical regions of America, Asia and Australia (Kolařík *et al*. 2007, 2008). Despite being regular scolytine beetle associates, the effects of *Geosmithia* on the beetles still remain vague (Kolařík *et al*. 2007, 2008). Kolařík and Kirkendall (2010) suggested that they may have nutritional value to scolytine beetles. Phytopathogens of this genus include *Geosmithia morbida* M. Kolarík, E. Freeland, C. Utley & Tisserat, which is a serious threat to black walnut trees (*Juglans nigra* L.) as it causes the thousand cankers disease (Tisserat *et al*. 2009). *Geosmithia morbida* is dispersed by the walnut twig beetle (*Pityophthorus juglandis* Blackman) between its host trees (Tisserat *et al*. 2009). A study by Čizkova *et al*. (2005) suggested that *G. langdonii* M. Kolarík, Kubátová & Pažoutová and *G. pallida* (G. Sm.) M. Kolarík, Kubátová & Pažoutová, isolated from *Scolytus intricatus* (Ratz.), has the ability to inhibit root formation in *Lepidium sativum* L. var. *capitatum* Hook. f., probably due to toxin production.

The Cape Floristic Region (CFR) of South Africa is home to the ornamental and ecologically important tree genus *Virgilia* Pior. (Fabaceae). *Virgilia* species are confined to riparian vegetation, thickets, hillsides and forest margins (Palgrave 1983, 2002; Palmer and Pitman 1972). Recent reports based on personal observations indicated bark beetle activity on *Virgilia* trees throughout the CFR. However, very little is known about these beetles and

their associated organisms. Our research sought to identify the bark beetles, mites and fungal species associated with these *Virgilia* trees. Specific objectives included to: (i) identify bark beetle species infesting *Virgilia* trees from a wide geographical range; (ii) identify mite species phoretic on these beetles; (iii) isolate and identify fungal taxa consistently associated with the bark beetles and their phoretic mites and (iv) test whether mites that are phoretic on bark beetles can feed on the fungi they consistently carry.

2.   MATERIALS AND METHODS

2.1   Bark beetle and mite collection

Bark beetles were collected from naturally infested *Virgilia* trees throughout the CFR during the course of 2011 and 2012 (Table 1). Samples of bark and branches colonised by scolytine beetles were randomly collected from stems and branches of affected trees. Bark samples were placed in labelled zipper lock bags, while masking tape labels were placed around branches, and the collected branches were placed in large black plastic bags.

Table 1: Study sites

Site	GPS coordinates	Host *Virgilia* species
HPNBG	S 34' 20.893 E 18' 55.519	V. *oroboides oroboides*
Jonkershoek	S 33°58'23.10" E 18°56'11.38"	*V. o. oroboides*
KNBG	S 33°59'11.3'' E 18°25'34.4''	*V. o. oroboides*
Table Mountain	S 33°57'17.76" E 18°25'29.64"	*V. o. oroboides*
SMNR	S 34°05'27.89" E 18°25'17.55"	*V. o. oroboides*
George	S 33°54'56.07" E 22°33'11.10"	*V. o. ferruginea*
Knysna	S 34°00'21.44" E 23°07'00.61"	*V. divaricata*
Storms River	S 33°05'15.54" E 18°25'06.96"	*V. divaricata*

HPNBG – Harold Porter National Botanical Garden; KNBG – Kirstenbosch National Botanical Garden; SMNR – Silver Mine Nature Reserve

In the laboratory, collected branches were placed in insect emergence cages constructed from sealed cardboard boxes (ca. 49 x 49 x 32.6 cm) fitted with two clear plastic bottles (5.7 cm diameters). Emerging beetles are attracted to light penetrating through these bottles and were thus easily collected. In addition, bark beetles were collected directly from galleries on a subset of bark and branch samples using metal tweezers under sterile conditions.

A Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan) was used to study the collected bark beetles and their gallery systems. Beetles were often associated with phoretic mites. Beetles and their phoretic mites were grouped into morpho-species. Phoretic mite numbers were noted and for the two most common bark beetle species, phoretic mite numbers were monitored over a seven week period. Each week, the number of mites per beetle was counted on 20 bark beetle individuals of each morpho-species. Normality of the

mite numbers data was tested using a Shapiro-Wilk test (Shapiro and Wilk 1965). The data was analysed using Kruskal-Wallis ANOVA and Median test procedures in Statistica 10 (Statsoft Corporation, USA). Significant differences are reported when $P \leq 0.05$.

A few individuals of each bark beetle and mite morpho-species were stored in 100% ethanol. Mites were mounted onto microscope slides for later identification by professional acarologists. Reference specimens of beetles and mites were deposited in the Stellenbosch University Insect Collection, Stellenbosch, South Africa.

2.2   Fungal isolation

Fifty individuals of each morpho-species of bark beetle and mite were washed separately in eppendorf tubes containing 0.1 ml ddH$_2$O. This water was subsequently spread onto malt extract agar (MEA: 20gL-1 malt extract and 20gL-1 agar, Biolab, South Africa) in petri dishes. Additionally, individual beetles were separately crushed in eppendorf tubes containing 0.1 ml ddH$_2$O, where after this solution was spread onto MEA. Plates were sealed with parafilm and incubated at room temperature (20 to 25°C) under normal day and night conditions until resultant fungi could be purified. To purify the growing fungi, hyphal tips of developing mycelia were transferred to fresh MEA plates under sterile conditions.

Fungal isolations were also made directly from bark samples containing fresh bark beetle galleries. Bark samples were placed in separate, labelled moisture chambers (clear plastic bag with moist filter paper) for 7 to 12 days to stimulate sporulation of fungi in the gallery systems. These were stored at room temperature (20 to 25°C) in the dark. Spores from fungal structures that formed within galleries were transferred to MEA plates using a sterile needle. Pure cultures from all isolated fungi were stored at 4°C on MEA until further use. The most consistent fungal morpho-types isolated from each mite and bark beetle morpho-species and their gallery systems were examined and their morphological characters recorded. Representative isolates of all fungal taxa used in this study will be deposited in the

culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at University of Pretoria, South Africa.

2.3   DNA extraction, amplification and sequencing

At least three pure cultures of representative isolates of each fungal morphologically similar isolates from all bark beetle and mite morpho-species and fresh bark beetle galleries were chosen for molecular identification. Fungal mycelia from these isolates were harvested using a sterile scalpel. DNA was extracted using Sigma-Aldrich™ plant PCR kit (USA) following the manufacturer's instructions. ITS1-f (Gardes and Bruns 1993) and ITS4 (White *et al*. 1990) primers were used to amplify the nuclear ribosomal internal transcribed spacer regions (ITS1, ITS2) including the 5.8S gene of the rDNA. 20 µL PCR reaction volumes were used, consisting of 5 µL REDExtract-N-Amp PCR ready mix (Sigma-Aldrich™, USA), 10 µL ddH$_2$O, 0.5 µL (10mM) of each primer and 4 µL extracted fungal DNA.

DNA from one representative of each bark beetle morpho-species was isolated from the head and 2 hind legs of individual beetles using a Qiagen DNeasy Blood & Tissue Kit (50) (QIANEN GmbH, Hilden) following the manufacturer's instructions. The lysis step lasted for 24 hours. The cytochrome oxidase gene 1 region of the DNA was amplified using universal insect primers Jerry (C1-J-2183) and Pat (TL2-N-3014) (Simon *et al*. 1994). PCR reaction volumes of 20 µL were used containing 10 µL of KAPA-Taq ReadyMix (KAPA BIOSYSTEMS, Cape Town), 4 µL of ddH$_2$O, 1 µL (10mM) of each primer, 2 µL of 25mM MgCl$_2$ and 2 µL extracted fungal DNA. Amplification of both gene regions was done using a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.).

PCR reaction conditions for ITS were: initial denaturing at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute 30 seconds and a final elongation step at 72°C for 8 minutes. PCR conditions for CO1 were: initial denaturing for 30 seconds at 95°C, followed by 35 cycles of denaturing for 30 seconds at 95°C, annealing at 50°C for 40 seconds and elongation at

72°C for 2 minutes and a final elongation step for 8 minutes at 72°C. All PCR products were visualised by gel electrophoresis. 5 µL of each PCR product was loaded onto 1.5 % agarose gel (Promega Corporation, Madison, U.S.A.) stained with 2.5 µL of ethidium bromide and placed into Tris-Borate-EDTA buffer (TBE). The products were visualised under ultraviolet light. Amplified PCR products were sent to the Stellenbosch University Sequencing Facility for subsequent purification and sequencing using the same primers as for PCR. Resulting sequences were manually edited using BIOEDIT v7. 0. 5 (Hall 2005).

2.4   Molecular analyses

Fungal sequences generated in this study were compared to published sequences using the BLAST (Basic Local Alignment Search Tool) algorithm search (Altschul *et al*. 1990) in GenBank (http://www.ncbi.nlm.nih.gov/genbank, Table 3). The closest matching fungal taxa based on ITS included *Geosmithia* spp*.* Existing *Geosmithia* sequences were downloaded from GenBank for the analyses and these were adopted from known phylogenies of *Geosmithia* (Kolařík *et al*. 2004, 2005, 2007, 2008; Kolařík and Kirkendall 2010). The dataset was aligned using Clustal W (Thompson *et al*. 1994) and manual adjustment in BioEdit v7. 0. 5 (Hall 2005)*. Glomerella acutata* Guerber & Correll (GenBank AF521210) and *Colletotrichum musae* (Berk. & M.A. Curtis) Arx (GenBank AY266401) were used as outgroup taxa (not shown in constructed tree) (Afanador-Kafuri *et al*. 2003). Phylogenetic analyses were conducted using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003) and PAUP (Phylogenetic Analysis Using Parsimony PAUP*4.0b10) (Swofford 2002). In PAUP, a Maximum Parsimony (MP) analysis was done using the heuristic search option with random addition of sequences (1 000 replications), tree bisection-reconnection (TBR) and MULTREES options ON. Bootstrap support values with 1 000 replications were calculated to assess the confidence of resultant nodes in the MP trees with the MULTREES option OFF and 10 random sequence additions in each of 1 000 pseudo-replications. In MrBayes, a Markov Chain Monte Carlo (MCMC) approach was used, using the most parameter rich model (GTR+I+K) as selected in jModelTest 0.1.1 (Posada 2008), using Akaike information criteria (Akaike 1974). Eight million generations were run, with a sampling frequency of 100 and burn-in trees set at the first 25%. The remaining trees were pooled into a 95% majority consensus tree.

2.5   Mite feeding studies

To test the ability of phoretic mites to feed on the fungi they were commonly associated with, 10 mite individuals were placed onto three isolates of each *Geosmithia* spp. isolated in this study. Plates with sterile MEA served as control and the experiment was replicated three times. Plates (6.4 cm diam.) with fungi that had grown to fully cover the surface of the MEA media were used in these assays to limit growth of potential contaminants. To prevent mites from escaping, plates were sealed with parafilm and placed in 15 L plastic containers that were halve-filled with water, allowing the plates to float. The lid of the containers was lined with petroleum jelly before closing to prevent entry of contaminating mites and other organisms. After 40 days at 25°C, we recorded the number of surviving mites in each plate using a stereo-microscope.

Mite feeding preferences were tested in dual choice assays. Ten adult mites were placed in petri dishes (6.4 cm diam.) that contained 1 cm diameter plugs of cultures of the various *Geosmithia* spp. isolated in this study. Sterile MEA plugs acted as controls. Pair-wise combinations were designed in such a way that all the fungal taxa were tested against each other, as well as against a sterile MEA control. The two plugs on each plate were placed opposite each other against the plate walls to ensure maximum spatial separation. Mites were released in the centre of each dish, and the number of mites and eggs at any of the plugs were counted and recorded at 4, 24, 48 and 72 hours. This experiment was replicated 3 times.

3.   RESULTS

3.1   Bark beetles

A total of 43 beetle infested *Virgilia* trees throughout the CFR were sampled for scolytid beetles. Four morpho-species of scolytine beetles were collected from these samples (Tables

2 and 3 and Fig. 1). This was confirmed by DNA sequence data (data not shown). The numbers of each beetle morpho-species collected were as follows: morpho-species A *ca*. between 4 500 and 5 000; morpho-species B *ca*. between 8 000 and 9 000; morpho-species C between 1700 and 2 500 and morpho-species D only 14.

Table 2: Summary of the scolytine beetle morpho-species collected from *Virgilia* trees from different localities throughout the CFR.

Site	*Virgilia* taxon	Bark beetle morpho-species collected	Trees sampled for bark beetles
HPNBG	V. *oroboides oroboides*	A, B, C and D	17
Jonkershoek	*V. o. oroboides*	C	1
KNBG	*V. o. oroboides*	A and B	4
Table Mountain	*V. o. oroboides*	A, B and C	5
SMNR	*V. o. oroboides*	A, B and C	4
Groenkop, George	*V. o. ferruginea*	A, B and D	4
Knysna	*V. divaricata*	A, B and C	4
Storms River	*V. divaricata*	A and B	4

HPNBG – Harold Porter National Botanical Garden; KNBG – Kirstenbosch National Botanical Garden; SMNR – Silver Mine Nature Reserve

Table 3: Summary of numbers of individual scolytine beetle morpho-species collected from *Virgilia* trees from different localities throughout the CFR.

	Morpho-species A	Morpho-species B	Morpho-species C	Morpho-species D
HPNBG	>200	>300	700-800	13
Jonkershoek			100-200	
KNBG	550-650	700-800		
Table Mountain	800-900	1 000-1 200	400-500	
SMNR	>400	300-400	600-700	
Groenkop, George	700-800	500-600		1
Knysna	600-700	800-900	*ca*. 300	
Storms River	>1 000	>2 000		

HPNBG – Harold Porter National Botanical Garden; KNBG – Kirstenbosch National Botanical Garden; SMNR – Silver Mine Nature Reserve
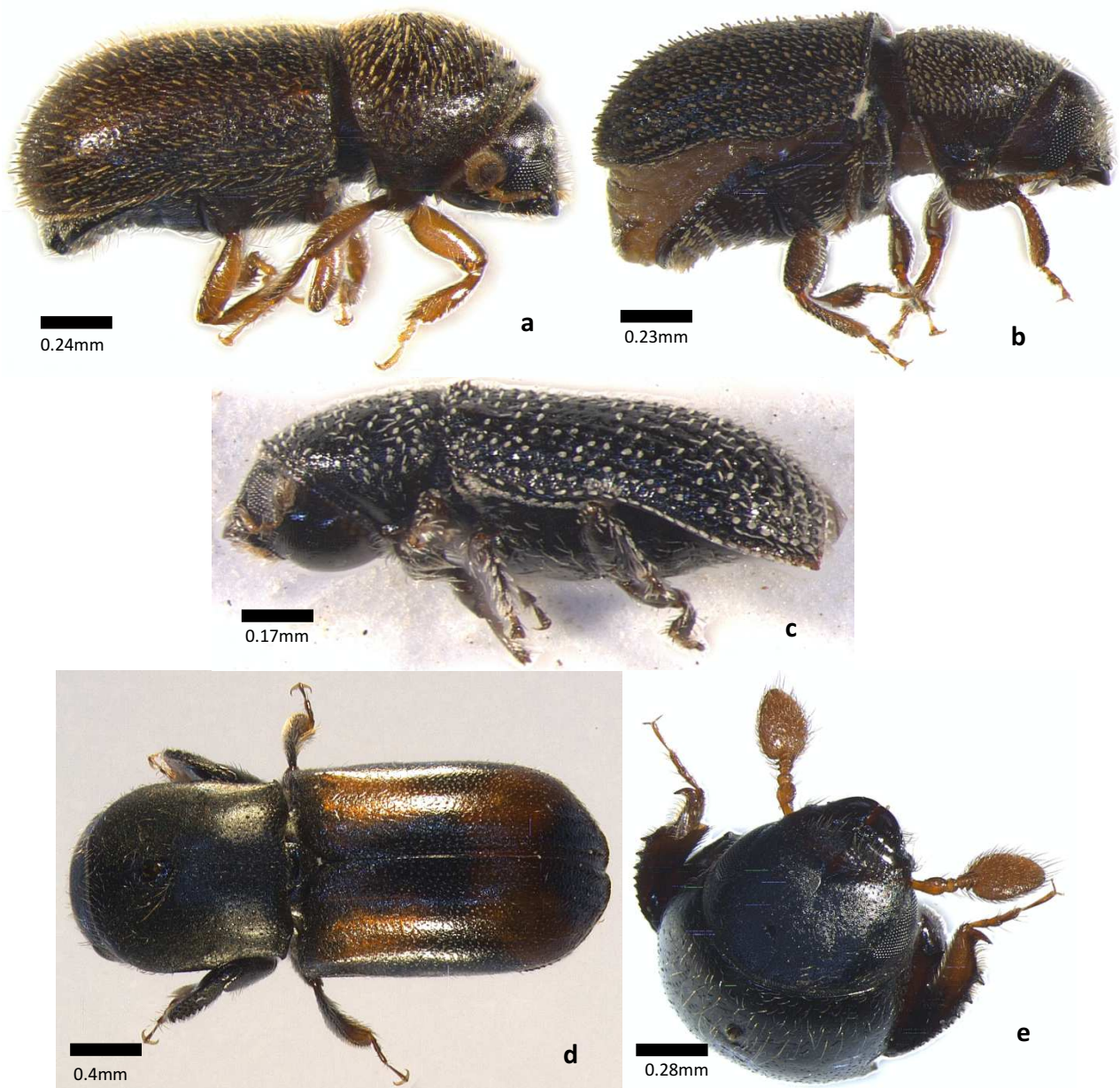
Fig. 1: Morpho-species of scolytine beetles associated with dead and dying *Virgilia* trees in the CFR. (a)-Morpho-species A (ca. 2 mm long); (b) morpho-species B (ca. 1.8 mm long); (c) morpho-species C (ca. 1.5 mm long); (d) morpho-species D (ca. 3 mm long); e close-up of head and thorax of morpho-species D.

Morpho-species A and B were common in all areas sampled, except in Jonkershoek (Fig. 1 and Table 2). Morpho-species C was found in most sampled areas, except KNBG, Groenkop

and Storms River, while morpho-species D was found in HPNBG and only one specimen in Groenkop (Fig. 1 and Table 2). Morpho-species A, B and C constructed their galleries in the cambium/inner bark, while morph-species D bore straight into the heartwood of its host (Fig. 2). Morpho-species C seemed to prefer smaller trees/branches, but was occasionally found inhibiting outer bark layers in larger trees/branches with thicker bark. All morpho-species co-existed with at least one other scolytine beetle at some stage during the course of the study period. However, each morpho-species had a distinct gallery system (Fig. 2). Parental galleries of morpho-species A are short, slightly thicker than those of morpho-species B and C and orientated horizontal to (against the grain of) vascular tissue. Larval galleries radiate at right angles from parental galleries extending parallel to the grain of the tree (vascular tissue). They are short, and in older hosts they contained frass. They terminate in large pupation chambers containing frass, which turned brown as the brood of beetles sclerotized. Morpho-species B has almost straight parental galleries that extend parallel to the grain of the host tree. They are clean, without frass. Larval galleries expand gradually at right angles from parental galleries, perpendicular to the grain of the host. They can be long, and gradually become thicker as the larvae feed and grow. They contain frass from about midway onwards, and terminate in a large feeding and pupation cell. Morpho-species C makes small parental galleries and larval galleries also diverge at right angles from the parental galleries. It has the thinnest larval galleries, and also contains frass. Morpho-species D bore deep into the wood of host trees. Each hole contained a pair of adults and had frass at the entrance. At HPNBG, morpho-species A, B and D occupied the same individual trees. In all study sites, except in Jonkershoek, morpho-species A and B were often collected from the same individual tree, with their galleries constructed in close proximity to one another (Fig. 3). In HPNBG, Table Mountain, SMNR and Knysna, morpho-species A, B and C were sometimes collected from the same individual hosts.

Fig. 2: Gallery systems of bark beetles underneath the bark of *Virgilia* spp. in the CFR. (a) gallery system of bark beetle morpho-species A, i – parental tunnels, ii – larval tunnels; (b) gallery system of bark beetle morpho-species B, i – parental tunnels; ii –larval tunnels; (c) gallery system of bark beetle morpho-species C, I – parental tunnels; ii – larval tunnels.
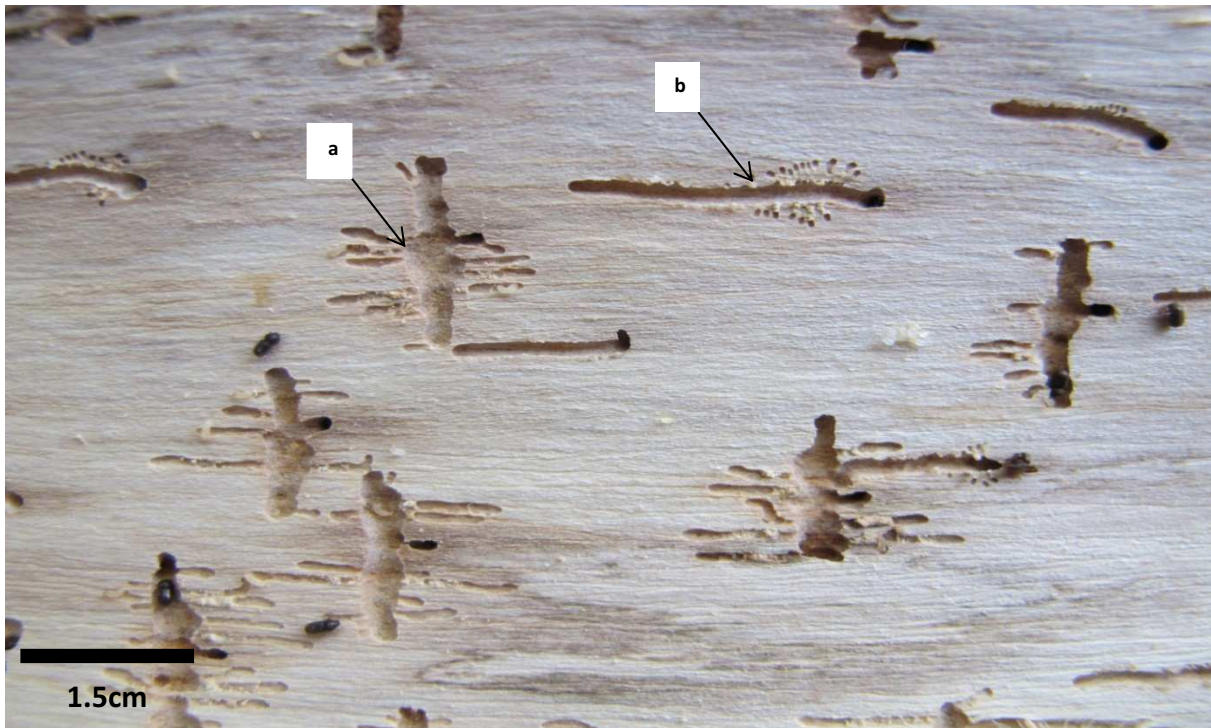
Fig. 3: Gallery systems of co-occurring bark beetles underneath the bark of *Virgilia* spp. in the CFR. (a) gallery system of bark beetle morpho-species A; (b) gallery system of bark beetle morpho-species B.

3.2 Phoretic mites

Bark beetle morpho-species A and B commonly carried a single morpho-species of Pygmephoridae mite (most likely from the genus *Elattoma*) phoretic on them (Fig. 4). The mites were gregarious and were always found in clusters. The numbers of mites per beetle varied from zero to as many as 217. This depended on the time at which the beetles emerged from the wood (Fig. 5). Bark beetle morpho-species C never carried phoretic mites. Morpho-species D was very rarely encountered (13 individuals in total) and phoretic mites were not usually seen on them. However, in one instance (during week 6 after collection) 217 mites were counted from a single individual. These mites were similar to those on bark beetle morpho-species A and B. Mite taxon identities based on morphological characters were confirmed by DNA sequencing results (data not shown).
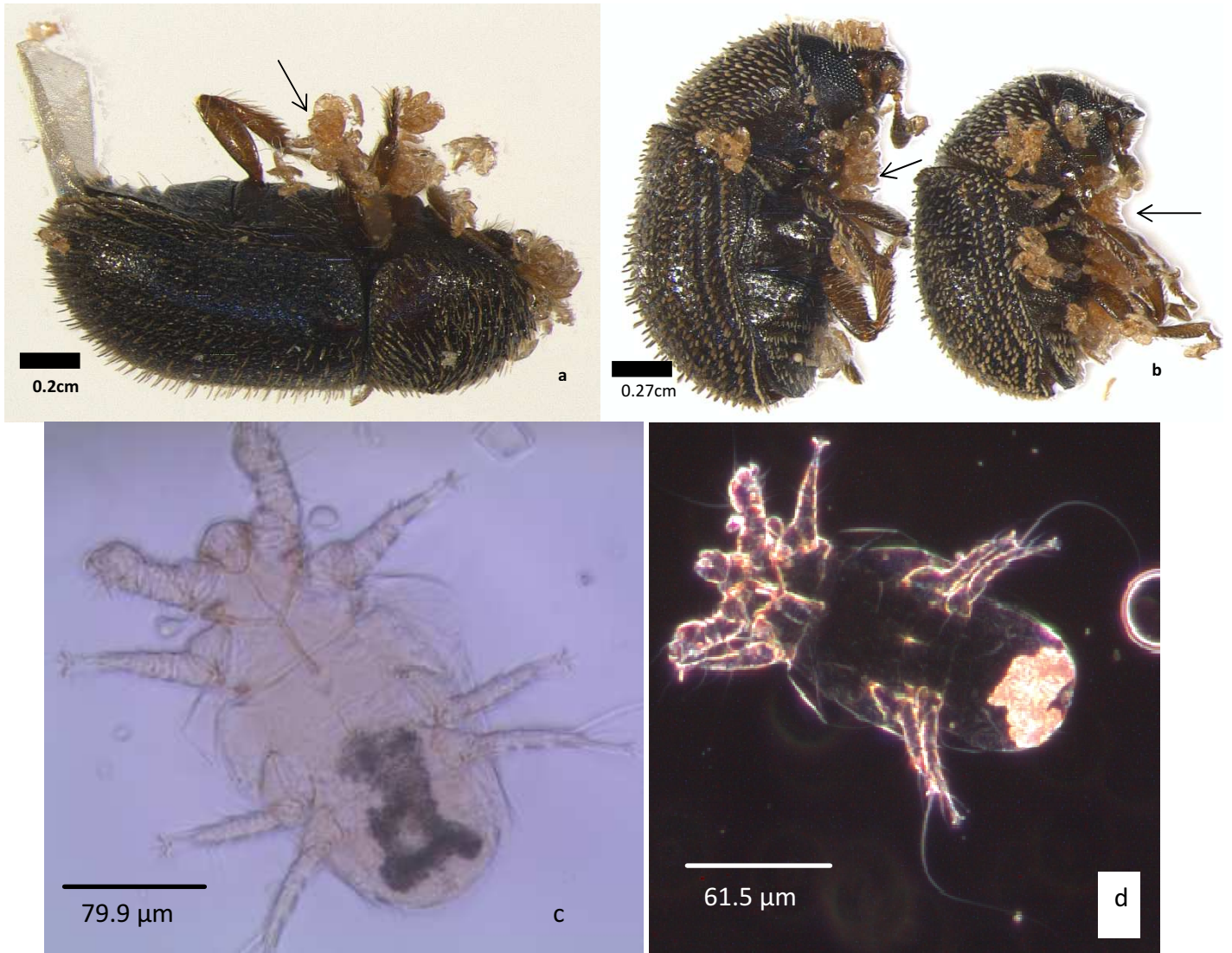
Fig. 4: Pygmephoridae mites phoretic on bark beetles collected from *Virgilia* spp. in the CFR; (a) and (b) mites were found in clusters on bodies of bark beetle morpho-species A and B; (c) and (d) close-up of the phoretic mites.
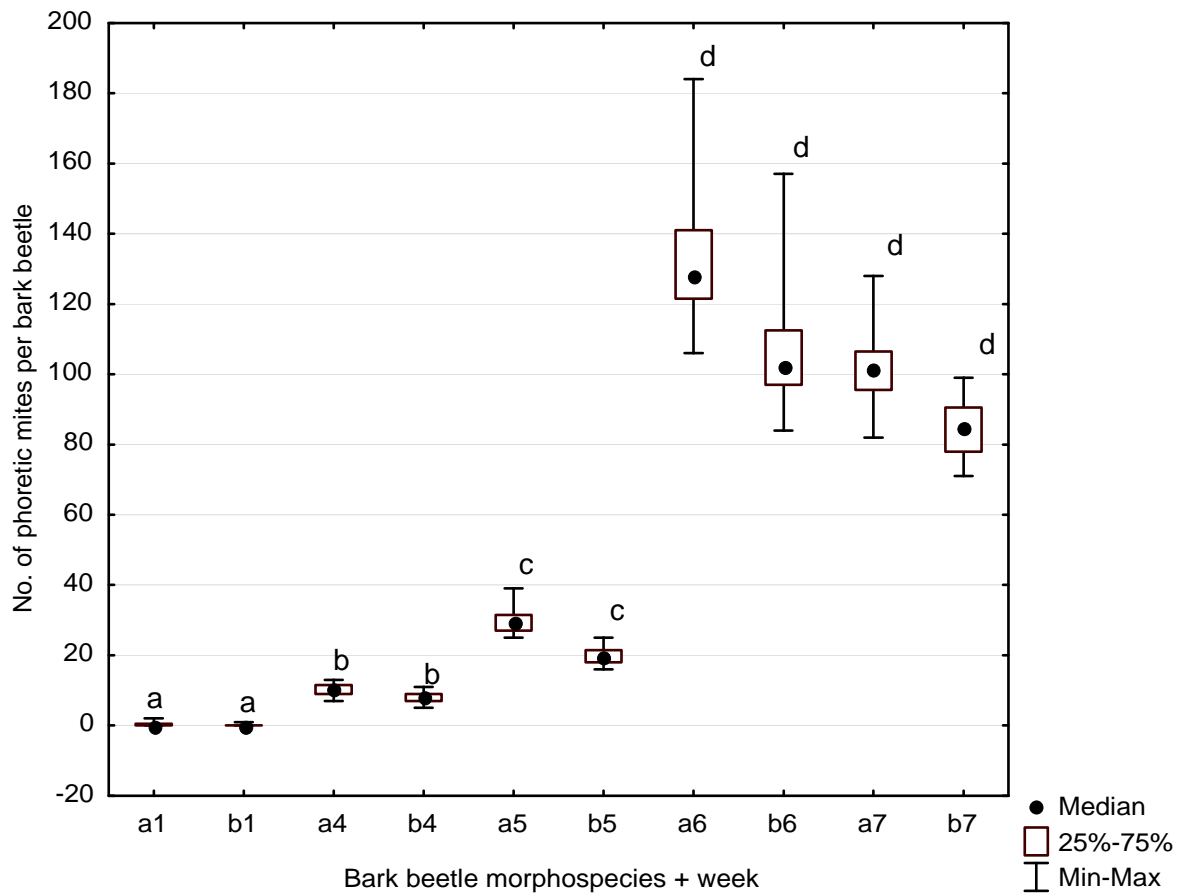
Fig. 5: Box and Whisker plot showing median numbers of phoretic mites on twenty individuals of two bark beetle morpho-species (A and B) as these emerged from *Virgilia* wood over a 7 week period (week 2 to 3 omitted from graph). A Kruskal-Wallis ANOVA indicated a significant relationship in the number of mites phoretic on the beetles (H (df=9, N=200) = 92.7595; p=0.00). Different letters above bars indicate significant differences in mite numbers.

No beetles emerged from branches placed in insect emergency cages from week 1 to week 3; they only started emerging in very low numbers at week 4. Data presented for week 1 in Figure 5 refers to the numbers of mites counted on beetles that were physically removed from their galleries in wood samples (by pealing the bark). From week 5 onwards both the numbers of beetles that emerged (data not shown) and their phoretic mite numbers increased (Fig. 5). At week 7, all mites encountered on the beetles were dead. Brood beetles that started to emerge after ca. five months had the same morpho-species of mites on them.

Some of these mites were dead, while others were still alive (data not shown). The numbers of mites from the respective beetle morpho-species differed significantly between weeks 5 to 7 compared to weeks 1 and 4.

3.3   *Geosmithia* species isolated from bark beetles and mites

A single fungal genus was consistently isolated from all crushed and washed individuals of all four morpho-species of bark beetles and their phoretic mites. Morphologically these isolates resembled *Geosmithia* spp (refer to Table 4)*.* These fungi sporulated copiously and were easily observed in both the maternal and pupal galleries of the bark beetles. Based on morphological and culture characteristics and ITS sequence data, both beetle morpho-species A and B and their phoretic mites were associated with four different *Geosmithia* taxa (*G. flava*, *G. microcorthlyi*, *G.pallida* and a *Geosmithia* sp. (here refered to as *Geosmithia* sp. *1*)). Beetle morpho-species C was associated with three of these same *Geosmithia* taxa (*G. flava*, *G.pallida* and *G.* sp. *1*). Beetle morpho-species D was associated with a single species of *Geosmithia* that differed from the *Geosmithia* species isolated from the other beetle species and their mites (*Geosmithia pallida*-like, here refered to as *G.* sp. *2*). The *Geosmithia* taxa isolated from beetles that were physically collected, those that were isolated from emerging beetles and those isolated from respective beetle galleries always remained consistent.

3.4   Phylogenetic analyses of isolated fungi

The aligned fungal ITS data set included 56 taxa and 611 characters. 393 of these characters were constant, 127 were parsimony-informative and 91 variable characters were parsimony-uninformative. Parsimony analyses retrieved a consensus tree with length of 384, a consistency index of 0.755 and retention index of 0.890. Both parsimony analysis and Bayesian inference of the ITS marker placed our fungal isolates into five operational taxonomic units (OTU) (Fig. 6) that corresponded to the five morpho-types based on morphological and culture characters. Three of these grouped with previously described OTUs; *Geosmithia microcorthlyi* M. Kolarík, *G. flava* Kolařík, Kubátová & Pažoutová and *G.*

pallida. Another OTU grouped with an un-described taxon, here referred to as *Geosmithia* sp. 1 (Fig. 6). The single isolate from beetle morpho-species D (NM105) also grouped with *G. pallida*, but based on its branch length and GenBank blast results (GenBank blast closest matching entry, AJ578488.3 Identities = 436/497 (88%); Gaps = 9/497 (2%)) it probably represents another un-described taxon, here referred to as *Geosmithia* sp. 2 (Fig. 6). Based on these data, beetle morpho-species A and B and their phoretic mites were associated with *Geosmithia flava*, *G. microcorthyli*, *G. pallida* and *Geosmithia* sp. *1*. Beetle morpho-species C was associated with *G. flava*, *G. pallida* and *Geosmithia* sp. *1*, while morpho-species D was only associated with *Geosmithia* sp. *2*.
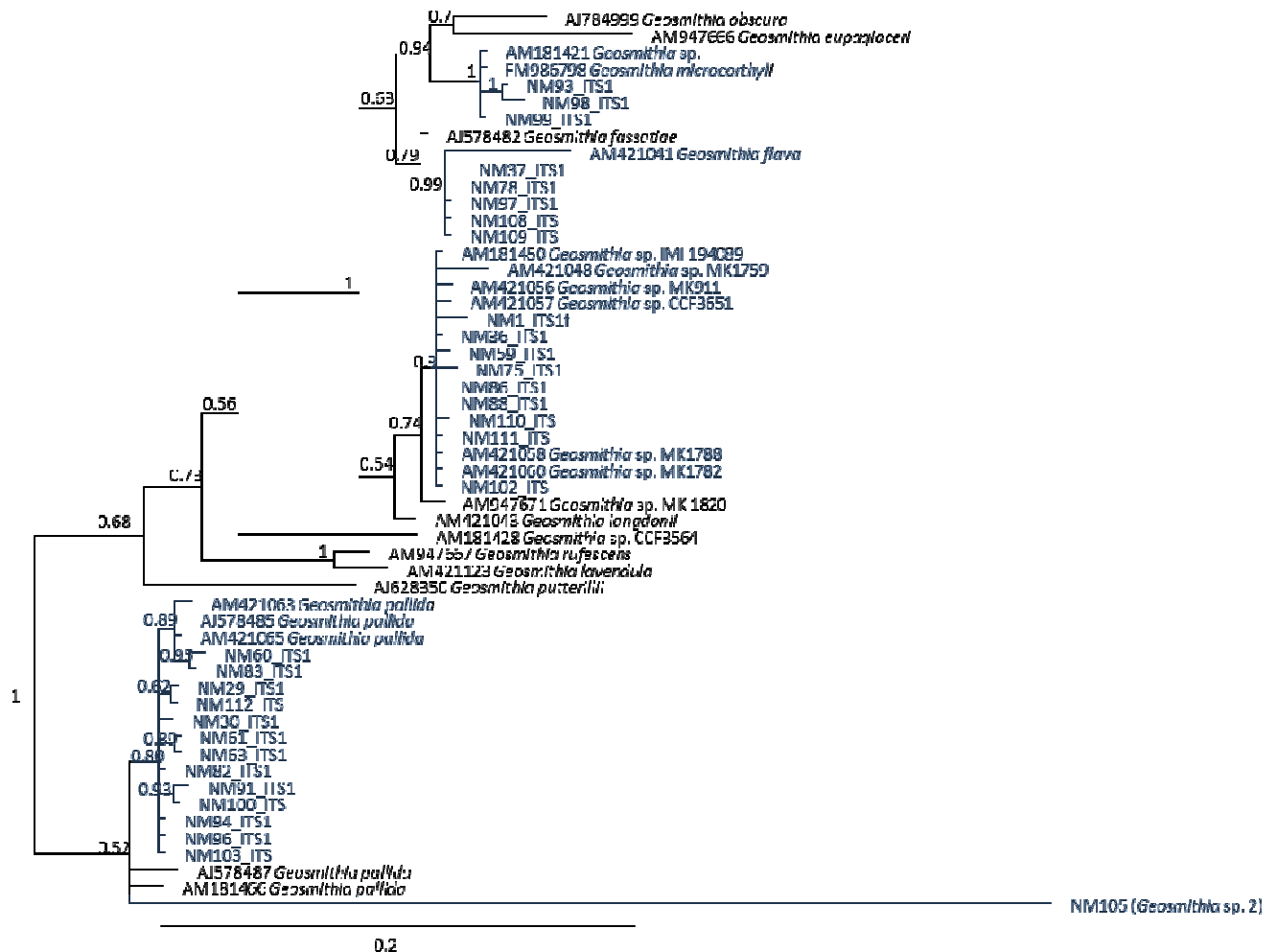
Fig 6: Strict concensus tree (MrBayes) based on ITS sequence data of described *Geosmithia* species available on GenBank and isolated from this study (highlighted in blue). Basian probabilities are shown above and bootstrap support values are shown below the branches. Only isolate NM105 from bark beetle morpho-species D did not group with any described species.

Table 4: Isolates of *Geosmithia* spp. and outgroups *(Glomerella acutata* and *Colletotrichum muses)* used in this study.

Species	Isolate no.	Bark beetle species	Host plant	Geographical origin	Reference	GenBank (ITS)
Colletotrichum musae	CMUBP1	NA	*Unknown*	Thailand	Unpublished	AY266401
Glomerella acutata	TOM-12	NA	*Tamarillo*	Israel	Afanador-Kafuri *et al*. (2003)	AF521210
Geosmithia eupagioceri	CCF 3754	*Eupagiocerus dentipes*	*Paullinia renesii*	Costa Rica	Kolařík & Kirkendall, (2010)	AM947666
G. fassatiae	AK 14/93		*Q. pubescens*	Czech Republic	Kolařík *et al*. (2005)	AJ578482
G. flava	MK1736	*Hypoborus ficus*	*Ficus carica*	Bulgaria	Kolařík *et al*. (2007)	AM421041
G. langdonii	CCF3637	*Chaetoptelius vestitus*	*Pistacia* sp.	Turkey	Kolařík *et al*. (2007)	AM421043
G. lavendula	MK968II	*Hypoborus ficus*	*Ficus carica*	Slovenia	Kolařík *et al*. (2007)	AM421123
G. microcorthyli	CCF3861	*Microcorthylus sp.*	*Cassia grandis*	Costa Rica	Kolařík & Kirkendall, (2010)	FM986798
G. obscura	CCF 3422	*Scolytus intricatus*		Czech Republic: Bohemia, Louny	Kolařík *et al*. (2005)	AJ784999
G. pallida	MK1623	*Scolytus kirschii*	*Ulmus minor*	Spain	Kolařík *et al*. (2007)	AM421063
G. pallida	CCF 3340	*Scolytus intricatus*	*Quercus robur*	Czech Republic	Kolařík *et al*. (2004)	AJ578485
G. pallida	MK91	*Scolytus rugulosus*	*Prunus sp.*	Czech Republic	Unpublished	AM421065
G. pallida	CCF 3341	*Scolytus intricatus*	*Quercus robur*	Czech Republic	Kolařík *et al*. (2004)	AJ578487

G. pallida	MK1722	*Scolytus multistriatus*	*Ulmus laevis*	Hungary	Kolařík *et al.* (2008)	AM181466
G. putterillii	CCF3442	*Liparthrum colchicum*		France: Aquitania	Kolařík *et al.* (2004)	AJ628350
G. rufescens	CCF:3751	*Cnesinus sp.*	*Paullinia*	Costa Rica	Kolařík & Kirkendall, (2010)	AM947667
Geosmithia sp.	CCF3358	*Scolytus intricatus*	*Quercus petraea*	Czech Republic	Kolařík *et al.* (2008)	AM181421
Geosmithia sp.	IMI 194089	NA (air sample)	NA	Israel	Kolařík *et al.* (2008)	AM181450
Geosmithia sp.	MK1759	*Hypoborus ficus*	*Ficus carica*	Israel	Kolařík *et al.* (2007)	AM421048
Geosmithia sp.	MK911	*Hypoborus ficus*	*Ficus carica*	Bulgaria	Kolařík *et al.* (2007)	AM421056
Geosmithia sp.	CCF3651	*Scolytus amygdali*	*Amygdalus*	Syria	Kolařík *et al.* (2007)	AM421057
Geosmithia sp.	MK1788	*Hypoborus ficus*	*Ficus carica*	Azerbaijan	Kolařík *et al.* (2007)	AM421058
Geosmithia sp.	MK1782	*Hypoborus ficus*	*Ficus carica*	Azerbaijan	Kolařík *et al.* (2007)	AM421060
Geosmithia sp.	MK 1820	*Cnesinus lecontei*	*Croton draco*	Costa Rica	Kolařík & Kirkendall, (2010)	AM947671
Geosmithia sp.	CCF3564	*Ips typographus*	*Picea abies*	Czech Republic	Kolařík *et al.* (2008)	AM181428
Geosmithia sp. 1	NM1	Bb morpho-sp. a	*Virgilia oroboides oroboides*	HPNBG, SA		
G. pallida	NM29	Bb morpho-sp. a	*V. o. oroboides*	Table Mountain, SA		
G. pallida	NM30	Bb morpho-sp. b	*V. o. oroboides*	Table Mountain, SA		
Geosmithia sp. 1	NM36	Bb morpho-sp. c	*V. o. oroboides*	Silver Mine, SA		
G. flava	NM37	Bb morpho-sp. c	*V. o. oroboides*	Silver Mine, SA		

Geosmithia sp. 1	NM59	Phoretic mite on Bb morpho-sp. a	*V. divaricata*	Storms River, SA
G. pallida	NM60	Phoretic mite on Bb. morpho-sp. b	*V. divaricata*	Storms River, SA
G. pallida	NM61	Bb morpho-sp. a	*V. o. ferruginea*	George, SA
G. pallida	NM63	Bb morpho-sp. b	*V. divaricata*	Storm River, SA
Geosmithia sp. 1	NM75	Bb morpho-sp. a	*V. o. oroboides*	HPNBG, SA
G. flava	NM78	Bb morpho-sp. a	*V. o. oroboides*	HPNBG, SA
G. pallida	NM82	Bb morpho-sp. a	*V. o. oroboides*	HPNBG, SA
G. pallida	NM83	Bb morpho-sp. a	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 1	NM86	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 1	NM88	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. pallida	NM91	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. microcorthyli	NM93	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. pallida	NM94	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. pallida	NM96	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. flava	NM97	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. microcorthyli	NM98	Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. microcorthyli	NM99	Phoretic mite on Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA

G. pallida	NM100	Phoretic mite on Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 1	NM102	Phoretic mite on Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
G. pallida	NM103	Phoretic mite on Bb morpho-sp. b	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 2	NM105	Bb morpho-sp. d	*V. o. oroboides*	HPNBG, SA
G. flava	NM108	Bb. Morpho-sp. c	*V. o. oroboides*	HPNBG, SA
G. flava	NM109	Bb. Morpho-sp. c	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 1	NM110	Bb. Morpho-sp. c	*V. o. oroboides*	HPNBG, SA
Geosmithia sp. 1	NM111	Bb. Morpho-sp. c	*V. o. oroboides*	HPNBG, SA
G. pallida	NM112	Bb. Morpho-sp. c	*V. o. oroboides*	HPNBG, SA

CCF- Culture Collection of Fungi, Prague; M. Kolařík; Bb- bark beetle; HPNBG- Harold Porter National Botanical Garden; SA- South Africa

3.5   Mite feeding studies

Mites did not feed on any of the *Geosmithia* fungal species and were all dead at the end of the 40 day period. Mites on the control plates were also dead. These results were corroborated by food choice experiments, as mites were never encountered on any of the *Geosmithia* plugs or sterile MEA.

---

4.   DISCUSSION

Virgilia trees in the CFR are associated with three morpho-species of bark beetles that are common throughout the region and one morpho-species (D) of ambrosia beetle, found at two sites, HPNBG and Groenkop in George, during this study. These beetles were only found on dead and dying *Virgilia* trees, weakened by primary pathogens (see Chapters 2 and 4) and storm damaged trees. They were never associated with healthy trees. The *Virgilia* population in Jonkershoek seemed healthy, but bark beetle morpho-species C was collected from a broken branch, which probably broke during a winter storm. This suggests that all of these beetles belong to the secondary group of bark beetles, also referred to as being "facultative parasitic" (Raffa *et al*. 1993). This is recognised as a survival strategy of these beetles, whereby they avoid tree defence chemicals (Raffa *et al*. 1993). They are thus unlikely to be of major pest concern, except when trees in large areas become stressed (through e.g. drought).

Scolytine beetle morpho-species A, B and D vectored a single morpho-species of Pygmephoridae (likely *Elattoma* Mahunka sp.) mite. Members of this family are parasitoids of bark beetles (Kaliszewski *et al*. 1995; Moser *et al*. 1971) and/or fungivorous (Klepzig *et al*. 2001). *Elattoma* sp. are true phoretic mites that have lost larval stages normal to non-phoretic mites (Moser and Cross 1975, Moser *et al*. 2005). These are commoly phoretic on beetles and other insects (Moser and Cross 1975). Moser *et al*. (2005) found *Elattoma* sp.

associated with *Scolytus multistriatus* (Marsham) and *Scolytus pygmaeus* (Fabricius) in Australia. In another study, *Elattoma bennetti* was observed feeding within the *Entomocorticium* sp. lining the galleries of *Ips avulsus* (Eichhoff) (Klepzig *et al*. 2001). In this study, we observed mites foraging in areas around dead beetle larvae in galleries. They could have been feeding on other fungi and/or microbes associated with dead and decaying larvae thereby playing a cleaning role in beetle galleries of detrimental microbes. This needs to be confirmed in future studies.

Very few phoretic mites were observed on beetles that still occupied their tunnels. However, after four weeks and when beetles started to emerge from tunnels, the number of phoretic mites significantly increased over time. This suggests that mites have co-evolved with their bark beetle associates, resulting in synchronization of their life histories and emergence times. A study by Lombardero *et al*. (2000) showed that *Tarsonemus* mites phoretic on the SPB populations grow rapidly. They first reproduce at a very early stage, and by the time of dispersal, the females would have mated already. Thus, by the time the SPB completes a generation (*ca*. 40-100 days), the mites would have reproduced, increased in numbers and grown fully, ready for dispersal (Lombardero *et al*. 2000). The same type of synchronization of life cycles appears to be operational on *Virgilla*. We also assume that mites respond to some queue released by beetles when they are ready to emerge and disperse. This encourages the mites to climb onto beetles. Previous studies have indicated that scolytine beetles release differing pheromones during host colonization (Raffa *et al*. 1993). In this study we only found dead mites on beetles after seven weeks. We assume that mites only leave the beetles when arriving at a new host trees, during the host selection and/or concentration phases of host colonisation, *sensu* Raffa *et al*. (1993). If the beetles fail to find hosts soon enough, the mites will probably die. It is possible that the beetles found in the plastic bottles during week seven had already emerged from bark much earlier, and that their phoretic mites may have died due to the long transportation time and failure to colonize new suitable hosts.

According to Kolařík and Kirkendall (2010), 23 recorded (some not yet described) taxa and 14 additional unpublished species of *Geosmithia* (recognising the *G. pallida* complex as a single

taxon) have been recorded from gymnosperms and angiosperms globally. Most *Geosmithia* taxa are exclusively associated with wood-boring insects such as scolytine and bostrichid (Coleoptera, Bostrichidae) beetles (Čizkova *et al*. 2005; Kolařík *et al*. 2004; 2008; Kolařík and Kirkendall 2010; Tisserat *et al*. 2009), as well as ambrosia beetles (Belhoucine *et al*. 2011; Kolařík and Kirkendall 2010; Six *et al*. 2009).   It has been suggested that *Geosmithia* spp. may serve as supplementary diet for beetles during development (Belhoucine *et al*. 2011; Kolařík and Kirkendall 2010; Six *et al*. 2009).

Considerable morphological dimorphism has been recorded for *Geosmithia* species associated with beetles. *Geosmithia microcorthyli*, for example, was shown to possess morphological characteristics typically present in ambrosial fungi such as the formation of dense palisades of cuffs of monilioid cells or hyphae, frequently with large and single conidia and/or chlamydospores acropetally formed (Kolařík and Kirkendall 2010). It has a yeast-like phase upon germination of conidia (sprout cells) that comprises of richly vacuolated and short-lived thin-walled cells (Kolařík and Kirkendall 2010). It is also said to lack morphological characters common to other *Geosmithia* species, such as persistent chains of small conidia and distinct penicillate conidiophores (Kolařík and Kirkendall 2010). In this study we isolated *G. microcirthyli* from bark beetle morpho-species A and B, but not from morpho-species D (an ambrosia beetle) as an ambrosial fungus. The ambrosial features we observed on this fungus in this study in culture was the formation of dense palisades of hyphae, a short-lived yeast-like phase after conidia germination, slightly larger conidia than other *Geosmithia* spp. and the absence of penicillate conodiophores. Chains of conidia were present, but not persistent. The association of this fungus with bark beetles probably suggests a nutritional value to its vector beetles. Similarly, *Geosmithia* sp. 2 from the ambrosia beetle (morpho-species D) could have nutritional advantages and is morphologically different to the rest of the *Geosmithia* isolates in our study. It had palisades of hyphae in culture, but other ambrosial features were lacking. These morphological differences could be seen as adaptive features for the ambrosial habitat.

Generally bark beetles on *Virgilia* trees presented similar *Geosmithia* communities with *G. pallida, G. flava* and *Geosmithia* sp. *1* as the most common associates of morpho-species A,

B and C. Bark beetles that co-occur had similar *Geosmithia* communities. We observed galleries of co-occurring beetles to overlap at times and with the beetles moving around galleries of neighbouring co-existing taxa (seen for morpho-species A and B). This would allow the fungi to easily come into contact with other beetle individuals and taxa, rendering it unsurprising that the communities strongly overlap. Our results agree with those of Kolařík *et al*. (2008) who found similar *Geosmithia* communities from bark beetles that shared similar host plants. This is also supported by the isolation of only *G.* sp. 2 from ambrosia beetle morpho-species D. This beetle species occupies an isolated niche (deep within wood), which does not allow it to frequently come into physical contact with the other *Geosmithia* spp. from other co-occurring beetles.

Geographical distance does not seem to affect the *Geosmithia* associates of the various bark beetle taxa identified in this study. The same *Geosmithia* communities were constantly isolated from the same bark beetle morpho-species over the entire sampling area (*ca*. 600 km). Bark beetles and their associated *Geosmithia* spp. were also not specific towards any particular *Virgilia* taxon. Kolařík *et al*. (2004, 2008) also observed that bark beetle populations from different geographic regions maintained their *Geosmithia* associates. In the study by Kolarik *et al*. (2008), bark beetle species had uniform *Geosmithia* associates over a large geographical area, ranging from southern Bulgaria to the Czech Republic. This was identified as potentially an effective method of dispersal of *Geosmithia* species (Kolařík *et al*. 2004, 2008). The maintenance of these constant *Geosmithia* communities over geographical ranges can also suggest a nutritional role to their vector beetle hosts.

The inability of the mites to feed on *Geosmithia* suggests a commensalistic association between the mites and fungi in this system. The *Geosmithia* species appear to gain no specific benefit for the mites, while the fungus benefits by being transported to new hosts. These mites must thus rely on other food sources. They were often observed foraging on dead beetle larvae and microbes growing on them in larval tunnels. The mites may thus perform a "cleaning service", ridding galleries of potentially detrimental microbes. It may thus have been purely coincidental that *Geosmithia* species were isolated from these mites. They may have accidentally picked up spores when moving around bark beetle galleries.

108

Mites in this system will benefit from the association with beetles by being transported to new suitable hosts.

The association between *Geosmithia* and their bark beetle associates collected in this study remain unknown. These *Geosmithia* spp. are not pathogenic to *Virgilia* trees (Chapter 2). They are thus not involved in overcoming tree defences to create more suitable habitats for beetles to utilise (see Six and Wingfield 2011). However, as these beetles colonise recently dead and dying *Virgilia* trees with very poor food quality and unpredictable resources (Raffa *et al*. 1993), the fungi may have a direct nutritional advantage to the beetles, as suggested by Kolarik *et al*. (2008).

This study presents the first record of *Geosmithia* species and their association with secondary bark beetles and their phoretic mites on *Virgilia* tree hosts in South Africa. There are few studies on scolytine beetles associated with non-phytopathogenic fungi (Kolařík *et al*. 2004; Six and Wingfield 2011). We have shown that *Geosmithia* communities are relatively similar for co-occurring scolytine beetles and different for those with isolated ecological niches. Geographic distance is not a determining factor for *Geosmithia* associates of the beetles. The relationship of bark beetles-mites-fungi on *Virgilia* trees is complex, and we suspect that more microbial taxa are involved besides the *Geosmithia* species identified here. We suggest that other microbial taxa involved (not included in this study) could have nutritional value to the phoretic mites, for the mites were here shown to be unable to feed on *Geosmithia* species they carry phoretically. We believe that this study will serve as platform for further scolytine beetle-*Geosmithia*-mite association studies, not only in South Africa, but globally.

REFERENCES

Afanador-Kafuri L, Minz D, Maymon M, Freeman S, 2003. Characterization of *Colletotrichum* isolates from tamarillo, passiflora, and mango in Colombia and identification of a unique species from the genus. *Phytopathology* **93** (5): 579-587.

Akaike H, 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**:716-723.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.

Avtzis DN, Bertheau C, Stauffer C, 2012. What is next in bark beetle phylogeography? *Insects* **3:** 453-472.

Barras SJ, 1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. *Annals of the Entomological Society of America* **63**: 1187-1190.

Belhoucine L, Bouhraoua RT, Meijer M, Houbraken J, Harrak MJ, Samson RA, Equihua-Martinez A, Pujade-Villar J, 2011. Mycobiota associated with *Platypus cylindrus* (Coleoptera: Curculionidae, *Platypodidae*) in cork oak stands of North West Algeria, Africa. *African Journal of Microbiology Research* **5**(25): 4411-4423.

Bridges JR, 1984**.** A quantitative study of the yeasts and bacteria associated with laboratory-reared *Dendroctonusfrontalis* Zimm (Coleopteran:Scolytidae). *Journal of Applied Entomology* **97**: 261.

Christiansen E, Waring RH, Berryman AA, 1987. Resistance of conifers to bark beetle attack: searching for general relationships. *Forest Ecology and Management* **22**: 89-106.

Čizkova D, Šrutka P, Kolarik M, Kubatova A, Pazoutova S, 2005. Assessing the pathogenic effect of *Fusarium*, *Geosmithia* and *Ophiostoma* fungi from broad-leaved trees. *Folia Microbiol*. **50**(1): 59-62.

Cardoza YJ, Moser JC, Klepzig KD, Raffa KF, 2008. Multipartite symbioses among fungi, mites, nematodes, and the spruce beetle, *Dendroctonus rufipennis. Environmental Entomology* **37**(4): 956-963.

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR, 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. *Plant Diseases* **92**: 215-224.

Franceschi VR, Krokene P, Christiansen E, Krenkling T, 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist* **167**(2): 353-375.

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

Hall T, 2005. BioEdit, biological sequence alignment editor for Win95/98/NT/2K/XP. Carlsbad, California: Ibis therapeutic.

Harrington TC, 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE, Blackwell M (eds.), *Ecological and Evolutionary Advances in Insect-Fungal Associations*, Oxford University Press, pp. 257-291.

Harrington TC, Fraedrich SW Aghayeva DN, 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* **104**: 399-404.

Harrington TC, Zambino PJ, 1990. *Ceratocystiopsis ranaculosis* not *Ceratocystis minor* var. *barrasii* is the mycangial fungus of the southern pine beetle. *Mycotaxon* **38**: 103-15.

Hofstetter RW, Cronin J, Klepzig KD, Moser JC, Ayres MP. 2006. Antagonisms, mutualisms and commensalisms affect outbreak dynamics of the southern pine beetle. *Oecologia* **147**: 679-691.

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

Jiri H, Dunn RR, 2011. The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. Proceedings of the Royal Society Biological Siences, p. 1130-1138.

Kirkendall LR, 1983. The evolution of mating systems in bark and ambrosia beetles (Coleoptera: Scolytidae). *Zoological Journal of the Linnean Society* **77**: 293-352.

Kaliszewski M, 1995. Parasitism and parasitoidism in Tarsonemina (Acari: Heterostigmata) and evolutionary considerations. *Advances in Parasitology* **35**: 335-377.

Klepzig KD, Moser JC, Lombardero MJ, Hofstetter RW, Ayres MP, 2001. Symbiosis and competition: complex interactions among beetles, fungi and mites. *Symbiosis* **30**: 83-96.

Kolařík M, Kirkendall LR, 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). *Fungal Biology* **114**: 676-689.

Kolařík M, Kubátová A, Hulcr J, Pažoutová S, 2008. *Geosmithia* fungi are highly diverse and consistent bark beetle associates: evidence from their community structure in temperate Europe. *Microbial Ecology* **55**(1): 65-80.

Kolařík M, Kostovčík M, Pažoutová S, 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. *Mycological Research* **111**(11): 1298-1310.

Kolařík M, Kubátová A, Cepicka I, Pazoutova S, Srutka P, 2005. A complex of three new white-spored, sympatric, and host range limited *Geosmithia* species. *Mycological Research* **109**(12): 1323-1336.

Kolařík M, Kubátová A, Pažoutová S, Srutka P, 2004. Morphological and molecular characterisation of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. *Mycological Research* **108**(9): 1053-1069.

Langstrom B, Hellqvist C, 1993. Induced and spontaneous attacks by pine shoot beetles on young Scots pine trees: tree mortality and beetle performance. *Journal of Applied Entomology* **115**: 25-26.

Lee J-S, Haack RA, Choi WI, 2011. Attack Pattern of *Platypus koryoensis* (Coleoptera: Curculionidae: Platypodinae) in relation to crown dieback of Mongolian Oak in Korea. *Environmental Entomology* **40**(6): 1363-1369.

Lieutier F, Yart A, Salle A, 2009. Stimulation of tree defences by Ophiostomatoid fungi can explain attack success of bark beetles on conifers. *Annals of Forest Science* **66** (8): 801-822.

Linnakoski R, de Beer ZW, Niemelä P, Wingfield MJ, 2012. Associations of Conifer-infesting bark beetles and fungi in Fennoscandia. *Insects* **3**: 200-227.

Lombardero MJ, Klepzig KD, Moser JC, Ayres M, 2000. Biology, demography and community interactions of *Tarsonemus* (Acarina: Tarsonemidae) mites phoretic on *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Agricultural and Forest Entomology* **2**: 193-2002.

Moser JC, Cross EA, Roton LM, 1971. Biology of *Pyemotes parviscolyti* (Acarina: Pyemotidae). *Entomophaga* **16**: 367-379.

Moser JC, Cross EA, 1975. Phoretomorph: a new phoretic phase unique to the Pyemotidae (Acarina: Tarsonemoidea). *Annals of the Entomological Society of America* **68**: 820-822.

Moser JC, Perry TJ, Bridges JR, Yin H.-F, 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus*, a mycangial fungus of the southern pine beetle. *Mycologia* **87**: 84-86.

Moser JC, Konrad H, Kiristis T, Carta LK, 2005. Phoretic mites and nematodes associates of *Scolytus multistriatus pygmaeus* (Coleoptera: Scolytidae) in Austria. *Agricultural and Forest Entomology* **7**: 169-177.

Ogawa H, Yoshimura A, Sugiyama J, 1997. Polyphyletic origin of species of the anamorphic genus *Geosmithia* and the relationships of the cleistothecial genera: evidence from 18S, 5S, and 28S rDNA sequence analysis. *Mycologia* **89**: 756-771.

Paine TD, Raffa KF, Harrington TC, 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Paine TD, Stephen FM, Taha HA, 1984. Conceptual model of infestation probability based on bark beetle abundance and host tree susceptibility. *Environmental Entomology* **13**: 619-24.

Palgrave KC, 1983. Keith Coates Palgrave Trees of Southern Africa. Edition 2. Struik, Cape Town.

Palgrave KC, 2002. Keith Coates Palgrave Trees of Southern Africa. Edition 3. Struik, Cape Town.

Palmer E, Pitman N, 1972. Trees of South Africa Vol. 2. Balkema. Cape Town.

Price TS, Doggett C, Pye JL, Holmes TP (eds.), 1992. A history of southern pine beetle outbreaks in the south-eastern United States. Sponsored by the Southern Forest Insect Work Conference. The Georgia Forestry Commission, Macon, GA, p. 65.

Posada D, 2008. Selection of models of DNA evolution with jModelTest. In: Posada D (ed.), *Bioinformatics for DNA sequence analysis*. Humana Press, Totowa, pp. 93-112.

Raffa KF, Phillips TW, Salom SM, 1993. Strategies and mechanisms of host colonization by bark beetles. In: Schowalter T, Filip G (eds.), *Beetle-Pathogen Interactions in Conifer Forests.* San Diego: Academic, pp. 102-128.

Raffa FR, Smalley EB, 1995. Interaction of pre-attack and induced monoterpene interactions in host conifer defense against bark beetle-fungal complexes. *Oecologia* **102**: 285-295.

Reed SE, Muzika RM, 2010. The influence of forest stand and site characteristics on the composition of exotic dominated ambrosia beetle communities (Coleoptera: Curculionidae: Scolytinae). *Environmental Entomology* **39**(5): 1482-1491.

Roets F, Crous PW, Wingfield MJ, Dreyer LL, 2006. Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom: *Environmental Entomology.* **36**: 1226-1237.

Roets F, de Beer ZW, Wingfield MJ, Crous PW, Dreyer LL, 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* **100**: 496-510.

Roets F, Crous PW, Wingfield MJ, Dreyer LL, 2009. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* **38**(1): 143-152.

Ronquist FR, Huelsenbeck JP, 2003. MrBayes: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**: 1572–1574. (doi:10.1093/bioinformatics/btg180).

Roux J, Kamgan Nkuekam G, Mbenoun M, Wingfield MJ, 2011. Fungus-Insect interactions and tree health in South Africa. Meeting of the Southern African Society of Botanists, Grahamstown, South Africa, January 2011. *South African Journal of Botany* **77**: 555.

Shapiro SS, Wilk MB, 1965. An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.

Simon C, Frati F, Beckenbach A, Crespi B, Lui H, Flook P, 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**: 651-701.

Six DL, 2003**.** Bark beetle-fungus symbioses. *In* Bourtzis K, Miller T (eds.), Insect symbioses. CRC, Boca Raton, FL, pp. 96-116.

Six DL, De Beer ZW, Beaver RA, Visser L, Wingfield MJ, 2005. Exotic invasive elm bark beetle, *Scolytus kirschii*, detected in South Africa: research in action. *South African Journal of Science* **101**(5/6): 229-232.

Six DL, Doug SW, de Beer ZW, Woolfolk SW, 2009. *Ambrosiella beaveri*, sp. nov., associated with an exotic ambrosia beetle, *Xylosandrus mutilatus* (Coleoptera: Curculionidae, Scolytinae), in Mississippi, USA. *Antonie Van Leeuwenhoek* **96**: 17-29.

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

Six DL, Wingfield MJ, 2011. The role of phytopathogenicity in bark beetle–fungus symbioses: A challenge to the classic paradigm. *Annual Review Entomology* **56**: 255-72.

Stark RW, 1982. Generalized ecology and life cycle of bark beetles. In: Mitton JB, Sturgeon KB (eds.), *Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology.* University of Texas Press, Austin, Tex, USA, pp. 21-45.

Swofford DL, 2002. PAUP*: Phylogenetic Analysis Using Parsimony. (* and Other Methods), Version 4.0b10. Sinauer Associates, MA (2002).

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Tisserat N, Cranshaw W, Leatherman D, Utley C, Alexander K, 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Online. Plant Health Progress doi:10.1094/PHP-2009-0811-01-RS.

Tribe GD, 1990. Phylogeny of *Pinus radiata* log colonization and reproduction by the European bark beetle *Orthotomicus erosus* (Wollaston) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa* **2**: 117-126.

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

Wermelinger B, 2003. Ecology and management of the spruce bark beetle *Ips typographus* - a review of the recent research. *Forest Ecology and Management* **202**: 67-82.

White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., New York, pp. 315-322.

Whitney HS, 1982. Relationships between bark beetles and symbiotic organisms. In: Mitton JB, Sturgeon KB (eds.)*, Bark beetles in North American Conifers.* Austin: University of Texas Press, pp. 183-211.

Wood DL, 1982. The role of pheromones, kairomones, and allomones in the host se lection and colonization behavior of bark beetles. *Annual Review Entomology* **27**: 411–446.

Young CWT, 1978 Sooty Bark Disease of Sycamore. Arboricultural Leaflet No. 3. HMSO, London.

Zhou XD, De Beer W, Wingfield BD, Wingfield MJ, 2002. Infection sequence and pathogenicity of *Ophiostoma ips, Leptographium serpens* and *L. lundbergii* to pines in South Africa. In: Hyde KD, Lones EBG (ds.), *Fungal Succession*. *Fungal Diversity* **10**: 229-240.

CHAPTER 4

DISEASES OF *VIRGILIA* TREES FROM NEAR PRISTINE ENVIRONMENTS IN THE CAPE FLORISTIC REGION OF SOUTH AFRICA

ABSTRACT

The Cape Floristic Region (CFR) of South Africa is renowned for its high plant species diversity, richness and endemism. However, little attention has been awarded to the pathogens from this region, even though they greatly influence plant distributions. The CFR is home to the endemic genus *Virgilia*, trees that are ecologically and economically important, but often show symptoms of disease. This study set out to document the major pathogens on *Virgilia* across the CFR. Bark, wood and root samples were collected from affected areas and fungi were isolated from the samples. Key fungal isolates were identified by sequencing of the internal transcribed spacer regions (ITS1, ITS2) including the 5.8S gene of the rDNA and compared to taxa available on GenBank. *Virgilia oroboides* subsp*. oroboides* showed three different disease symptoms: (1) stems with several small cankers, (2) root rot disease with white mycelial fans underneath the bark and (3) cankers in association with small bracket fungi on stems. The most consistent fungus isolated from the several small cankers was a *Fusarium acuminatum*-like species*. Armillaria mellea* was associated with the root rot disease and the small bracket fungus was identified as *Schizophyllum commune*. Dead and diseased *Virgilia oroboides* subsp. *ferruginea* and *V. divaricata* showed symptoms of rapid wilting before death, with stems damaged by tunnelling larvae of an unidentified cerambycid and the ghost moth (*Leto venus*). Tunnels were often associated with severe wood discoloration. Fungi isolated from these tunnels included the ophiostomatoid fungi *Ceratocystis tsitsikammensis*, *Ophiostoma plurianulatum* and *O. querci*. Pathogenicity trials showed that *F. acuminatum*, *S. commune* and *C. tsitsikammensis* are pathogenic to *Virgilia* trees. Our results indicate that natural populations of *Virgilia* are hosts to numerous

117

destructive pathogens, some of which are non-native (e.g. *A. mellea*) and a cause for special concern.

---

1.   INTRODUCTION

The Cape Floristic Region (CFR, Goldblatt and Manning 2000) of South Africa ranks among thirty-five global biological diversity hotspots (Williams *et al*. 2011). It comprises of an entire floral kingdom, the smallest of the world's six floral kingdoms, yet the richest per unit area, with almost 69% of its flowering plant species endemic to the area (Goldblatt and Manning 2000). Linder (2003) suggested that geographical and ecological isolation of the CFR had given rise to the exceptionally high levels of endemism recorded in the region. Biomes in the CFR include, amongst others, the Fynbos, Thicket and Afromontane Forest Biomes (Goldblatt and Manning 2002; Mucina and Rutherford 2006). Fynbos is dominated by the families Proteaceae, Ericaceae and Restionaceae and grows on infertile sandy or rocky soils (Linder 2003; Goldblatt and Manning 2002). Thickets mostly occur in river valleys and are characterised by a mixture of shrubs, vines and evergreen succulent or sclerophyllous trees (Cowling 1984; Everard 1987; Volk *et al*. 2003). Afromontane forests, with trees 10 - 30 meters in height, are outliers of the tropical African Afromontane forests associated with high East African mountains (Turpie *et al*. 2003) and exist in small fragmented areas on mountains, foothills, coastal platforms, river valleys, coastal scarps and dunes and along the mountainous arc in the southwest of the Western Cape Province (Geldenhuys 2010; Morgenthal and Cilliers 2000). They are found in well-watered areas with relatively fertile, well-drained soils that are deep in valleys, but shallower on steeper slopes (Geldenhuys 1997).

Virgilia is a fast growing tree genus often found in association with Fynbos, Thicket and Afromontane forest margins. It is a mass-flowering, fast-growing genus, which makes it a common ornamental (Palmer and Pitman 1961, 1972; Smith 1966). It is endemic to the southwestern and southern coastal regions of South Africa and includes three taxa; *Virgilia oroboides* (Berg.) Salter subsp. *oroboides*, distributed from the Cape Peninsula to

Swellendam, *Virgilia oroboides* (Berg.) Salter subsp. *ferruginea* B-E van Wyk only present around George as a geographic intermediate taxa and *Virgilia divaricata* Adamson found in the southern Cape to Port Elizabeth (Van der Bank *et al*. 1996; Van Wyk 1982). *Virgilia* trees are known to be short-lived; the average lifespan ranges from 12 to 20 years (Mbabezeli and Notten 2003). Despite their short life expectancy, both species produce long lived-seed banks (up to 150 years) (Goldblatt and Manning 2000). *Virgilia* trees are important breeding sites for the rare ghost moth (*Leto venus* Cramer), which lays its eggs only at the base of these trees. The stems of *Virgilia* trees are the only known source of food for the caterpillars (monophagy) (Nielsen *et al*. 2000). The caterpillars bore into the trunk of the tree, where they feed and live and only emerge as adults (Palmer and Pitman 1972; Van Wyk and Van Wyk 1997). *Virgilia* trees grow well in the open and are wind tolerant, which make them important pioneer species in forest plant succession (Geldenhuys 1994; Phillips 1926; Van Daalen 1981).

Pathogens and insect pests naturally co-exist with their hosts in natural ecosystems, usually causing insignificant damage to their hosts (Brasier 2008; Loo 2009). They only cause devastating effects on their hosts when there is a sporadic outbreak due to natural or human disturbances that weaken the hosts (Allard *et al*. 2003; Gilbert 2002). Pests and pathogens influence the survival, growth rate and health status of hosts, thereby determining the abundance and distribution of plant species (Abdurahman 1992; Speight and Wylie 2001). Several global studies have shown the ability of pests and diseases to impact forest structure and composition, diversity and succession (Anagnostakis 1987; Brasier 1990; Von Broembsen 1989). Very little is currently known about the pest and diseases of natural forest trees in South Africa and nothing has been published on those associated with *Virgilia* in the CFR.

With regards to pathogenic fungi of native trees worldwide, the ophiostomatoid fungi (Wingfield *et al*. 1993) are probably the best known group (Heath *et al*. 2009; Montoya and Wingfield 2006; Kamgan Nkuekam *et al*. 2009, 2012). Members of this group have been recorded on a variety of gymnosperms and angiosperms in several natural ecosystems around the world (Montoya and Wingfield 2006). They are characterised by dark-coloured,

globuse ascomatal bases with elongated necks, at the tips of which sticky ascospores assemble (Malloch and Blackwell 1993; Montoya and Wingfield 2006). They have adapted to an entomochoric lifestyle, whereby their sticky conidia or ascospores easily adhere to the exoskeleton of arthropods for dispersal (Malloch and Blackwell 1993; Montoya and Wingfield 2006). Many ophiostomatoid fungi are opportunists that infect their hosts when their arthropod associates create wounds or visit wounds made by other organisms (Kamgan Nkuekam *et al*. 2012a, 2012b). Bark beetles are the best studied mutualistic and commensalistic associates of ophiostomatoid fungi (Six 2003; Kirisits 2004). Other arthropod associates include cerambycid beetles (Jacobs and Kirisits 2003; Jacobs and Wingfield 2001), weevils (Jacobs and Wingfield 2001; Kirisits 2004), nitidulid beetles (Kamgan Nkuekam *et al*. 2012a, 2012b) and phoretic mites on bark beetles (Bridges and Moser 1986; Moser 1995, 2005; Malloch and Blackwell 1993) and other beetles (Roets *et al*. 2007, 2009).

Ophiostomatoid fungi can exist as saprophytes, some causing blue-staining of timber (Harrington 2005; Seifert 1993; Uzunovic and Webber 1998), or as pathogens that can cause cankers, wilting, vascular staining and rot diseases, often leading to death of infected plants (Barnes *et al*. 2005; Brasier 2008; Bretz 1952; Kile 1993; Roux *et al*. 2005; Sinclair *et al*. 1987; Wingfield *et al*. 1993, 1999). Saprophytes include the blue-stain fungus *Ophiostoma minus* (Hedgcock) H. & P. Sydow and *O. pluriannulatum* (Hedgcock) H. & P. Sydow, renowned for reducing the commercial value of timber in the Northern Hemisphere (Harrington 2005; Seifert 1993). Phytopathogens include *Ophiostoma ulmi* (Buismann) Nannf and *O. novo-ulmi* Brasier that cause Dutch Elm disease (Brasier 2008; Loo 2009; Tainter and Baker 1996; Wingfield *et al*. 1993), *Ceratocystis fagacearum* (Bretz) Hunt responsible for oak wilt disease (Bretz 1952; Sinclair *et al*. 1987; Wingfield *et al*. 1993) and *C. albifundus* M.J. Wingf., De Beer & M.J. Morris, which is an important wilt pathogen of *Acacia mearnsii* De Wild. trees (Barnes *et al*. 2005; Roux *et al*. 2005; Wingfield *et al*. 1996) that cause significant economic losses in South African plantations (Roux *et al*. 1999).

In South Africa, little is known about the occurrence and impacts of ophiostomatoid fungi on native trees. Recent studies have been motivated by this gap and have led to the discovery of numerous undescribed species and newly recorded hosts. In a study by Roux *et al*. (2007),

Ceratocystis albifundus was found on seven native tree genera, including the species *Acacia caffra* (Thunb.) Wild., *Burkea africana* Hook, *Combretum molle* R.Br. ex G.Don, *Ochna pulchra* Hook, *Protea gaguedi* J.F.Gmel. and *Terminalia sericea* Burch. ex DC. Pathogenicity tests revealed the ability of *C. albifundus* to cause disease and kill three year old *Combretum molle* and *Acacia caffra* saplings, as the fungus produced significant lesions and some of the saplings were dying at the end of the experiment. Kamgan Nkuekam *et al*. (2008) isolated C. *albifundus*, *Ophiostoma quercus* (Georgévitch) Nannfeldt and *Pesotum fragrans* (Math.-Käärik) G. Okada & Seifert from eight tree species, including *Acacia nigrescens* Oliver (Leguminosae), *Sclerocarya birrea* (A.Rich.) Hochst. (Anacardiaceae), *Faurea saligna* Harvey (Proteaceae), *Ocotea bullata* (Burch.) Baill. (Lauraceae), *Rapanea melanophloeos* (L.) Mez. (Myrsinaceae) and *Terminalia sericea* (Combretaceae). In the same study, three new species were described, namely *Ceratocystis tsitsikammensis* Kamgan & Jol. Roux, infecting *Rapanea melanophloeos* and *Ocotea bullata* (Burch.) Baill. trees, *Ceratocystis savannae* Kamgan & Jol.Roux, infecting *Acacia nigrescens* Oliver and *Combretum zeyheri* Sond. trees, and *Ophiostoma longiconidiatum* Kamg. Nkuek., K. Jacobs & Jol. Roux. infecting *Faurea saligna* Harvey and *Terminalia sericea*. *Ceratocystis tsitsikammensis* may cause disease in *Rapanea melanophloeos* trees, as greenhouse inoculation trials resulted in serious lesions (Kamgan Nkuekam *et al*. 2008).

Fusarium Link (Hypocreales) is another well-known, often pathogenic fungal taxon. Its phytopathogenic members cause root rots, wilts, cankers, blights and damping-offs (Thrane and Seifert 2000). Renowned nursery damping-off pathogens of pine seedlings include *Fusarium acuminatum* Ellis & Everh.*, F. avenaceum* (Fr.) Sacc.*, F. moniliforme* Sheldon*, F. oxysporum* Schltd.:Fr. f. and *F. solani* (Mart.) Sacc. (Viljoen *et al*. 1994). In South Africa, as in other parts of the world, *Fusarium subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marasas is a common pathogen of Pines, causing the pitch-canker disease (Jacobs *et al*. 2006; Wingfield *et al*. 2002).

The pathogenic nature of some fungal taxa is unclear. *Schizophyllum commune* Fr. (Agaricales: Schizophyllaceae), for example, colonizes a diverse range of living and dead trees and even infects humans with a compromised immune system (Castro *et al*. 2010;

Chowdhary *et al*. 2012; Sigler *et al*. 1999). *Schizophyllum commune* seems to be an opportunistic fungus and requires wounds like fire scars (Erwin *et al*. 2008; Schmidt 2006; Sinclair *et al*. 1987), pruning wounds (Schmidt 2006; Snieskiene and Juronis 2001) and sunscald lesions (Sinclair *et al*. 1987) to invade living trees. It has a worldwide distribution and is also recorded from South Africa (Abdullah and Rusea 2009; Castro *et al*. 2010). Some researchers consider it as a heart rot fungus (Oprea *et al*. 1994; Visarathanonth 1990), some as a sap rot fungus (Schmidt 2006; Sinclair *et al*. 1987), some as a saprophyte and/or saproparasite (Snieskiene and Juronis 2001), while others regard it as a phytopathogen that causes Scizophyllum rot (Kishi 1998; Snieskiene and Juronis 2001).

Accidental introduction of exotic pathogens pose great threats to natural ecosystems (Manion 1991). A good example of this threat is the introduction of the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, to the eastern USA, where it almost eliminated American chestnut trees (Quimby, 1982). Studies by Coetzee *et al*. (2001, 2003) and Wingfield *et al*. (2010) have shown that *Armillaria mellae* (Vahl. Fr.) Kummer was introduced into South Africa more than 300 years ago by early Dutch settlers in the Company Gardens, Cape Town. It has since become an important killer pathogen of the Proteaceae, attacking species such as *Leucadendron argenteum* (L.) R.Br., *L. gandogeri* Schinz ex Gand., *L. grandiflorum* (Salisb.) R.Br., *Protea longifolia* Andrews, *P. eximia* (Salisb. ex Knight) Fourc. and *P. scolymocephala* (L.) Reichard, oaks (*Quercus* L.) and other ornamental trees and native shrubs in the Company Gardens and Kirstenbosch National Botanical Garden (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010).

Research in the CFR has mainly focused on botanical diversity and uniqueness (Linder 2003; Taylor 2001). Little attention has been given to pathogens on native trees (Taylor 2001), despite the critical importance of these organisms in ecosystem function (Castello *et al*. 1995). The need for research focussed on the interactions between pests, pathogens and the plant species that host them within the CFR has been the driving force behind this project. In recent years it has been noted that *Virgilia* trees in many natural populations in the CFR are declining, with seedlings sometimes also displaying signs of ill-health. The primary objective of this study was to undertake a disease survey of *Virgilia* trees in near-natural areas within

the CFR. Specific objectives were to isolate, identify and determine the relative pathogenicity of key fungal species associated with diseased *Virgilia* trees. Where needed we also evaluated possible interactions between various arthropods and the identified pathogens.

2. MATERIALS AND METHODS

2.1 Sample collection in the filed

During 2011 and 2012, field surveys were conducted across the CFR to assess the health status of *Virgilia* trees. Individual trees were visually inspected for disease symptoms. When present, branches, stems, bark, root and wood samples were aseptically collected from diseased sites along with those damaged by wood boring insect larvae. These were placed in separate labelled sealable plastic bags (to retain moisture) and stored in the fridge pending processing. Where all individuals in populations seemed healthy or all trees were long dead (dry and brittle), no samples were collected.

Table 1: Study areas from where samples were collected in the CFR

Site	GPS coordinates	*Virgilia* species
Table Mountain	S 33°57'17.76" E 18°25'29.64"	*V. oroboides oroboides*
Kirstenbosch National Botanical Garden	S 33°59'11.3'' E 18°25'34.4''	*V. oroboides oroboides*
Jonkershoek	S 33°58'23.10" E 18°56'11.38"	*V. oroboides oroboides*
Groenkop, George	S 33°54'56.07" E 22°33'11.10"	*V. oroboides ferruginea*
Knysna	S 34°00'21.44" E 23°07'00.61"	*V. divaricata*
Storms River	S 33°05'15.54" E 18°25'06.96"	*V. divaricata*

2.2   Association between wood boring insect larvae and fungi

In cases where potentially pathogenic fungi were obtained from the areas surrounding the tunnels of ghost moth and cerambycid larvae, we investigated whether these insect phases could be involved in vectoring the pathogens. This was necessary, as adults of these insects are not wood borers and thus unlikely vectors of the pathogenic fungi. We also tested if other organisms attracted to the gum oozing from wounds created by these larvae may be involved in disease spread. Artificial wounds were randomly made on branches of 10 healthy individuals using a 5 mm diameter cork borer. After 6 weeks, wood and bark samples were collected from these wounds and were placed in sealable plastic bags. Any arthropod individuals found in and around these wounds were collected and stored in aseptic vials until fungal isolation was done.

2.3   Fungal isolations

Branches, stems and root samples were washed under flowing tap water and pat-dried with filter paper prior to surface sterilisation. This was done by soaking them in 70% ethanol for five minutes and placing it in a laminar flow cabinet to dry. The outer bark was removed with a sterile scalpel to expose any lesions and/or mycelial fans. Small pieces (ca. 2 mm²) of wood and root tissues from the edges of fresh necrotic lesions were plated onto malt extract agar (MEA: 20gL-1 malt extract and 20gL-1 agar, Biolab, Midrand, South Africa and 1 000 ml sterile distilled water) and incubated at room temperature (20-25°C) for 5 to 10 days under natural day and night conditions. Isolates were purified by transferring single hyphal tips of developing mycelium onto fresh MEA plates. In cases where large fruiting structures of fungi were obtained, small pieces were directly removed with a sterile scalpel and plated onto MEA.

Wood samples from tunnels of boring insect larvae often showed streaking patterns typical of *Ceratocystis* infection (Heath *et al*. 2009). A subsample of these was thus assessed for this fungal genus using the carrot baiting technique of Moller and De Vay (1968). Fresh carrots were washed under running tape water and cut into discs (*ca*. 2cm thick). They were

immersed in 0.001 g/vol streptomycin sulphate solution (SIGMA, Steinheim, Germany) for 10 minutes and air dried on filter paper for 2 minutes. Small wood pieces (*ca*. 2 x 0.5 x 2 cm) with vascular streaking were sandwiched between two discs of carrots and wrapped with parafilm to prevent desiccation and contamination. These were incubated at room temperature (20-25°C) for 5 to 10 days. The rest of these wood and bark samples were placed in moisture chambers (plastic bags with moistened tissue paper) for up to 10 days at room temperature to induce sporulation. After the incubation period, spore masses at the tips of fruiting structures were collected with a sterile needle and plated onto MEA supplemented with streptomycin sulphate. Plates were incubated at room temperature under natural day and night conditions and isolates were purified as mentioned earlier.

Collected arthropods were studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan) and grouped into morpho-species. Representatives of each morpho-species were preserved in 70% ethanol pending identification by specialists. Reference arthropods were stored in 100% ethanol and were submitted to the Stellenbosch University Insect Collection, Stellenbosch, South Africa. Fungi were isolated from the exoskeletons of these insects by either washing them in sterile distilled water (0.1 ml) and plating the water onto MEA or crushing them between two carrot slices. Insects were washed separately in eppendorf tubes containing 0.1 ml ddH$_2$O and the water was spread onto MEA in petri dishes. Alternatively, individual arthropods were squashed between two slices of carrots (*ca.* 2cm thickness) washed and immersed in 0.001 g/vol streptomycin sulphate solution for 10 minutes (Moller and De Vay 1968). These were wrapped with parafilm to prevent desiccation and contamination and placed in labelled plastic bags and incubated at room temperature (20-25°C) for 5 to 10 days. Spores at the tips of fruiting bodies growing on the carrots were transferred on to MEA containing 0.05 g/l streptomycin sulphate.

Representative isolates of all fungal taxa used in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Institute (FABI) at the University of Pretoria, South Africa.

2.4   DNA isolation, amplification and sequencing

Fungal mycelium from randomly chosen representative isolates of each morpho-species was harvested from colonies of pure cultures on MEA with a sterile scalpel. DNA was extracted using a Sigma-Aldrich™ plant PCR kit (USA) following the manufacturer's instructions. The nuclear ribosomal RNA Internal Transcribed Spacer (ITS) regions 1 and 2, and the 5.8S operon, were amplified using primers ITS1-f (Gardes and Bruns 1993) and ITS4 (White *et al*. 1990). 20 μL PCR reaction volumes consisting of 10 μL ddH$_2$O, 5 μL REDExtract-N-Amp PCR ready mix (Sigma-Aldrich™, USA), 4 μL extracted fungal DNA and 0.5 μL (10mM) of each primer. The gene regions were amplified in a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.) machine. PCR reaction conditions were: 2 minutes of initial denaturing at 95°C, followed by 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, 90 seconds elongation at 72°C and a final elongation step at 72°C for 8 minutes. DNA amplification was confirmed under ultraviolet light illumination using gel electrophoresis with 1.5 % agarose gel containing ethidium bromide. Amplified PCR products were purified and sequenced at the Stellenbosch University Central Analytical Facility, Stellenbosch University, South Africa.

2.5   Phylogenetic analysis

Sequences generated in this study from the suspected pathogenic fungal taxa were compared to published sequences using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al*. 1990) in GenBank (http://www.ncbi.nlm.nih.gov/genbank). Ideally we would have wanted to construct phylogeny trees for other fungal taxa but due to time constrains, we ended up constructing one for *Armillaria mellea* s. s. We isolated it from *Virgilia* roots in near pristine vegetation. *Armillaria mellea* is known to be an introduced species in South Africa (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010). Clarifications of the origins of this taxon was thus of particular interest. Additional sequences of *A. mellea,* isolated from various sources during other studies (Table 2) (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010) were downloaded from GenBank, aligned using Clustal W (Thompson *et al*. 1994), and manually adjusted in BioEdit v7. 0. 5 (Hall, 2005). The taxa used and the out-group taxon selected (*Armillaria osteyae* (Romagn.) Herink) were adopted from previous

phylogenetic studies on *A. mellea* by Coetzee *et al*. (2000, 2001, 2003) and Wingfield *et al*. (2010).

Reconstruction of molecular phylogenetic trees was done using PAUP (Phylogenetic Analysis Using Parsimony PAUP*4.0b10 (Swofford, 2002) and MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003). In PAUP, phylogenetic trees were constructed using a heusteric search option via stepwise addition of 1 000 replicates. Confidence intervals were calculated using 1 000 bootstrap pseudo-replicates. In MrBayes, 5 million random trees were generated using the Markov Chain Monte Carlo (MCMC) procedure, using the GTR+I+K (most parameter rich) model as selected in jModelTest 0.1.1 (Posada 2008) using Akaike information criteria (Akaik 1974), sampled at every 1000th generation. To avoid inclusion of trees sampled before convergence, burn-in procedure for the first 1 million generations was implemented and these trees were discarded. The remaining trees from both data sets were pooled into a 95% majority rule consensus tree. Newly generated sequence data from this study will be deposited in GenBank and alignments and phylogenetic trees will be deposited in TreeBASE (http://www.treebase.org/treebase/index.html).

2.6 Pathogenicity tests

The pathogenicity of the fungal taxa consistently isolated from diseased *Virgilia* trees was assessed on healthy *Virgilia* trees in the regions were the pathogens were isolated from. Eight trees were inoculated with each test strain and sterile MEA served as control. Three representative isolates of each suspected pathogenic fungal morpho-species isolated from roots, three isolates from cankers and six from the tunnels of ghost moth and cerambycid beetle larvae were used in these pathogenicity tests (Table 3).

A 7 mm cork borer was used to make artificial wounds in the bark of similar sized stems (ca. 1.5 cm in diameter) of *Virgilia*, ensuring that a bark disc was removed to expose the xylem. Similar sized disks from actively growing fungal colonies on MEA were inserted into these

wounds with mycelium facing the xylem. These were sealed with masking tape to prevent contamination by other organisms and desiccation. After 6 weeks, masking tape and outer bark were removed from the wounds and resultant lesion lengths were measured. Re-isolations of fungi were done from these lesions to confirm that the inoculated fungi were responsible for lesion development. For this, wounds were surface sterilized with ethanol and the outermost wood tissue on the wounds was removed. Small pieces of inner wood (2 mm²) were plated onto MEA and incubated at room temperature (20 - 25°C) in the dark. Fungal cultures growing in the plates were purified and identified based on morphological characteristics. All stems used in inoculation studies were removed from the trees after the completion of these experiments and burnt to avoid accidental spread of diseases in natural populations. Lesion length data for fungal isolates were compared to those of the control using Kruskal-Wallis procedures in Statistica 10 on the non-parametric data (Statsoft Corporation, USA). A Tukey HSD post-hoc test as implemented in Statistica was used to test differences in median group lesion length.

3.   RESULTS

3.1   Field observations

Three disease symptoms were observed in the Table Mountain region on dead and diseased *Virgilia oroboides* subsp. *oroboides* trees. Symptoms observed in the area surrounding the lower cable car station were leaf yellowing and dropping in individuals (saplings and mature) with numerous small stem cankers. These cankers were scattered along the main stem and side branches of trees (usually associated with axillary buds of side branches) and often had gum oozing from them (Fig. 1a). No arthropods were found associated with these cankers. Another symptom commonly observed in this area was wilted and dead trees (saplings and mature) without any sign of cankers. These individuals showed root rot disease symptoms. Removal of outer bark from roots and the base of stems exposed white mycelial fans and in some cases the leading edges of the mycelial fans were moist, the wood appearing as though soaked (Fig. 1b). Similar symptoms of white mycelial fans underneath the bark were

observed in the forests that form part of the Kirstenbosch National Botanical Garden, but at the base of the main trunk of long dead (mature) trees. Hence, samples could not be collected from these for identification purposes. The third disease symptom often observed on mature trees at Table Mountain was the presence of small bracket fungi on the bark of dying trees (Fig. 1c). These symptoms were not associated with any of the aforementioned diseases.
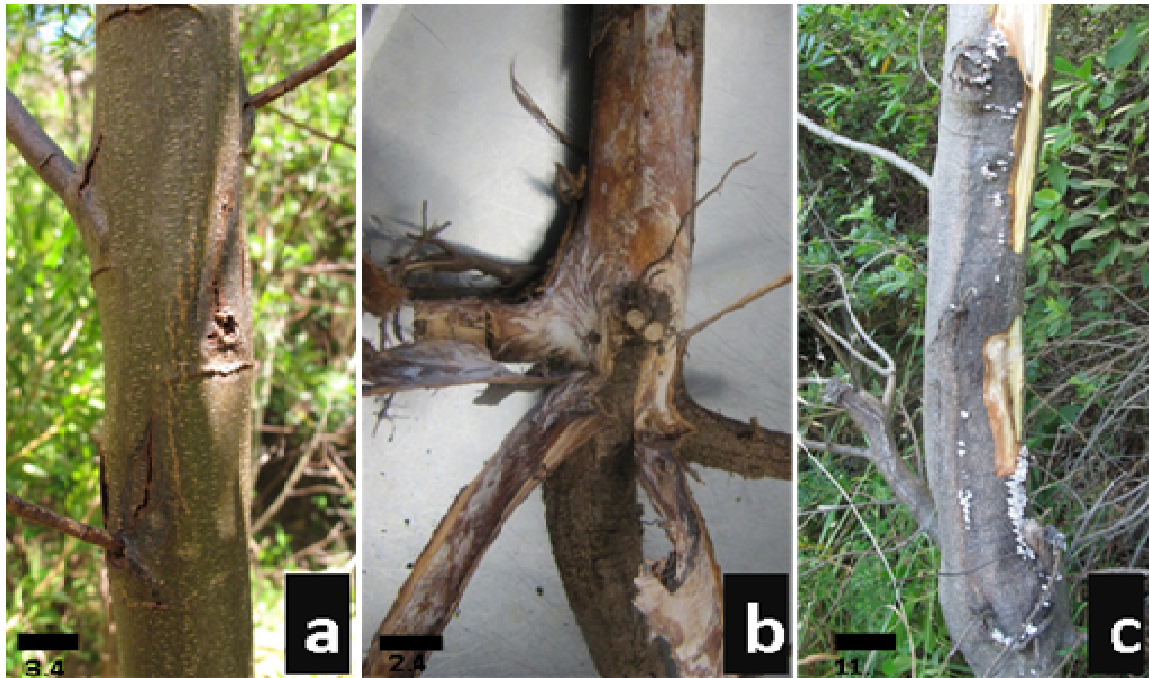

Fig. 1: Disease symptoms observed on *Virgilia* spp. in the vicinity of Table Mountain, South Africa. a – Numerous small stem cankers filled with exuding gum (only revealed after bark removal) on a dying tree; b - White mycelial fans exposed after removal of outer bark of roots of dying trees; c – Fruiting structures of a bracket fungus associated with a stem canker (some bark removed) on the trunk of a dying tree.

Virgilia oroboides subsp. *ferruginea* in the George region and *V. divaricata* in the Knysna and Storms River regions are commonly associated with ghost moths, which utilize the trees as breeding sites. Their larvae usually bore into the trunks close to ground level. The hosts react by exuding gum from the resultant wounds (Fig. 2a). The larvae feed on wood inside the trunks and emerge as adults, leaving large exit holes (Nielsen 2000) (Fig. 2b-d). The areas around these exit wounds often showed signs of disease in the form of cankers and cracked wood (Fig. 2b). Trees with tunnels of ghost moth larvae often showed serious wilting symptoms and were often found dead. However, numerous trees with old exit holes of

ghost moths appeared healthy. In addition to ghost moth larvae, larval tunnels of unidentified cerambycid beetles were often found at breast height on wilting and dead *Virgilia* trees (Fig. 2e).
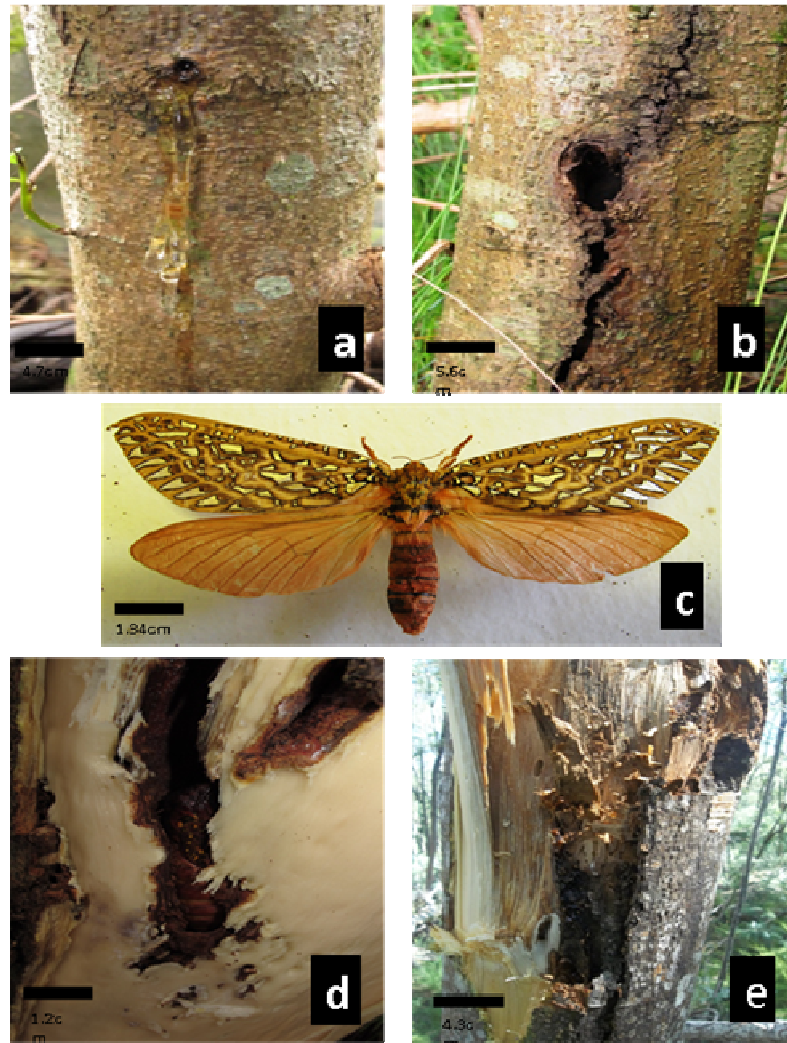
Fig. 2: Damage on the stems of *Virgilia* spp. made by ghost moth and cerambycid larvae, both of which are often colonised by ophiostomatoid fungi. a – Entrance hole of ghost moth larvae with gum exudate; b – Exit hole of ghost moth adult after completing its life-cycle within the trunk; c – Ghost moth adult; d – Tunnel exposed after removal of bark containing the pupa of a ghost moth. Also obvious is the discoloration of wood within the tunnel; e – Exposed tunnel of cerambycid larvae showing dead and severely discoloured wood.

The bark surrounding the wounds created by both types of boring insects had gum oozing from them. Removing the bark from these revealed wood discoloration and gum exudate inside the tunnels as well. The discoloured wood had a streaked appearance typical of

130

infection by ophiostomatoid fungi. Nitidulid beetles were often found in association with the oozing gum in and around ghost moth and cerambycid tunnels and in many instances their larvae were found within the tunnels of the ghost moth larvae.

3.2 Association of arthropods with ophiostomatoid fungi

Artificial wounding of *Virgilia* trees to attract and collect associated nitidulid larvae was not successful as only two nitidulid larvae were found on a single wound. Out of all the wounds made, only three oozed with gum and the wood was discoloured and had streaking as observed in tunnels of cerambycid and ghost moth larvae. The other wounds were dry without obvious stress symptoms. All three previously isolated ophiostomatoid fungi, *Ceratocystis tsitsikammensis*, *Ophiostoma plurianulatum* and *O. querci* were successfully isolated from the three artificial wounds. None were isolated from the two nitidulid larvae.

3.3   Fungal identification

Generally, specific fungal morpho-species were consistently isolated from individuals showing specific disease symptoms. Fungi commonly isolated from root samples of *Virgilia oroboides* subsp. *oroboides* from Table Mountain with the white mycelial mats underneath bark formed unusual strings (rhizomorphs) in MEA culture. It was provisionally identified as *Armillaria mellea* (GenBank HQ232290.1; Identities = 823/830 (99%); Gaps = 2/830 (0%)) using the Blast algorithm as implemented in GenBank on its ITS sequence data. Disease symptoms of numerous small cankers on bark, branches and stems of *V. o. oroboides* from Table Mountain were commonly associated with a fungus identified as *Fusarium acuminatum* (HM068320.1; Identities = 553/557 (99%), Gaps = 4/557 (1%)). Isolates from the small bracket fungus from the Table Mountain area produced colonies with an offensive smell in culture. It was identified as *Schizophyllum commune* (GenBank JF439509.1; Identities = 614/616 (99%); Gaps = 1/616 (0%)) as closest taxon. Three fungal taxa were commonly isolated from the larval tunnels of the ghost moth and cerambycid beetle on *Virgilia* trees in Groenkop, Knysna and Stroms River. These were *Ophiostoma plurianulatum* ((FJ437230.1; Identities = 660/669 (99%); Gaps = 2/669 (0&)), *Ceratocystis tsitsikammensis* (EF408555.1; Identites = 417/428 (97%); Gaps = 1/428 (0%)) and *Ophiostoma querci*

(AF493251.1; Identities = 653/656 (99%); Gaps = 3/656 (0%)). Fungi from artificial wounds made on bark later proved to be similar to those from tunnels of ghost moth and cerambycid beetle larvae.

3.4   Phylogenetic analyses

All isolates of *A. mellea* collected from root samples from *Virgilia* on Table Mountain had almost identical ITS sequences. We aligned our sequences with those from other *A. mellea* isolates collected from around the world, including South Africa, that were available from GenBank (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010, Table 2). The aligned ITS data set consisted of 27 taxa and 914 characters. Eighty-three of these were parsimony informative, 78 variable characters were parsimony uninformative, and 753 characters were constant. Parsimony analyses using heusteric searches retained 69 trees. The strict consensus tree had a Tree Length of 215, Consistency Index of 0.823 and Retention Index = 0.885. Both parsimony and Bayesian inference analyses of the ITS data resolved 4 main clades (Fig. 3). Each clade represented a main distinct geographical region; Europe, Asia, Western North America and Eastern North America. Our isolates (NM42 and NM43) grouped with isolates from Europe, with a strong Bayesian posterior probability of 1 and MP bootstrap support value of 97% (Fig. 3). They grouped with isolates from the Company Gardens and Kirstenbosch Botanical Garden of South Africa in a strongly supported sub-clade. Our sequences were almost identical to those originating from isolates collected from Kirstenbosch in previous studies (Coetzee *et al*. 2003; Wingfield *et al*. 2010). These South African isolates group in a clade along with isolates from England (Fig. 3).
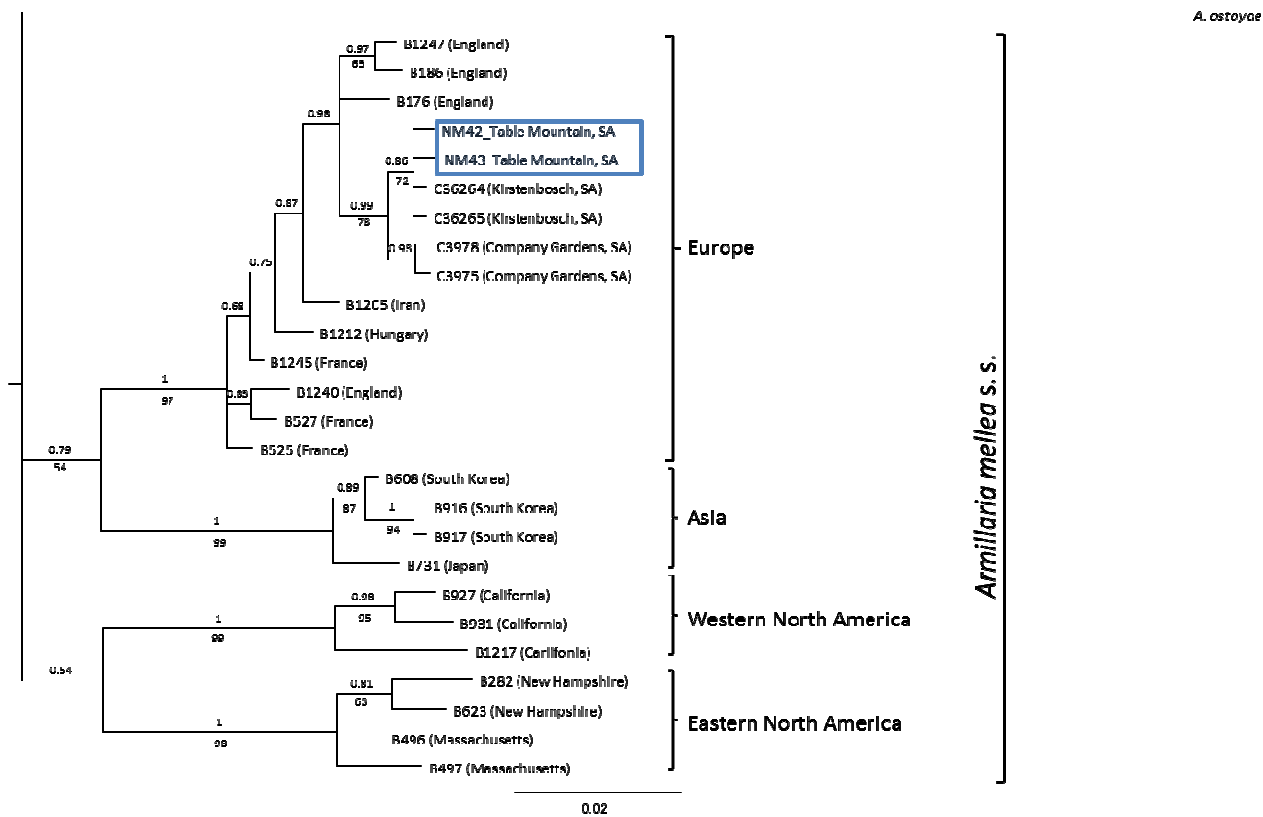
Fig. 3: Bayesian phylogenetic tree of *Armillaria mellea* s. s. using ITS data. Bayesian posterior probabilities are provided at the top of branches at nodes, while maximum parsimony bootstrap support values are provided below the branches (only values above 50%). The tree was rooted using *A. ostoyae.* Isolates from this study are shaded in blue, they grouped together with other isolates found in South Africa, in a well-supported clade with England isolates.

Table 2: Isolates of *Armillaria mellea* used in this study.

Culture no.	Alternative No.*	Host tree	Origin	Collector	Reference	GenBank (ITS)
B176	CMW3179, M1*	*Rosa* sp.	Cambs Co., England	Rishbeth, J. B.	Coetzee *et al*. (2005)	AF163578
B186	CMW4603, BQ5F*	Unknown	Straiton, England	Gregory, S.	Coetzee *et al*. (2000)	AF163582
B927	CMW3964	*Quercus* sp.	Orinda, CA, USA	Bruns, T. D.	Coetzee *et al*. (2005)	AF163595
B931			Califonia, USA			AF163596
B1217	CMW4619, 216*	unknown	CA, USA	Raabe, B.	Coetzee *et al*. (2000)	AF163597
B1240	CMW4624, AM1*	unknown	England	Sierra, A-P	Coetzee *et al*. (2000)	AF163580
B282	CMW4605, TCH-2-1*	*Betula populifolia*	Durham, NH, USA	Harrington, T. C.	Coetzee *et al*. (2005)	AF163587
B496		unknown	Provincetown, MA,	Anderson, J. B.	Coetzee *et al*. (2005)	AF163589
B497	CMW3956, NABS V1*, 97-1*	*Acer saccharum*	USA	Anderson, J. B.	Coetzee *et al*. (2005)	AF163590
B623	CMW4609, PJZ-87-1A*	unknown	Provincetown, MA,	Zambino, P. J.	Coetzee *et al*. (2005)	AF163588
B525	CMW3957, KD1*	unknown	USA	Guillaumin, J-J.	Coetzee *et al*. (2005)	AF163585
B527	CMW4607, PM8*	unknown	Durham, NH, USA	Guillaumin, J-J.	Coetzee *et al*. (2000)	AF163584
B1212	CMW4615, 94056/1*	unknown	France	Szato, M.	Coetzee *et al*. (2005)	AF163581
B1245	CMW4627, D5*	*Prunus* sp.	France	Unknown	Coetzee *et al*. (2000)	AF163586
B1247	CMW4628, P-5580*	*Chamaecyparis* sp.	Hungary	Sierra, A-P.	Coetzee *et al*. (2000)	AF163579
B608	CMW3966, AF*	unknown	France	Sung, J-M.	Coetzee *et al*. (2005)	AF163591
B916	CMW4610, A-4*	unknown	England	Sung, J-M.	Coetzee *et al*. (2005)	AF163592
B917	CMW4611, A-2*	unknown	South Korea	Sung, J-M.	Coetzee *et al*. (2005)	AF163593

B931			Carlifonia, USA	Bloomer, P.	Coetzee *et al*. (2005)	AF163596
B731	CMW3967, 1003"*	*Chamaecyparis* sp.	Japan	Shaw, C. G.	Coetzee *et al*. (2005)	AF163594
B1205	CMW4613, 86009/1*	Unknown	Iran	Saber, M.	Coetzee *et al*. (2005)	AF163583
	CMW3975		Company Gardens, SA		Coetzee et al. (2001)	AF310329
	CMW3978		Company Gardens, SA		Coetzee *et al*. (2001)	AF310328
	CMW36264		Kirstenbosch, SA		Wingfield *et al*. (2010)	HQ441179
	CMW36265		Kirstenbosch, SA		Wingfield *et al*. (2010)	HQ441180
Armillaria ostoyae	TrAo19042011	*Pinus* sp.	Sinop, Boyabat, Turkey	Lehtijarvi, A & Aday, G.	Lehtijärvi *et al*. (2012)	JN859184
	NM42	*Virgilia oroboides*	Table Moutnain, SA	Machingambi, N. M.		
	NM43	*Virgilia oroboides*	Table Mountain, SA	Machingambi, N. M.		

CMW numbers refer to the collection numbers in the fungal culture collection of the Tree Protection Co-operative Programme (TPCP), FABI (Pretoria).

* Alternative numbers refer to culture numbers used for isolates in previous publications.

3.5 Pathogenicity tests

All stems inoculated with fungal isolates of *Fusarium acuminatum*-like and *Schizophyllum commune* had gum exuding from the wounds after 6 weeks. Gum production was absent from control wounds. Three isolates of the *F. acuminatum*-like fungus, *C. tsitsikammensis*, *O. plurianulatum* and a single isolate of *S. commune* were used in inoculation trials. All isolates were able to cause lesions on stems of their host *Virgilia* trees, while control inoculations caused very small or no lesions. Kruskal-Wallis tests revealed significant differences in the length of lesions caused by isolates compared to the control (H (7, N = 64) = 27.99783 p = 0.0002). Post hoc analyses showed that all lesion lengths were significantly different from the control, with the exception of one of the *F. acuminatum*-like isolate (F2) (Fig. 4).
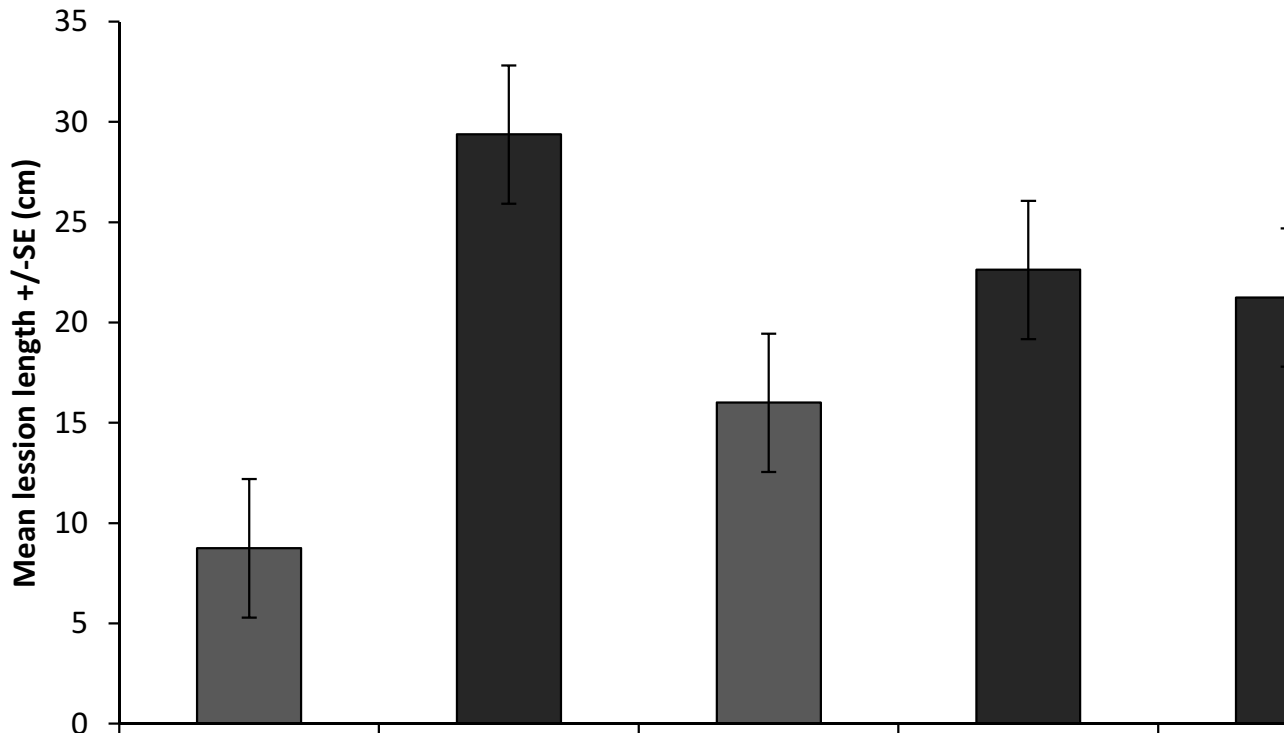
Fig. 4: Mean lesion length caused by by fungal isolates from Table Mountain. One-way ANOVA on the lesion lengths showed significant differences of lesion lengths caused by fungal isolates as compared to those of control (f = 6.9, df = 4, p < 0.00001). Post Hoc test showed that only isolates F1, F3 (*Fusarium acuminatum*-like fungus) and M1 (*Schizophyllum commune*) caused significantly larger lesions than the control. Lessions of isolate F2 (*Fusarium acuminatum*-like fungus) are not significantly different from controls.

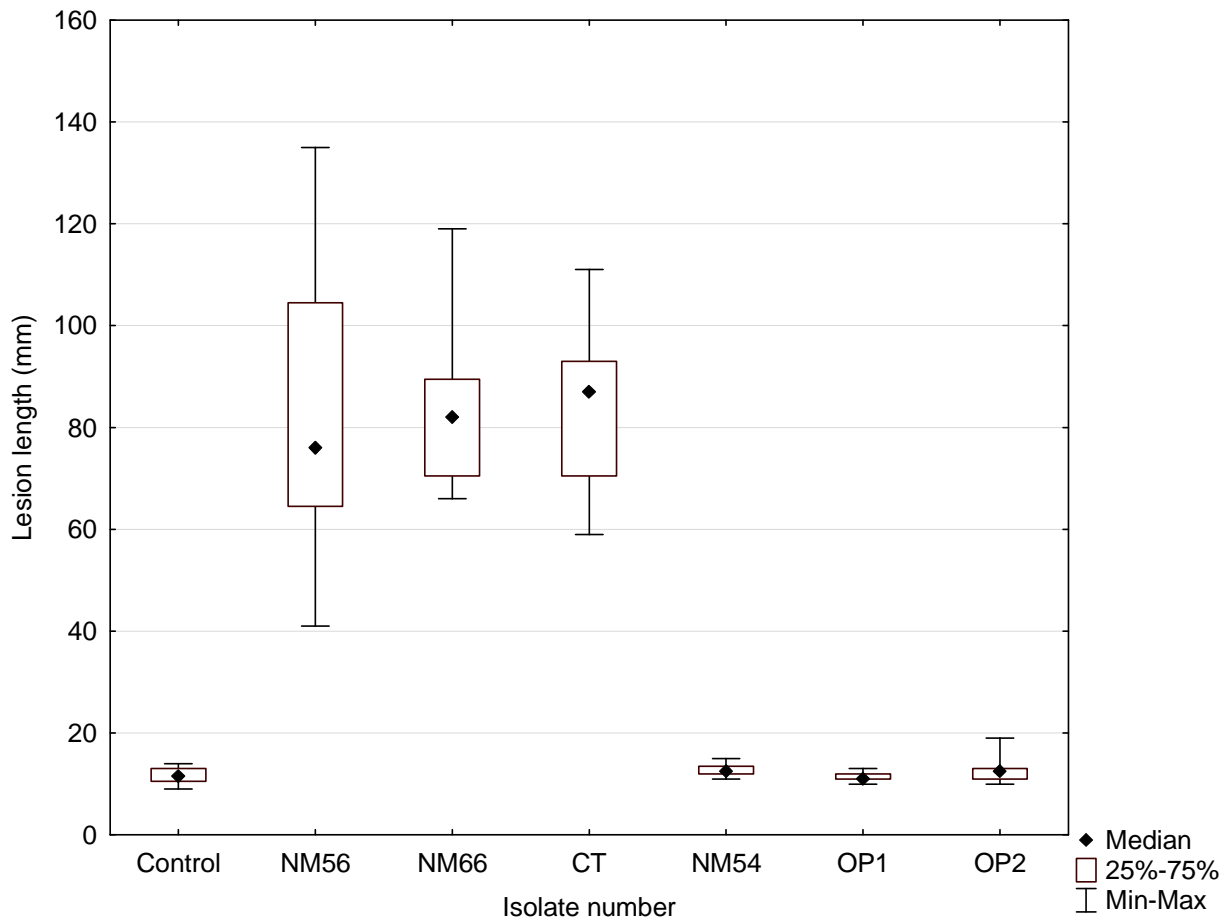Fig. 5: Box and Whisker plot showing lesion lengths caused by ophiostomatoid fungal isolates from Groenkop, Knysna, and Storms River. Kruskal-Wallis ANOVA on the lesion lengths showed significant differences of lesion lengths caused by fungal isolates as compared to those of control (H (df = 6, N= 56) = 42.22; p =.0000). Post Hoc test showed that only isolates of *Ceratocystis tsitsikammensis* (NM56, NM66 and CT) caused significantly different lesion lengths to the control, while lesions of isolates of *Ophiostoma plurianulatum* (NM54, OP1 and OP2) are not significantly different to those of control.

4.   DISCUSSION

This study has shown that *Virgilia* trees in near-pristine areas are commonly infected with pathogens. Some of these are known to be exotic and are cause for serious conservation concern. Three strongly pathogenic fungal species were isolated from *Virgilia oroboides* subsp. *oroboides* in the Table Mountain area: *Fusarium acuminatum*-like fungus, *Armillaria mellea* and *Schizophyllum commune*. In the George, Knysna and Storms river areas where *V.*

oroboides subsp. *ferruginea* and *V. divaricata* are found respectively, various Ophiostomatoid fungal taxa were found in association with damage caused by boring insects. *Ceratocystis tsitsikammensis* is primarily responsible for the death of the *Virgilia* trees in that area, as confirmed by pathogenicity tests. Other taxa are not pathogenic (that is *Ophiostoma plurianulatum* and/or not tested *O. querci*.

The multiple cankers disease on *Virgilia* at Table Mountain was commonly associated with a *F. acuminatum*-like fungus. *Fusaruim acuminatum* is a common damping-off pathogen of nursery pine seedlings in severalparts of the world, including South Africa (Viljoen *et al*. 1994), but our *F. acuminatum*-like fungus causes different disease symptoms. Gum was often found exuding from these cankers. All trees with these cankers also had die-back of main stems. These disease symptoms are similar to those of the pitch canker disease of pine trees caused by *F*. *subglutinans* worldwide (Coutinho et *al*. 2007). Symptoms of pitch canker disease include resinous cankers and die-back of main stem. The major difference between the two diseases is that cankers of the *F. acuminatum*-like fungus are smaller and occurs in high numbers on infected individuals, while pitch cankers are large and not as numerous on pine individuals. *Fusaruim* species that cause stem cankers usually produce air-borne spores that require wounds for infection (Blakeslee *et al*. 1978; Gebeyehu and Wingfield 2003). It is assumed that several cankers seen on stems of *Virgilia* trees are the entry points of this fungus and that it is closely associated with leaf axils. As diseased plants are entirely covered with cankers, it is unlikely that insect damage causes initial infection with this fungus.

Schizophyllum commune was isolated from the bark of *Virgilia oroboides* subsp. *oroboides* trees at Table Mountain. Pathogenicity tests revealed that it can be pathogenic to *Virgilia* trees. It is considered an opportunistic fungus that requires wounds to invade living trees after they have lost their vigour (Erwin *et al*. 2008; Schmidt 2006; Sinclair *et al*. 1987; Snieskiene and Juronis 2001). Vigour loss may be due to harsh conditions like extreme heat and cold, drought and very high humidity (Sinclair *et al*. 1987; Snieskiene and Juronis 2001). However, this fungus has been recorded as pathogen in various studies (Dai 2005; Snieskiene and Juronis 2001). The entry point of this fungus into *Virgilia* trees still remains unknown. It also remains unclear whether it is a secondary or primary pathogen of *Virgilia*.

Three ophiostomatoid fungi were found to invade tunnels made by larvae of ghost moths and unidentified cerambycid beetles on *V. o. ferruginea* from Groenkop and *V. divaricata* from Knysna and Storms River. These were *Ceratocystis tsitsikammensis, Ophiostoma plurianulatum* and *O. querci.* It is most probable that these fungi are vectored by nitidulid beetles as they visit the tunnels. Nitidulid beetles are sap-feeding insects and this explains their presence on oozing tunnels on *Virgilia* trees. They are attracted to gum oozing on stems as the trees respond to tunnelling. Upon visiting the gum, they are thought to inoculate the tree with ophiostomatoid fungi. Kamgan Nkuekam *et al*. (2012a) isolated *O. quercus*, among other ophiostomatoid fungi, from nitidulid beetles on *Eucalyptus* hosts in South Africa and they suggested that nitidulid beetles spread these fungi. In another study, Kamgan Nkuekam *et al*. (2012b) found the association of nitidulid with *Ceratocystis* species on *Eucalyptus* hosts in Australia. The artificial wounds made on *Virgilia* stems attracted nitidulid beetles as marked by the presence of their larvae, although not very successfully. The same ophiostomatoid fungi were isolated from the artificial wounds, but not from the nitidulid larvae. Our findings support the idea of adult nitidulid beetles as vectors of ophiostomatoid fungi.

Other studies suggest cerambycid beetles are vectors of Ophiostomatoid fungi. *Ophiostoma kryptum* K. Jacobs & Kirisits was commonly isolated from breeding galleries of *Tetropium gabrieli* Weise on European larch (*Larix decidua* Mill.) in Australia, although the mode of transportation of this fungus still remains unclear (Jacobs and Kirisits 2003). In another study, several ophiostomatoid fungi were isolated from bodies and galleries of *Tetropium* species on Norway spruce (*Picea abies* (L.) H.Karst) in Poland, including genera *Grosmannia* Goid., *Ophiostoma*, *Graphium* Corda and *Leptographium* Lagerberg and Melin (Jankowiak and Kolařík 2010). In the same study, *Grosmannia piceiperda* (Rumbold) Goid. and *O. bicolor* R. W. Davidson & D. E. Wells proved to be pathogenic to spruce trees, suggesting an important role played by the cerambycid beetles in the death of spruce trees as possible vectors of these fungi. In our study, however, we did not isolate ophiostomatoid fungi directly from the cerambycid adults or larvae. We cannot rule out nitidulid beetles as primary vectors of the ophiostomatoid fungi, because of their presence on the oozing cerambycid tunnels.

In this study, pathogenicity tests of *O. plurianulatum* and *C. tsitsikammensis* on *Virgilia* trees in the field confirmed the virulence of *C. tsitsikammensis*, while *O. plurinanulatum* is not pathogenic. The pathogenicity of *O. querci* was not tested because it is known as a common contaminant. Stems inoculated with *C. tsitsikammensis* had gum exudate on and around the inoculation wounds, but inoculation wounds of *O. plurinanulatum* were not oozing. Isolates of *C. tsitsikammensis* caused exceptionally long lesions (135 mm maximum length). *O. plurinanulatum* is regarded a non-pathogenic fungus, only capable of staining wood of its hosts (Harrington 2005; Seifert 1993). It also proved to be non-pathogenic in our study. *Ceratocystis tsitsikammensis* was first encountered in Groenkloof forest (Tsitsikamma, South Africa) in 2008 on *Rapanea melanophloeos* and *Ocotea bullata* (Kamgan Nkuekam *et al*. 2008). Artificial inoculations were done under greenhouse conditions and this fungus caused significantly long lesions on stems of *R. melanophloeos*, proving to be highly pathogenic to that species (Kamgan Nkuekam *et al*. 2008). At the end of the pathogenicity trials, many individuals had epicormic shoots below the inoculation points as a sign of stem girdling (Kamgan Nkuekam *et al*. 2008).

Armillaria mellea was found killing *Virgilia* trees close to the lower cable station on Table Mountain. The presence of white mycelial fans, characteristic of *A. mellea*, was also observed underneath the bark at the base of trunks of long dead *Virgilia* trees in forests at the Kirstenbosch National Botanical Garden. *Armillaria mellea* is a non-host specific, aggressive, soil-borne pathogen (Baumgartner *et al*. 2011; Gregory *et al*. 1991) native to the northern hemisphere (Coetzee *et al*. 2001). Previous studies have shown that it was introduced to South Africa from Europe (Coetzee *et al*. 2001, 2003; Wingfield 2010). The history of *Armillaria mellea* in South Africa was described by Coetzee *et al*. (2001, 2003) and Wingfield *et al*. (2010). Death of oak trees caused by *Armillaria mellea* was first reported in 1996 in the Company Gardens. Older stumps of dead oaks and other unidentified trees were seen, indicating that *A. mellea* may have been present for many years. In 2001, 2003 and 2010, more reports of *Armillaria* root rot deaths were reported in and around the Company Gardens and in the Kirstenbosch National Botanical Garden (Coetzee *et al*. 2001, 2003; Wingfield *et al*. (2010). Some of the plant genera killed by *A. mellea* included *Protea* L., *Leucadedron* R.Br., *Aesculus* L*., Albizia* Durraz*., Ficus* L*., Hydrangea* L., *Morus* L., and *Strelitizia* Ait.. In all cases the presence of the fungus was noted in anthoropogenically

transformed habitats. In the present study we have isolated it from recently dead and dying *Virgilia oroboides* subsp. *oroboides* trees in near-pristine Fynbos habitats on Table Mountain. Symptoms indicative of *A. mellea* disease were also observed on long dead *Virgilia* trees in natural Afromontaine forests next to the Kirstenbosch National Botanical Garden. It has thus escaped into the natural areas on the Cape Peninsula and may become very problematic in the future. This was already predicted *ca*. 10 years ago (Coetzee *et al*. 2003).

Armillaria mellea likely spread to the natural areas of Table Mountain from the Kirstenbosch Botanical Garden through basidiospores. It is known to sporulate profusely in autumn at the onset of the first rains in these areas (Wingfield *et al*. 2010). Even though the Kirstenbosch National Botanical Garden is located at the foot of Table Mountain to the south and our isolates were collected from the northern side of the mountain, our phylogenetic analyses suggest that it is the same *A. mellea* strain isolated in the Kirstenbosch National Botanical Garden in previous studies (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010). This shows that this fungus was able to spread *ca*. 5 to 10 km with relative ease. As *Virgilia* trees are distributed across the peninsula, this fungus may have serious future impacts on the natural distribution of this ecologically important plant species. It may thus also impact the floral composition of these natural ecosystems, similar to that of *Phytophthora cinnamomi* Rands within the Western Australian Jarrah forests (Davison and Shearer 1989).

This study provides the first report of pathogenic fungi attacking *Virgilia* trees in the near pristine environments in the CFR of South Africa. The findings are interesting, in that they reveal different pathogens attacking different species of *Virgilia* in different geographical areas. The Table Mountain area has three different pathogens that are not taxonomically related. *Virgilia* trees in Knysna, George and Storms River are being attacked by taxonomically related pathogens through the aid of associated insects. The interaction of insects, pathogens and their host trees (*Virgilia*) is quite interesting, as it entails a cascade of events involving interdependence of the organisms for survival. The discovery of an exotic pathogen, *Armillaria mellea*, has management implications when it comes to the movement of rooted plant material to new areas, where serious quarantining must be implemented, or transport of rooted material should be prohibited completely.

REFERENCES

Abdullah F, Rusea G, 2009. Documentation of inherited knowledge on wild edible fungi from Malaysia. *Blumea* **54**: 35-38.

Abdurahman A, 1992. Research need in forest entomology. In, *Proceedings of the National Workshop on Setting Forestry Research Priorities in Ethiopia*, April 27-30 1992. Forestry Research Centre, Addis Ababa, Ethiopia, pp. 113-121.

Akaike H, 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716-723.

Allard GB, Fortuna S, See LS, Novotny J, Baldini A, Courtinho T, 2003. Global information on outbreaks and impact of major forest insect pests and diseases. XII World Forestry Congress, Canada.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Anagnostakis SL, 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* **79**: 23-37.

Baumgartner K, Coetzee M, Hoffmeister D, 2011. Pathogen profile: Secrets of the subterranean pathosystem of *Armillaria. Molecular Plant Pathology*: 1-20.

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

Blakeslee GM, Oak SW, Gregory W, Moses CS, 1978. Natural association of *Fusarium moniliforme* var. *subglutinans* with *Pissodes nemorensis*. *Phytopathology News* **12**: 208. (Abstract).

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

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

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

Bridges JR, Moser JC, 1986. Relationship of phoretic mites (Acari: Tarsonemidae) to the bluestaining fungus, *Ceratocystis minor*, in trees infested by southern pine beetle (Coleoptera, Scolytidae). *Environmental Entomology* **15**: 951–953.

Castello JD, Leopold DJ, Smallidge PJ, 1995. Pathogens, Patterns and Processes in Forest Ecosystems. *BioScience* **45**(1): 16-24.

Castro LÁ, Álvarez MI, Martinez E, 2010. Case report of *Schizophyllum commune* sinusitis in an immunocompetent patient. *Colombia Médica* **41**(1): 71-75.

Chowdhary A, Randhawa HS, Gaur SN, Agarwal K, Kathuria S, Roy P, Klaassen CH, Meis JF, 2012. *Schizophyllum commune* as an emerging fungal pathogen: a review and report of two cases. Mycoses Diagnosis, Therapy and Prophylaxis of Fungal Diseases, pp 1-10.

Coetzee MPA, Wingfield BD, Harrington TC, Dalevi D, Coutinho TA, Wingfield MJ, 2000. Geographical Diversity of *Armillaria mellea* s. s. based on phylogenetic analysis. *Mycologia* **92**(1): 105-113.

Coetzee MPA, Wingfield BD, on Harring TC, Steimel J, Coutinho TA, Wingfield MJ, 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* **10**: 387-396.

Coetzee MPA, Wingfield BD, Roux J, Crous PW, Denman S, Wingfield MJ, 2003. Discovery of two nothern hemisphere species on Proteaceae in South Africa. *Plant Pathology* **52**: 604-612.

Coetzee MP, Wingfield BD, Bloomer P, Wingfield MJ, 2005. Phylogenetic analyses of DNA sequences reveal species partitions amongst isolates of *Armillaria* from Africa. *Mycological Research* **109** (11): 1223-1234.

Coutinho TA, Steenkamp ET, Mongwaketsi K, Wilmot M, Wingfield MJ, 2007. First outbreak of pitch canker in a South African pine plantation. *Australasian Plant Pathology* **36**: 256-261.

Cowling RM, 1984. A syntaxonomic and synecological study in the Humansdorp region of the Fynbos Biome. *Bothalia* **15**(1): 177-227.

Davison EM, Shearer BL, 1989. Phytophthora spp. in indigenous forests in Australia. *New Zealand Journal of Forestry Science* **19**: 277-289.

Dai YC, 2005. First report of sapwood rot of peach caused by *Schizophyllum commune* in China. *Plant Disiseases* **89**: 778.

Erwin, Takemoto S, Hwang W-J, Takeuchi M, Itoh T, Imamura Y, 2008. Anatomical characterization of decayed wood in standing light red meranti and identification of the fungi isolated from the decayed area. *Journal of Wood Science* **54**: 233-241.

Everard DA, 1987. A classification of the subtropical transitional thicket in the Eastern Cape, based on syntaxonomic and structural attributes. *South African Journal of Botany* **53**(5): 329-340.

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

Gebeyehu S, Wingfield MJ, 2003. Pine weevil *Pissodes nemorensis*: threat to South Africa pine plantations and options for control. *South African Journal of Science* **99**: 531-536.

Geldenhuys CJ, 1994. Bergwind fires and the location pattern of forest patches in the southern Cape landscape, South Africa. *Journal of Biogeography* **21**: 49-62.

Geldenhuys CJ, 1997. Composition and biogeography of forest patches on the inland mountains of the southern Cape. *Bothalia* **27**(1): 57-74.

Geldenhuys CJ, 2010. Distribution, Size and Ownership of Forests in the Southern Cape. *South African Forestry Journal* **158**: 51-66.

Gilbert GS, 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology* **40**: 13-43.

Gregory SC, Rishbeth J, Shaw CG, 1991. Pathogenicity and virulence. In: Shaw CG, Kile GA, eds. *Armillaria Root Disease*, Agriculture Handbook no. 691. Washington, DC, USA: Forest Service, US Department of Agriculture, pp. 76–87.

Goldblatt P, 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.

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

Hall T, 2005. BioEdit, biological sequence alignment editor for Win95/98/NT/2K/XP. Carlsbad, California: Ibis therapeutic.

Harrington TC, 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE & Blackwell M (eds.) *Ecological and Evolutionary Advances in Insect-Fungal Associations*, Oxford University Press, pp. 257-291.

Heath RN, Wingfield MJ, Wingfield BD, Meke G, Mbaga A, 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.

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

Jacobs A, Coutinho TA, Wingfield MJ, Ahumaba R, Wingfield BD, 2006. Characterization of the pitch canker fungus, *Fusarium circinatum*, from Chile. *South African Journal of Science*. **102**: 1-5.

Jankowiak R, Kolařík M, 2010. Diversity and pathogenicity of ophiostomatoid fungi associated with *Tetropium* species colonizing *Picea abies* in Poland. *Folia Microbiologica* **55**(2): 145-154.

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

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

Kile GA, 1993. Plant diseases caused by species of Ceratocystis sensu strica and Chalara. In: Wingfiled MJ, Seifert KA, Webbe JF (eds.), *Ceratocystis* and *Ophiostoma: Taxonomy, ecology and pathogenicity*. APS Press, St. Paul, Minnesota, pp. 173-183.

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

Kishi K, 1998. Plant diseases in Japan. Zenkoku-Noson-Kyoiku Kyokai Publishing Co. Ltd., Tokyo, Japan.

Linder HP, 2003. The radiation of the Cape flora, southern Africa. *Biological Review* **78**: 597-638.

Logrieco A, Altomare C, Moretti A, Bottalico A, 1992. Cultural and toxigenic variability in *Fusarium acuminatum*. *Mycological Research* **96** (6): 518-523.

Logrieco A, Moretti A, Castella G, Kostecki M, Golinski P, Ritieni A, Chelkowski J, 1998. Beauvericin Production by *Fusarium* Species. Applied And Environmental Microbiology **64**(8): 3084-3088.

Loo JA, 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* **11**: 81-96.

Malloch D, Blackwell M, 1993. Dispersal biology of the ophiostomatoid fungi. In: Wingfield MJ, Seifert KA, Webber JF (eds), *Ceratocystis and Ophiostoma: taxonomy, ecology, and pathogenicity*. American Phytopathological Society, St. Paul, pp. 195-206.

Manion PD, 1991. Tree disease concepts. Prentice Hall, Engelwood Cliffs, NJ (USA), pp. 402.

Mbabezeli G, Notten A, 2003. *Virgilia divaricata* Adamson and *Virgilia oroboides* (Berg.) Salter. South African National Biodiversity Institute, South Africa.

Moller WJ, Devay JE, 1968. Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499-1507.

Montoya MM, Wingfield MJ, 2006. A review of *Ceratocystis* sensu stricto with special reference to the species complexes *C. coerulescens* and *C. fimbriata*. *Revista Facultad Nacional de Agronomía – Medellín* **59**(1): 3045-3075.

Morgenthal TL, Cilliers SS, 2000. Species composition and phytogeographical significance of an afromontane forest fragment in the Mpumalanga province, South Africa. *The Southern African Forestry Journal* **189**(1): 81-102.

Moser JC, Perry TJ, Bridges JR, Yin H.-F, 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus*, a mycangial fungus of the southern pine beetle. *Mycologia* **87**: 84-86.

Moser JC, Konrad H, Kiristis T, Carta LK, 2005. Phoretic mites and nematodes associates of *Scolytus multistriatus pygmaeus* (Coleoptera: Scolytidae) in Austria. *Agricultural and Forest Entomology* **7**: 169-177.

Mucina L, Rutherford M, 2006. The vegetation of South Africa, Lesotho and Swaziland. *Streletzia* **19**, Pretoria: *South African National Biodiversity Institute*: **77**: 93-94: 181-182.

Nielsen ES, Robinson GS, Wagner DL, 2000. Ghost moths of the world: a global inventory and bibliography of the Exoporia (Mnesarchaeoidea and Hepialoidea) (Lepidoptera). *Journal of Natural History* **34**(6): 823-878.

Oprea M, Şesan T, Bălan V, 1994. *Schizophyllum commune* – canker and dieback disease of apricot trees in orchards of southeastern Romania. *Rev. Roum. Biol. – Biol. Végét.* **39**: 35–40.

Palmer E, Pitman N, 1961. Trees of South Africa. Balkema. Cape Town.

Palmer E, Pitman N, 1972. Trees of South Africa volume two. Balkema. Cape Town.

Phillips JFV, 1926. *Virgilia capensis* Lamk. ("Keurboom"): a contribution to its ecology and silviculture. *South African Journal of Science* **23**: 435-454.

Posada D, 2008. Selection of models of DNA evolution with jModelTest. In: Posada D (ed.), *Bioinformatics for DNA sequence analysis*. Humana Press, Totowa, pp. 93–112.

Quimby PC, Jr. 1982. Impact of diseases on plant populations. In: Charudattan R, Walker HL, (eds.), *Biological Control of Weeds with Plant Pathogens* John Wiley & Sons, New York, pp. 47-60.

Roets F, Crous PW, Wingfield MJ, Dreyer LL, 2007. Discovery of fungus-mite mutualism within a unique niche of the Cape Floral Kingdom. *Environment Entomology* **36**: 1226-1237.

Roets F, Crous PW, Wingfield MJ, Dreyer LL, 2009. Mite-mediated Hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. *Environmental Entomology* **38**(1): 143-152.

Ronquist FR, Huelsenbeck JP, 2003. MrBayes: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574. (doi:10.1093/bioinformatics/btg180).

Roux J, Dunlop R, Wingfield MJ, 1999. Susceptibility of elite *Acacia mearnsii* families to Ceratocystis wilt in South Africa. *Journal of Forestry Research* **4**: 187–90.

Roux J, Heath RN, Labuschagne L, Kamgan Nkuekam G, Wingfield MJ, 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 GC, Nakabonge G, Heath RN, Wingfield MJ, 2005. Diseases of plantation forestry trees species in Eastern and Southern Africa. *South African Journal of Science* **101**: 409-413.

Schmidt O, 2006. Wood and tree fungi: biology, damage, protection, and use. Springer-Verlag, Germany.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield MJ, Seifert KA, Webber JF (eds.), *Ceratocystis and Ophiostoma: taxonomy,*

ecology, and pathogenicity. American Phytopathological Society, St. Paul, pp. 141-151.

Shapiro SS, Wilk MB, 1965. An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.

Sigler L, Bartley JR, Parr DH, Morris AJ, 1999. Maxillary sinusitis caused by medusoid form of *Schizophyllum commune*. *Journal of Clinical Microbiology* **37(**10): 3395-3398.

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

Six DL, 2003. Bark beetle-fungus symbioses. In: Bourtzis K, Miller TA (Eds.) *Insect Symbiosis Contemporary Topics in Entomology*. CRC Press, Boca Raton, London, New York, Washington D. C, pp. 97-114.

Smith CA, 1966. Common names of South African plants. *Memoirs of the Botanical Survey of South Africa* No. 35.

Snieskiene V, Juronis V, 2001. Disrtibution of the fungus *Schizophyllum commune* Fr. in plantings of trees in the Kaunas city. *Biologija* **3**: 45-47.

Speight MR, Wylie FR, 2001. Insect Pests in Tropical Forestry. CABI Publishing, pp. 307.

Swofford DL, 2002. PAUP*: Phylogenetic Analysis Using Parsimony. (* and Other Methods), Version 4.0b10. Sinauer Associates, MA (2002).

Tainter FH, Baker FA, 1996. Principles of Forest Pathology. New York: John Wiley & Sons, Inc, p. 805.

Taylor JE, Lee S, Crous PW, 2001. Biodiversity in the Cape Floral Kingdom: fungi occurring on *Proteaceae. Mycological Research* **105** (12): 1480-1484.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice**.** *Nucleic Acids Research* **22**: 4673-4680.

Thrane U, Seifert K, 2000. *Fusarium* Link: Fr. In Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (eds.), *Introduction to food- and airborne fungi*. Centraalbureau Voor Schimmecultures, Utrecht, pp. 120-175.

Turpie JK, Heydenrych BJ, Lamberth SJ, 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.

Uzunovic A, Webber JF, 1998. Comparison of bluestain fungi grown in vitro and in freshly cut pine billets. *European Journal of Forest Pathology* **28**: 322-334.

Van Daalen JC, 1981. The dynamics of the indigenous forest-fynbos ecotone in the southern Cape. *South African Forestry Journal* **119**: 14-23.

Van der Bank M, van der Bank FH, van Wyk B-E, 1996. Speciation in *Virgilia (Fabaceae):* allopatric divergence followed by introgression? *Plant Systematics and Evolution* **201**: 57-73.

Van Wyk BE, 1982. *Virgilia* (Keur): Some Preliminary Results Of Species And Provenance Differences In Establishment And Early Growth. *South African Forestry Journal* **123**: 24-28.

Van Wyk B, Van Wyk P, 1997. Field guide to trees of Southern Africa. Struik. Cape Town.

Viljoen A, Wingfield MJ, Marasas WF0, 1994. First report of *Fusarium subglurinans* f. sp. *pini* on pine seedlings in South Africa. *Plant Diseases* **78**: 309-312.

Visarathanonth N, 1990. A survey of some temperate fruit diseases in Thailand. *Acta Horticulturae* **279**: 609–618.

Volk JHJ, Euston-Brown DIW, Cowling RM, 2003. Acock's Valley Bushveld 50 years on: new perspectives on the delimitation, characterisation and origin of subtropical thicket vegetation. *South African Journal of Botany* **67**(1): 27-51.

Von Broembsen SL, 1989. Invasions of Natural Ecosystems by Plant Pathogens. In: Drake JA, Mooney HA, di Castri F, Groves RH, Kruger FJ, Rejmanek M, Williamson M, (eds.), *Biological Invasions: A Global Perspective* **37**: 77-83*.*

White TJ, Bruns T, Lee S, Taylor, JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., New York, pp. 315-322.

Williams KJ, Ford A, Rosauer D, De Silva N, Mittermeier R, Bruce C, Larson FW, Margules C, 2011. Forests of East Australia: the 35th biodiversity hotspot. In Zachos FE, Habel JC (eds.), *Biodiversity hotspots: distribution and protection of conservation priority areas*. Springer, Heidelberg, Baden-Württemberg.

Wingfield MJ, Coetzee MPA, Crous PW, Six D, Wingfield BD, 2010. Fungal phoenix rising from the ashes? *International Mycological Association Fungus* **1**(2): 149-153.

Wingfiled MJ, Jacobs A, Coutinho TA, Ahumada R, Wingfield BD, 2002. New disease report: First report of the pitch canker fungus, Fusarium circinatum, on pines in Chile. *Plant Pathology* **51**: 397.

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

Wingfield MJ, Seifer KA, Webber JF, 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Society Press, St. Paul Minnestota, USA.

Zeller KA, Summerell BA, Leslie JF, 2003. *Gibberella konza* (*Fusarium konza*) sp. vov. From praire grasses, a new species in the *Gibberella fujikuroi* species complex. *Mycologia*. 95: 943-954.

CHAPTER 5

CONCLUSIONS

This study revealed that the death of *Virgilia* trees, both in managed botanical gardens and in near pristine environments in the CFR of South Africa, is primarily caused by various species of pathogenic fungi with subsequent scolytine beetle attack. Many of these fungi are not yet formally described, which highlights the general lack of information on fungal pathogens on native hosts in South Africa. This study can therefore be seen as a pioneer study in the Cape Floristic Region, an area with exceptional botanical (Chen *et al*. 2012; Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010) and fungal (Crous 2005) biodiversity. This is especially important as pathogens play pivotal roles in ecosystem functioning (Abdurahman 1992; Anagnostakis 1987; Gilbert 2002; Speight and Wylie 2001; Brasier 1990; Von Broembsen 1989). As an example, *Virgilia* trees are vulnerable to numerous fungal pathogens and scolytine beetles wherever they exist and this may be one of the reasons for the short life expectancy of these trees (Mbambezeli and Notten 2003). This short life expectancy would make them ideal pioneers in forest tree succession (Geldenhuys 1994; Phillips 1926; Van Daalen 1981).

The fungal pathogens identified in this study are interesting in terms of their possible origins and the diseases they can cause. These origins range from being probably native (*Ceratocystis tsitsikammensis*), to unknown (*Fusarium acuminatum*-like, *Phomopsis virgiliansis* and *Schizophyllum commune*) to exotic (*Armillaria mellea*). This is the first record of the un-described *Phomopsis* root pathogen in HPNBG in South Africa. The rapid rate at which the *Virgilia* trees are dying from this pathogen suggests that it represents a new encounter of the pathogen with *Virgilia* hosts. The possibility that this pathogen is native to South Africa can, however, not be ruled out. The garden has an active nursery and the pathogen might have come from other parts of the country undetected. Given the time and resources, it would be beneficial to determine the origins of this pathogen and to monitor

the population of *Virgilia* trees in HPNBG over a longer period of time. This will determine if there will be any significant changes in the abundance and occurrence of these trees over time due to Phomopsis root disease.

Phomopsis root disease in HPNBG and Armillaria root rot, caused by the non-native *Armillaria mellea* in KNBG and Table Mountain are of particular importance to botanical garden and natural ecosystem managers, because they can be accidentally introduced to new areas. Several studies have shown that botanical gardens are common introductory points of non-native pests and pathogens (Coetzee *et al*. 2001; Wingfield *et al*. 2010; Von Broembsen 1989). Humans have accelerated such introductions through global trade (Brasier 2008; Desprex-Loustau *et al*. 2007; Jones and Baker 2007; Loo 2009; Pimental *et al*. 2001; Skarpaas and Okland 2009; Tatem *et al*. 2006; Von Broembsen 1989). Root pathogens are easily introduced to potted plants or nursery seedlings and to compost. *Armillaria mellea* was probably introduced to South Africa on rooted plants (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010) and has since spread from its point of introduction to KNBG and Table Mountain. *Virgilia* seedlings and/or organic compost are most likely to be moved from these gardens, as they are common ornamental trees. There is need for strict and updated quarantine measures if the seedlings and/or compost are to be moved from infected areas. The best option will be to prohibit the movement of any rooted *Virgilia* plants and organic matter from HPNBG, KNBG and Table Mountain. This will prevent unnecessary spread of *A. mellea* and *Phomopsis virgiliansis* and thereby help conserve the integrity of natural populations of *Virgilia*. There is a distinct possibility that the *Phomopsis* sp. and *A. mellea* can infect other leguminous hosts with even greater ecological consequences.

Numerous examples of invasion of natural ecosystems by non-indigenous pathogens, with negative impacts to ecosystem functions, exist worldwide (Anagnostakis 1987; Brasier 2008; Davison and Shearer 1989; Desprex-Loustau *et al*. 2007; Fraedrich *et al*. 2008; Gilbert and Miller 1952; Harrington *et al*. 2008; Loo 2009; Tribe 1995; Osborne 1985; Quimby 1982). In South Africa, previous studies have shown that *Armillaria mellea* is able to infect several native host plants (Coetzee *et al*. 2001, 2003; Wingfield *et al*. 2010), and our study has added yet another. It is now clear that *A. mellea* is able to successfully invade natural ecosystems in the CFR. Despite this, both previous studies and our study have only focused on the effects of this pathogen on hosts at the individual level. There is thus a lack of vital information on

the long-term ecological impacts of this pathogen including studies at the population and ecosystem level. It is recommended that future studies on *A. mellea* should consider its long-term ecological impacts such as on food webs, nutrient cycling, forest tree succession and fire regimes. This also applies to other possibly exotic pathogenic fungi we found on *Virgilia* trees in this study.

The mortality of *Virgilia* trees from George through to the Tsitsikamma area seems to be due to a cascade of natural events that involve interdependent organisms. We have shown that ghost moth larvae and those of an unidentified cerambycid beetles can be linked directly to tree death. They create wounds through which pathogenic fungi can enter. Nitidulid beetles may be the carriers of the pathogenic *Ceratocystis tsitsikammensis* and other ophiostomatoid fungi that subsequently invade these wounds. Future studies should focus on the ecology of these interactions and on determining the timing of events. Key future questions include: Can trees be killed by the fungus/larvae alone or will tree death only result after both have impacted on tree health? What are the benefits/negative impacts for the various organisms in these symbioses? Timing of tree death may also be important, as premature tree death would likely lead to the premature death of larvae.

Secondary scolytine beetles and their fungal and mite associates on native South African hosts have not attracted much research interest, as these do not have apparent economic implications. Our study is thus a first record of secondary scolytine beetles-mites-*Geosmithia* fungi interactions in South Africa. The classic paradigm (Six and Wingfield 2011) hypothesise the role of fungi in scolytine beetle-mite-fungi association as (1) virulent, directly leading to death of host trees by primarily blocking water conduction in the vascular tissue and (2) to stimulate and exhaust host tree defences, allowing the beetles to successfully invade their hosts (Six and Wingfield 2011). The non-pathogenic nature to *Virgilia* of the *Geosmithia* spp. associated with the scolytine beetles and their mites that were isolated in this study suggests different roles of the *Geosmithia* spp. from those hypothesised under the classic paradigm. One possibility is that the fungus is involved in beetle nutrition, but this requires future study. A few other fungal taxa were also isolated from scolytine beetles and their phoretic mites from *Virgilia* trees. These were ignored in this study, because they were not as consistently isolated as the *Geosmithia* spp. However, these other taxa may also have pivotal roles to play in these interactions. Mites also consistently carried *Geosmithia*

phoretically, but unexpectedly were unable to feed on these fungi. They probably either feed on other fungal taxa or on decaying organic matter and may thus play a "cleaning" role within beetle galleries. This should be an interesting field for future study, especially as it is becoming more obvious that *Geosmithia* spp. are common associates of bark beetles and their phoretic mites across the world (Jiri and Dunn 2011; Kolařík and Kirkendall 2010; Kolařík *et al*. 2007, 2008).

REFERENCES

Abdurahman A, 1992. Research need in forest entomology. In: *Proceedings of the National Workshop on Setting Forestry Research Priorities in Ethiopia*, April 27-30 1992. Forestry Research Centre, Addis Ababa, Ethiopia, pp. 113-121.

Anagnostakis SL, 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* **79**: 23-37.

Atkinson PR, Laborde, de RM, 1996. Estimates of the extent of infestation and potential losses caused by common forestry pests in South Africa. In: Nair KSS, Sharma JK, Varma RV (eds.), *Impact of Diseases and Insect Pests in Tropical Forests*. Proceedings of the IUFRO symposium, 23-26 November 1993, Peechi, India, pp. 274-288.

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

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

Chen SF, Wingfield MJ, Roets F, Roux J, 2012. A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea melanophloeos* in South Africa. *Plant Pathology* (In press).

Čizkova D, Šrutka P, Kolarik M, Kubatova A, Pazoutova S, 2005. Assessing the Pathogenic Effect of *Fusarium*, *Geosmithia* and *Ophiostoma* Fungi from Broad-Leaved Trees. *Folia Microbiol*. **50**(1): 59-62.

Coetzee MPA, Wingfield BD, on Harring TC, Steimel J, Coutinho TA, Wingfield MJ, 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* **10**: 387-396.

Coetzee MPA, Wingfield BD, Roux J, Crous PW, Denman S, Wingfield MJ, 2003. Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa. *Plant Pathology* **52**: 604-612.

Davison EM, Shearer BL, 1989. *Phytophthora* spp. in indigenous forests in Australia. *New Zealand Journal* of Forestry Science **19**: 277-289.

Desprez-Loustau ML, Robin C, Buée M, Courtecuisse R, Garbaye J, Suffert F, Sache I, Rizzo DM, 2007. The fungal dimension of biological invasions. *TRENDS in Ecology and Evolution* **22**(9): 472-480.

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR, 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. *Plant Diseases* **92**:215–224.

Geldenhuys CJ, 1994. Bergwind fires and the location pattern of forest patches in the southern Cape landscape, South Africa. *Journal of Biogeography* **21**: 49-62.

Gilbert GS, 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology* **40**: 13-43.

Gilbert JM, Miller LW, 1952. An outbreak of *Sirex noctilio* (F.) in Tasmanis. *Australian Forestry* **16**: 63-69.

Harrington TC, Fraedrich SW Aghayeva DN, 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* **104**: 399-404.

Jiri H, Dunn RR, 2011. The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. Proceedings of the Royal Society Biological Siences, p. 1130-1138.

Kolařík M, Kirkendall LR, 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). *Fungal Biology* **114**: 676-689.

Kolařík M, Kubátová A, Hulcr J, Pažoutová S, 2008. *Geosmithia* fungi are highly diverse and consistent bark beetle associates: evidence from their community structure in temperate Europe. *Microbial Ecology* **55**(1): 65-80.

Kolařík M, Kostovčík M, Pažoutová S, 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. *Mycological Research* **111**(11): 1298-1310.

Jones DR, Baker RHA, 2007. Introductions of non-native plant pathogens into Great Britain, 1970–2004. *Plant Pathology* **56**: 891-910.

Loo JA, 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* **11**: 81-96.

Mbabezeli G, Notten A, 2003. *Virgilia divaricata* Adamson and *Virgilia oroboides* (Berg.) Salter. South African National Biodiversity Institute, South Africa.

Osborne P, 1985. Some effects of the Dutch Elm Disease on the birds of a dorset dairy farm. *Journal of Applied Ecology* **22**: 681-691.

Phillips JFV, 1926. *Virgilia capensis* Lamk. ("Keurboom"): a contribution to its ecology and silviculture. *South African Journal of Science* **23**: 435-454.

Pimentel D, McNair S, Janecka J, Wightman J, Simmonds C, O'Connell C, Wong E, Russel L, Zern J, Aquino T, Tsomondo T, 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture, Ecosystems and Environment* **84**: 1-20.

Podger FD, 1972. *Phytophthora cinnamomi*, a cause of lethal disease in indigenous plant communities in West Australia. *Phytopathology* **62**: 972-981.

Quimby jr PC, 1982. Impact of diseases on plant populations. In: Charudattan R, Walker HL (eds.), *Biological Control of Weeds with Plant Pathogens*, John Wiley & Sons, New York, pp. 47-60.

Six DL, Wingfield MJ, 2011. The Role of Phytopathogenicity in Bark Beetle–Fungus Symbioses: A Challenge to the Classic Paradigm. *Annual Review Entomology* **56**: 255-72.

Skarpaas O, Økland B, 2009. Timber import and the risk of forest pest introductions. *Journal of Applied Ecology* **46**: 55-63.

Speight MR, Wylie FR, 2001. Insect Pests in Tropical Forestry. CABI Publishing, pp. 307.

Tatem AJ, Hay SI, Rogers DJ, 2006. Global traffic and disease vector dispersal. *PNAS* **103**(16): 6242-6247.

Tribe GD, 1995. The woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), a pest of Pinus species, now established in South Africa. *African Entomology* **3**: 215-217.

Van Daalen JC, 1981. The dynamics of the indigenous forest-fynbos ecotone in the southern Cape. *South African Forestry Journal* **119**: 14-23.

Von Broembsen SL, 1989. Invasions of Natural Ecosystems by Plant Pathogens. In: Drake JA, Mooney HA, di Castri F, Groves RH, Kruger FJ, Rejmanek M, Williamson M (eds.), *Biological Invasions: A Global Perspective* **37**: 77-83.