FUNGI ASSOCIATED WITH THE DIE-BACK OF *PTEROCARPUS ANGOLENSIS* (KIAAT) IN SOUTH AFRICA

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Fungi associated with the die-back of *Pterocarpus angolensis* (kiaat) in South Africa

by

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DECLARATION

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

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James William Montague Mehl

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This thesis is dedicated to the memory of my late earthly father, Jonathan Baden Mehl (16/5/1951 – 16/3/2008) and grandmother, Aletta Cornelia Greyling Senekal née Goosen (10/10/1924 – 24/3/2010), in acknowledgement and thanks to my parents, supervisors, colleagues, fellow students and friends who kept motivating and encouraging me, and to the glory of my heavenly Father.

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PREFACE

Pterocarpus angolensis (kiaat) is one of 20 species of trees in the pantropical genus *Pterocarpus*. The species occurs in Southern Africa and has a broad distribution over several African countries, including Tanzania, Democratic Republic of Congo, Angola, Zambia, Mozambique, Namibia, Botswana, Zimbabwe and South Africa. The tree is commercially used in several traditional medicinal applications and is well-known as a source of timber for woodcarving and furniture. A single serious disease known as mukwa has been reported on *P. angolensis*, and the cause has been attributed to the generalist fungus *Fusarium oxysporum*. Reports of this disease date back to the late 1950s and appear localized in the Livingstone distinct, between Livingstone in Zambia and Bulawayo in Zimbabwe. Recent studies indicate that mukwa disease has begun to subside. There are more recent reports, however, of *P. angolensis* trees dying in South Africa, in the Mpumalanga Province.

The aim of the research presented in this dissertation was to establish the nature of the disease affecting *P. angolensis* trees in the Mpumalanga Province in South Africa. Trees were sampled from several areas in the province, both from reported disease areas and from areas sustaining healthy trees. Field observations and fungal isolations provided a means to determine whether the disease was attributable to a biotic factor or the result of environmental factors affecting the tree. Isolations concentrated on obtaining species of the Botryosphaeriaceae and of the so-called ophiostomatoid fungi because these fungi are well-known pathogens of trees in the area, have been fairly well studied and the die-back symptoms suggested that they could be involved.

The first chapter of this dissertation presents a review of the literature pertaining to *P. angolensis*, with a focus on mukwa disease. Aspects of the review introduce the genus *Pterocarpus*, the ecology of the environment wherein *P. angolensis* occurs and cover the biology of the species. Commercial aspects of the tree as well as the need for conservation are also addressed, providing the foundation for further studies in this dissertation.

Many of the symptoms of diseased trees studied were indicative of infection by the Botryosphaeriaceae, a group of several genera of endophytic plant pathogens. These fungi were thus isolated from *P. angolensis* trees growing in different areas and were subsequently identified. Pathogenicity tests were used to evaluate the possible role that these species might play in the die-back of *P. angolensis*.

The value of *P. angolensis* trees in medicinal applications predisposes them to harvesting and physical damage by humans. A stem wounding trial on *P. angolensis* trees resulted in the collection of several isolates of *Ceratocystis*. These isolates were characterized based on morphology, phylogenetic analyses and biological ability to mate with other closely related *Ceratocystis* spp. Pathogenicity trials were also conducted to determine the role of these isolates in disease of *P. angolensis*. These results provide the focus of chapter three.

The final chapter of this dissertation reviews both mukwa disease and the disease reported in South Africa. It summarizes field observations and fungal isolations done from diseased *P. angolensis* trees in South Africa. It also considers possible contributory factors impacting on these trees.

Studies that make up this dissertation expand our understanding of the role of various fungi in disease of *P. angolensis*. Apart from contributing to our knowledge regarding the fungal biodiversity associated with this species, these results provide a base for future studies on diseases of *P. angolensis*. Knowledge of the fungi that potentially affect trees in South Africa and precipitate or contribute to their decline has implications for the management of this species. In addition, results obtained provide a foundation for comparison should similar studies be undertaken on *P. angolensis* trees in the Livingstone district that may or may not be affected by mukwa disease.

CHAPTER 1

PTEROCARPUS SPECIES WITH SPECIFIC REFERENCE TO *PTEROCARPUS ANGOLENSIS* (KIAAT) AND ITS HEALTH STATUS IN SOUTHERN AFRICA

1. INTRODUCTION

Approximately seven biomes occur in southern Africa. These include the forest, savanna, grassland, Nama Karoo, Succulent Karoo, desert and fynbos biomes. Of all the biomes, the most diversified and the most abundant (covering 46% of the total land area) is the savanna biome (Van Rooyen 1999, Van Wyk & Van Wyk 1997, White 1983). This biome is also home to most of Africa's population (Campbell *et al*. 1996).

Nine different types of savanna are mentioned in the treatise of Van Wyk and Van Wyk (1997). The most common are the miombo woodland (Campbell *et al*. 1996) and the undifferentiated woodland spanning South Africa, Namibia, Botswana, Zimbabwe, Zambia, and Angola (Van Wyk & Van Wyk 1997). Both vegetation types are found in the Zambezian regional centre of endemism, a region containing the most diverse flora in southern Africa with the broadest range of vegetation types (White 1983).

An estimated 8 500 higher plant species occur in the miombo woodland of which 54% (4 590 species) are endemic and 334 species are trees (Rogers *et al*. 1996). At least 32 plant families are found but the most frequently encountered is the super-family Leguminosae. The members of the Leguminosae make up 25% of all the genera and 22% of all the species (Tuite & Gardiner 1990a). One species that occurs in the miombo woodland across a variety of ecosystems that differ with respect to drought, fire and frost tolerance is *Pterocarpus angolensis* D. C. This is a well-known tree among the indigenous people of Southern Africa and is commonly known as kiaat, mukwa or umbila. *Pterocarpus angolensis* serves as a source of timber for the woodcarving and furniture industries of several African countries (Lowore 1993) and forms the focus of this review.

Pterocarpus angolensis is found in several vegetation types in the Zambezian regional centre of endemism according to the vegetation map of Africa by White (1983). These vegetation types are mainly located in the Zambezian woodland, although the Itigi thicket and related types of vegetation are located in the Zambezian thicket. Specifically these vegetation types are: Zambezian miombo woodland which includes both wetter and drier miombo, North Zambezian undifferentiated woodland and wooded grassland, South Zambezian undifferentiated woodland and scrub woodland, Zambezian “chipya” woodland and wooded grassland, Zambezian Kalahari woodland and Itigi thicket and related types of vegetation (White 1983).

This review provides a comprehensive summary of the large amount of available information that

deals with *P. angolensis*. The genus *Pterocarpus* (L.) Jacq. is introduced and its taxonomy and morphological characteristics discussed. Then the species *P. angolensis* is discussed. Aspects discussed include the taxonomy, morphological characteristics, biology, distribution and habitat. The commercial importance of *P. angolensis* for humans is discussed. Both the use of virtually all parts of the plant for medicinal use by the indigenous people as well as the use of the species as a source of timber is considered. The impact of abiotic factors such as mechanical damage, fire and rainfall on *P. angolensis* is discussed. The impact of biotic factors such as plants, animals, insects and fungi is discussed. Conservation of *P. angolensis* and its implications for the future survival of the species are considered. This review thus presents a foundation for the subsequent chapters of this thesis that consider what fungi are associated with *P. angolensis* as potential endophytic pathogens, stem wound pathogens and leaf spot-causing agents.

2. THE GENUS *PTEROCARPUS*: GENERIC CHARACTERS AND DISTRIBUTION

2. 1. Introduction

Pterocarpus angolensis is a member of the super-family Leguminosae Juss. that includes three families; the Mimosaceae R. Br. (thorn-tree family), the Caesalpiniaceae R. Br. (the *Cassia* L./*Bauhinia* L. family) and the Papilionaceae Giseke/ Fabaceae Lindl. (pea family). All three families produce a legume/pod as the fruit type and are easily distinguished from each other. The Mimosaceae produce small flowers that are grouped together in spikes or heads with petals that are either inconspicuous or absent. The Caesalpiniaceae produce large and showy flowers with, at most, ten stamens. The Fabaceae produce flowers that resemble the shape of a butterfly; the uppermost petals are the largest, the two side petals are small and stalked and the last two basal petals are joined to form a keel (Polhill 1994, Van Wyk & Van Wyk 1997).

Within the Fabaceae, *P. angolensis* falls into the tribe Dalbergieae D. C., subtribe Geoffroeinae Wright & Arn. (Bentham 1860, Lavin *et al*. 2001). The Dalbergieae includes tropical trees with fused floral parts and pods that are indehiscent (Bentham 1860, Lavin *et al*. 2001). The tribe was split into three sub-tribes by Bentham (1860) namely Pterocarpeae, Lonchocarpeae Benth. and Geoffroyeae D. C. but was later revised by Polhill (1971, 1981, 1994) to include 19 genera of woody tropical plants, mostly derived from the Pterocarpeae and Geoffroyeae. The Lonchocarpeae were grouped with other legumes that accumulated nonprotein amino acids in their seeds (Lavin *et al*. 2001). In 2001, Lavin *et al*. (2001) redefined the tribe Dalbergiae to include three subtribes; Adesmiinae Benth. & Hook, Geoffroeinae and Dalbergiinae Wright & Arn.

The majority of plants in the Fabaceae are herbs and shrubs, although a number of tall shrubs and trees also occur (Von Breitenbach 1973). In southern Africa, more trees belong to the Leguminosae than to any other family of plants (Vermeulen 1990). The pods or legumes of many of the herbaceous species serve as a source of nutrition for people and examples include the peas (*Pisum* L.), beans (*Phaseolus* L.) and peanuts (*Arachis hypogea* L.). Some of the trees are used as ornamentals in South African gardens and these include the coral trees (*Erythrina* L.), *Bolusanthus speciosus* (Bolus) Harms and *Millettia grandis* (E. Meyer) Skeels (Van Wyk & Van Wyk 1997).

The genus *Pterocarpus* (L.) Jacq. is pantropical and consists of 20 species that are located in South-Eastern Asia, tropical South America, and tropical Africa (Rojo 1972) (Fig. 1, Table 1), although a later, more generalist source (Dyer 1975) mentions 100 species. Most species are trees, but a few shrubs are also known. *Pterocarpus* species are generally found in savannahs and the open dry country (Rojo 1972) and favour a seasonal climate. At least half of the species in this genus possess deciduous foliage (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973). The rest are considered as either "semi-deciduous" or "mostly evergreen" (Rojo 1972). Even in dry areas, these evergreen species are leafless for a brief period of time (Rojo 1972).

2. 2. Generic relationships

Pterocarpus has, as its closest relatives, the tropical South American genera *Tipuana* Benth., *Platypodium* Vog. and *Centrolobium* Mart. *ex* Benth. (Bentham 1860, Rojo 1972, Von Breitenbach 1973). The relationship has recently been confirmed by phylogenetic analysis of members of the Dalbergiae based on the nuclear ribosomal 5.8S gene and the flanking internal transcribed spacer regions; ITS1 and ITS2, as well as the chloroplast *trnK* intron (including *matK*) and *trnL* intron (Lavin *et al*. 2001).

2. 3. Generic morphological characteristics

Several generic characters for *Pterocarpus* spp. are well-known. The type species is *P. officinalis* Jacq. The leaves are both alternate and imparipinnately compound (Coates Palgrave 1977, Rojo 1972, Sousa 1951, Van Wyk & Van Wyk 1997, Von Breitenbach 1973). Leaflets or pinnae are entire and alternate or are sub-opposite or, rarely, unifoliate (as in the case of an impoverished *P. lucens* Lepr.) (Rojo 1972, Sousa 1951, Van Wyk & Van Wyk 1997, Von Breitenbach 1973). The flowers are mostly yellow or yellow to orange and can constitute an inflorescence that is either a

panicle or raceme and can be either terminal or axillary (Rojo 1972, Sousa 1951, Van Wyk 1972, Von Breitenbach 1973). Not all generic characters are mentioned here as this falls outside the scope of this review. A complete list and discussion of all generic characters of *Pterocarpus* can be found in Rojo (1972). However, of all the generic characters present in *Pterocarpus*, fruit is the primary character used to distinguish species (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973).

The name *Pterocarpus* is derived from the Greek words "pteros" meaning "wing" and "karpos" meaning "fruit". Of the 20 *Pterocarpus* species, only three (*P. amazonum* (Benth.) Amshoff, *P. officinalis* and *P. santilinoides* D. C.) have typical pods, either narrowly winged or keeled over on one side (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973). The remaining 17 are dependent mainly on wind dispersal and, therefore, their fruits resemble winged nuts. Of these, only two (*P. lucens* and *P. rotundifolius* (Sonder) Druce) have elliptical pods surrounded by a narrower wing. The other 15 have round pods surrounded by a broad membraneous wing (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973).

As the fruit is a pod and not a nut, one side tends to grow faster than the other. The result is a falcate or spirally contorted shape. Examples of other legumes that possess such fruits include *Acacia karroo* Hayne and *A. tortilis* Hayne. The asymmetry in the round pods of *Pterocarpus* spp. is seen in the position of the beak or style (Table 2 and Fig. 3) which is used to distinguish between the different species. Additional morphological characteristics of the fruit used to distinguish species include whether the beak/style is protracted or not; the direction in which the beak is pointed; whether the margin between the beak and the stipe/stalk is concave, convex, or sinuous; the pod diameter; and whether the pod is merely thickened or has bristles (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973).

3. *PTEROCARPUS ANGOLENSIS* (KIAAT)

3. 1. Taxonomy

Pterocarpus angolensis (kiaat) was first described by A. P. De Candolle (D. C.) in 1825 in the second volume of his work "*Prodromus Systematis Naturalis Regni Vegetabilis*" (Prodrome to a Natural System of the Plant Kingdom) based on a type specimen gathered by an unknown collector in Angola. Five synonyms are known for the species, namely *P. adansonii* D. C. (Sousa 1951, Vermeulen 1990), *P. bussei* Harms based on a specimen from Tanzania, *P. dekindtianus* Harms based on a specimen from Angola (Vermeulen 1990, Von Breitenbach 1973), *P. echinatus* (Pers.)

Rojo and *P. erinaceus* Poir (Sousa 1951, Vermeulen 1990). Only one variety of *P. angolensis*, *P. dekindtianus* var. *latifoliolatus* De Wild., is mentioned in the literature, based on a single specimen collected from the Democratic Republic of Congo (DRC) in 1913 by De Wildeman (*P. dekindtianus*) (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973). The apparent lack of subdivision indicates that the species shows little, if any, botanical variation (Von Breitenbach 1973). The variety has, however, not been confirmed from collections elsewhere and thus has not been commonly accepted (Vermeulen 1990, Von Breitenbach 1973).

3. 2. Alternative/Common names

Common names for *P. angolensis* include muninga, mukwa, bloodwood (based on the colour of the sap that is exuded when a tree is wounded), lakboom and Transvaal/Wild Teak. Numerous other names exist depending on the area e.g. Dolfhout in Namibia, Umbila, etc. The names kiaat and teak refer to the properties of the wood that were considered equal to those of real Teak, *Tectona grandis* L., a member of the family Verbenaceae (Vermeulen 1990, Von Breitenbach 1973). The Malay name, "Dyati", was transformed to the Malay-Dutch "Kajate". In the Cape, the name was transformed into the Afrikaans "kiaat" and first applied to *Strychnos decussata* (Pappe) Gilg, or the so called Cape Teak (Chudnoff 1984, Comrie-Greig 1990, Farmer 1972, Piearce 1979, Von Breitenbach 1973).

3. 3. Morphology

The bark of *P. angolensis* is rough and fissured, being grey to brown in colour (Coates Palgrave 1977, Groome *et al*. 1957a, Orpen 1982, Rojo 1972, Van Wyk & Van Wyk 1997) and the lower part of the stem can be blackened due to fire damage and discoloured from earth tunnels made by termites (Groome *et al*. 1957a, Orpen 1982, Vermeulen 1990, Von Breitenbach 1973). The leaves taper to a bristle-tipped point and the flowers are orange-yellow in colour and constitute a panicle (Coates Palgrave 1977, Van Wyk & Van Wyk 1997, Vermeulen 1990, Von Breitenbach 1973) or an axillary raceme (Boaler 1966, Rojo 1972). The pods are encircled by a broad membranous wing extending all around that ends in a basal beak. The centre is covered by thick, dense bristles and contains one or two red-brown seeds (Rojo 1972, Vermeulen 1990, Von Breitenbach 1973).

3. 4. Biology

3. 4. 1. Seed germination

Boaler (1966), Bleys *et al*. (1982) and Munyanziza and Oldeman (1996) noted that the seeds of *P. angolensis* have a period of dormancy. Repeated wetting and drying during the later part of the first rainy season or in the second rainy season while resting on the soil can break this dormancy and induce the pod to open along one side. Epigeal/above ground germination then occurs inside the pod (Boaler 1966, Groome *et al*. 1957a, Van Daalen 1991, Vermeulen 1990, Von Breitenbach 1973).

3. 4. 2. Seedling development

The root system develops rapidly underground by rapid downward growth of the taproot and scant growth of side roots while root nodules that contain endomycorrhizae with confirmed nitrogenase activity appear within a few weeks (Boaler 1966, Groome 1955, Högberg 1986). Annual die-back (the so-called suffrutex stage) of the seedling occurs until the root system grows sufficiently large enough to collect enough water and nutrients for the production of a stem strong enough to withstand the harsh environmental conditions of the dry season (Lowore 1993, Vermeulen 1990, Von Breitenbach 1973). Usually the shoots produced in a rainy season die back 2.54 mm below the surface in the following dry season. This results in annual renewal of the shoots and continual growth of the root system. Until the seedling overcomes the suffrutex stage and becomes a sapling (a period averaging about seven to seven and a half years although it can last as long as 14 years), annual burning is not recommended (Boaler 1966, Groome 1955, Högberg 1986). In the nursery, annual die-back does not occur or, at least, has not been observed (Munyanziza & Oldeman 1995) as the seedlings remain sheltered from drought by persistent watering and from fires by man-made shelter. However, iron deficiency has been noted by Munyanziza, Kuyper and Oldeman (1998) when attempts to raise seedlings in a nursery were unsuccessful and die-back has been observed and documented by Mwitwa (2003).

Boaler (1966) noted that as many as 96% of all seedlings die before emerging from the suffrutex stage. The high mortality rate can be attributed to a combination of environmental factors including drought, frost, annual burning, nutrient deficiency (especially of boron), damage by animals, and competition (both intraspecific and interspecific) (Boaler 1966, Lowore 1993, Orpen 1982, Vermeulen 1990). Mwitwa (2003) investigated the physiological parameters involved in the growth of seedlings. He noted that the root concentration of all mineral nutrients (nitrogen, potassium, calcium, manganese, copper, zinc and boron) increased when shoot die-back occurred along with an increase in the leaf concentration of phosphorous, calcium, manganese, iron, copper, and boron. As

the concentration of nutrients in the stem and roots increased, leaves begin to yellow and fall off. He concluded that three types of die-back occur:

- Complete shoot die-back that only occurs in the field involves the shoots ageing from the apical bud to the base. This can give the appearance that the seedling is dead.
- Partial shoot die-back involves less than 50% of the top part of the shoot ageing. The remaining living portion of the shoot survives but only plays a supportive role in the growth of the following season's shoot. The new shoot must therefore suppress the growth of the previous season's shoot and compete with branches/secondary stems for dominance.
- Incomplete shoot die-back involves more than 50% of the top part of the shoot ageing and only a small portion of the shoot protrudes from the soil. This portion lacks the lateral branches that can compete with the new shoot for dominance.

3. 4. 3. Flowering and fruit production

Flowering of *P. angolensis* trees occurs in early summer, any time from August up to December depending on the area and environmental conditions (Boaler 1966, Coates Palgrave 1977, Vermeulen 1990, Von Breitenbach 1973). It can occur either before, concurrently (a phenomenon termed “precocious”) (Boaler 1966, Groome *et al*. 1957a, Palmer 1977, Van Wyk 1972, Von Breitenbach 1973), or after the production of leaves (Boaler 1966).

Pterocarpus angolensis begins producing fruit annually at the age of 20 years (about 15 years after the formation of a permanent stem/shoot) although fruiting is light until the age of 35 years (Boaler 1966, Groome *et al*. 1957a). Fruit can grow and develop over November and December but only ripens between April to June of the following year (Boaler 1966, Sousa 1951) persisting until October to enable quick recognition of the species in winter (Groome *et al*. 1957a, Storrs 1980, Vermeulen 1990). In dry areas, fruit is dry and brown by April but remains green in areas where the rainy season stretches over a longer period (Groome *et al*. 1957a, Orpen 1982, Vermeulen 1990).

Fruit of *P. angolensis* remains closed during a fire, unlike other woodland species, and the wings and bristles of the pods are burnt off. The seeds in the pods survive the fire undamaged and resist the effects of termites (Von Breitenbach 1973) although not every pod contains seeds. As Boaler (1966) noted, about 50% of fruit contains seed but the remainder is devoid of any seeds. Bleys *et al*. (1982), in contrast, put the figure at below 50%.

3. 5. Age

Pterocarpus angolensis is known to live as long as 100-120 years (Boaler 1966) however, based on a diameter-time curve, trees could live as old as 200 years (Calvert 1986). Both radiometry, employing the use of carbon-14 dating (Van Daalen *et al*. 1992), and dendrochronology (Stahle *et al*. 1999) have been used to date *P. angolensis*. Dendrochronology is simpler and cheaper than radiometry and has been used to indicate drought, fire damage, insect attack and fungal infection. The oldest tree discovered by Stahle *et al*. (1999) was 205 years old from a logging concession in western Zimbabwe (Stahle *et al*. 1999). Later evidence also points to a correlation between the formation of the annual rings and the climatic conditions of temperature and relative humidity (Fichtler *et al*. 2004). As *P. angolensis* approaches death, progressive die-back of both the branches and twigs (crown-shrinking) is observed along with a shrinking root-disc due to rapid development of root rot. Eventually the trees lean over and fall down to be consumed by fire (Von Breitenbach 1973).

3. 6. Distribution

The distribution of *P. angolensis* has been detailed by Rojo (1972) and Von Breitenbach (1973). According to Rojo (1972), the species is widespread south of Lake Victoria; to the east to Tanga and southwesterly to Cuanza Norte in Angola. The southernmost limit in Angola is Huila. From there, it stretches south, slightly easterly, to Grootfontein in Namibia (formerly South West Africa), and easterly to Sebungwe, found in the Zambezi River Basin south of the Kariba Dam (Hoare & Du Toit 1999), in Zimbabwe (Rojo 1972). Southerly the species extends between 31-32ºE to 27ºS up to Northern Kwazulu-Natal (Rojo 1972). A map of the distribution has also been produced by Boaler (1967, Fig. 2) but it lacks clearly defined geographic boundaries as to just where the species occurs.

3. 7. Habitat – the miombo woodland

Pterocarpus angolensis frequently occurs in miombo woodland dominated by species of *Brachystegia* Benth. (commonly known as mfuti or mountain acacia trees) or deciduous *Burkea africana* Hook (wild syringa) and *Baikiaea plurijuga* Harms (Rhodesian teak) (Coates Palgrave 1977, Farmer 1972, Munyanziza & Oldeman 1996, Rojo 1972, Von Breitenbach 1973). Savannas are the most widespread vegetation type in tropical Africa and miombo woodland is a form of the savanna that refers to woodlands occurring in south, central, and eastern Africa that are dominated by species of the genera *Brachystegia*, *Julbernardia* Pellegr., and/or *Isoberlinia* Craib & Stapf

(Campbell *et al*. 1996, White 1983). Miombo woodland is home to relatively small populations of humans when compared to other savannas, probably because of the lack of nutrients in the soil. There is also a lack of herbivores, except for elephants and buffalo that have been observed by Bell (1982). The lack of herbivores can be extended to include cattle. Hence predators, including humans, are not easily sustained in these areas (Bell 1982, Campbell *et al*. 1996). However, human population densities are set to increase primarily because humans are responsible for the removal of the cover (trees) present in these woodlands for both agricultural use and for use as firewood (Campbell *et al*. 1996, Chidumayo 1989). Despite this, *P. angolensis* dominates at a height of 15-20 metres and is often a remnant in areas cleared for cultivation. It also occurs in areas with cleared forests, in savannahs and on sandy soils (Comrie-Greig 1990, Farmer 1972, Munyanziza & Oldeman 1996, Rojo 1972, Von Breitenbach 1973).

The soils on which miombo woodland occurs are poor in organic matter, nitrogen and phosphorous (Högberg 1992). Trees that occur in miombo woodlands, therefore, have either vesicular-arbuscular (VA) mycorrhizae or root nodules to allow survival on the nutrient poor soils (Högberg 1992). The presence of root nodules in *P. angolensis* has been noted by Boaler (1966) and Corby (1974) but later proof of the presence of VA mycorrhizae along with nodulation in *P. angolensis* was provided by Högberg and Piearce (1986) and confirmed by Munyanziza and Oldeman (1995, 1996). Thus, *P. angolensis* is neither limited by a low supply of phosphorous inherent to tree species that form root nodules, which VA mycorrhizae accumulate, nor is it limited by a low supply of nitrogen inherent to tree species that have VA mycorrhizae (Högberg 1992).

The rainfall in areas where miombo woodland occurs is often low and the temperature and altitude can vary. *Pterocarpus angolensis* is known to survive in climatic conditions similar to the Dry Sub-humid Regions (Von Breitenbach 1973); an annual rainfall of as low as 500 mm (Groome *et al*.1957a, Van Wyk 1972) and as high as 1 250 mm (Vermeulen 1990, Von Breitenbach 1973), an average temperature of as low as 4ºC and as high as 20ºC and an altitude ranging from sea-level to 1 650 m above sea-level (Von Breitenbach 1973).

4. COMMERCIAL IMPORTANCE

The importance of *P. angolensis* is emphasized by the number of uses humans have found for it. Indigenous people have found medicinal use from all parts of the tree and their use of the species for timber is also important.

4. 1. Medicinal uses

The sticky, red sap is used as a dye (Coates Palgrave 1977, Van Wyk 1972) and can be used to treat nosebleeds (Coates Palgrave 1977). Although the sap can be used to treat cataracts, it does not possess any antimicrobial activity. Lactic acid has, however, been recovered from it along with several unknown compounds with a notably high degree of carbohydrates. It has been suggested that all the compounds collectively provide the antimicrobial activity of the sap (Van der Riet *et al*. 1998). Cunningham (1996) notes that the sap "dries like a scab" hence its use in treating menstrual disorders and in the treatment of wounds resulting from circumcision rituals. The bark can be heated and mixed with figs to produce an ointment that is applied to the breasts to induce lactation. A cold infusion containing the bark can be used to treat nettle rash, stomach upset, mouth ulcers and headaches (Coates Palgrave 1977). Either the bark or the root, in combination with boiled fresh meat, can be used to accelerate the preliminary treatment of gonnorhea. A deconcoction of the root is believed to cure malaria and blackwater fever. Corneal ulcers can be treated with the essence of a solution wherein the roots have soaked or the flowers are boiling (Coates Palgrave 1977).

4. 2. Use as a timber

The inner heartwood colour varies from light or medium brown through to a reddish-brown, even extending to a coppery-brown (Alfaro Cardosa 1962, Cox 1939, Farmer 1972, Francis Kukachka 1970, Hartwig 1964). Bands or streaks of a darker purple or gold have also been reported running through the heartwood (Cox 1939, Farmer 1972, Francis Kukachka 1970, Hartwig 1964, Von Breitenbach 1973). Small white spots can occassionally occur due to the deposition of crystalline deposits (Farmer 1972, Kromhout 1969, Vermeulen 1990) of calcium that do not impair the technical properties of the wood. The deposits are inorganic and thus do not "spread" through the wood (Kromhout 1969, Anon 1969). Some authors have noted that the fibres running through the wood can be irregular or straight (Francis Kukachka 1970, Hartwig 1964) but generally they are irregular to the eye and are interlocked to create the effect of curling (Alfaro Cardosa 1962, Chudnoff 1984, Farmer 1972, Hartwig 1964, Sousa 1951). These combined features add to the attractiveness of the timber of *P. angolensis* and increase its value as a commodity for woodcarvers.

The heartwood is extremely durable (Cox 1939, Farmer 1972, Von Breitenbach 1973) and resistant to termites (Duff 1944, Farmer 1972, Hartwig 1964), most beetles (Boaler 1966, Chudnoff 1984), and ants (Vermeulen 1990). It is also resistant to preservative treatments (Chudnoff 1984) although extraction with organic solvents can result in susceptibility of the heartwood to degradation by the

brown rot fungi *Coniophora cerebella* Alb. & Schwein., *Lenzites striata* (Sw.) Fr., and *Polysticus sanguineus* D. A. Reid (Anon 1966). The ability of the heartwood to withstand water, termites and wood borers enables its use for canoe construction (Chudnoff 1984, Comrie-Greig 1990, Cox 1939, Farmer 1972, Lowore 1993). Resistance to fungal decay can be attributed to the presence of pterostilbene, a compound extracted from an unknown species of the genus (Karrer 1958, Scheffer & Cowling 1966). In direct contrast, the outer sapwood is quite susceptible to attack from beetles, especially of powder-post and borers (Chudnoff 1984, Cox 1939, Vermeulen 1990) and is also damaged by bees (Vermeulen 1990). In colour, it is white (Van Wyk 1972, Von Breitenbach 1973), pale (Farmer 1972, Groome *et al*. 1957b), pale yellow or oatmeal coloured (Anon 1961, Chudnoff 1984, Vermeulen 1990).

The timber as a whole dries very well although it does so rather slowly and there is little, if any, tendency for it to split or distort (Chudnoff 1984, Cox 1939, Farmer 1972, Francis Kukachka 1970, Van Wyk 1972). It is easily worked and can be made into high quality furniture and attractive ornaments. It is also used in joinery and takes beautifully to polish (Chudnoff 1984, Comrie-Greig 1990, Cox 1939, Hartwig 1964, Lowore 1993). To determine whether a tree is suitable for harvesting for timber, the thickness of the heartwood and sapwood has to be determined. In Angola (Krynauw 1998) and Bushbuckridge (Shackleton 2002) this is achieved by axing through the bark. High demand for the attractive and durable timber has ensured that *P. angolensis* is under pressure and, amongst other trees of the miombo woodland, requires management for purposes of conservation (Munyanziza & Oldeman 1996).

5. ABIOTIC FACTORS INFLUENCING GROWTH

Abiotic factors are environmental agents that have a negative effect on the growth of a plant. This negative effect includes injury to the plant tissues leading to a breakdown in function and resulting in impaired plant growth and development. Environmental agents that can affect a plant include the climate, soil type, water availability, pollutants (in the air, soil and water), pesticides and utensils used by humans to either cultivate or obtain parts of the plant for some use (Bos & Parlevliet 1995). The most prominent abiotic factors that have an effect on *P. angolensis* are mechanical damage caused by humans and environmental fire and rainfall.

5. 1. Mechanical damage

The majority of mechanical damage inflicted on *P. angolensis* has largely been due to attempts to

cultivate the species and therefore most of the damage reported pertains to the seeds and seedlings. To extract the seeds from the pods, the latter were "clubbed" but the result was often seed that was either badly damaged or possibly suffering from slight cracks in the testa or seedcoat. Therefore the seedcoat became mouldy and inhibited successful seed germination (Bleys *et al*. 1982, Boaler 1966, Munyanziza & Oldeman 1996). In seedlings, damage or pruning of the taproot induced formation of secondary taproots and reduced the overall growth confining the root system to the upper soil surface where the roots were exposed to bush fires and drought (Munyanziza & Oldeman 1995, 1996).

5. 2. Fire and rainfall

Fire and rain are essential components of savanna ecology and both have an impact on *P. angolensis*. Fire decreases the level of nitrogen available to the vegetation present which favours the recruitment of legumes that develop root nodules (Edroma 1984, Frost & Robertson 1987). In addition, root nodule formation is induced by the release of moist rather than dry heat (Cushwa *et al*. 1968, Edroma 1984) indicating a rapidly developing fire that burns itself out. One would then expect that leguminous trees such as *P. angolensis* would be adapted to survive short-lived fires. Unlike many other trees that occur in miombo woodland, *P. angolensis* is tolerant to the effects of fire and the interaction is rather complex.

5. 2. 1. Ecology

Removing competing plant species, specifically trees, either by burning or clearing for cultivation promotes the growth and development of *P. angolensis* (Boaler 1966, Cooling 1959, Von Breitenbach 1973). Growth is inhibited by the presence of roots or crowns of other trees or a lack of genetic fitness. This implies that a tree is incapable of taking advantage of both its site of establishment and the surrounding environment. The result is a slower growth, increased susceptibility to disease and earlier death of trees (Von Breitenbach 1973).

Fire can be used to avoid an accumulation of undergrowth/thicket thus stimulating the regeneration of *P. angolensis* (Harrington & Ross 1974). Factors such as overgrazing by domestic livestock, drought and low atmospheric temperatures have an effect on the intensity of the fire and complicate its effect on the vegetation (Harrington & Ross 1974). The earliest account of the effect of fire on miombo woodland was by Trapnell (1959). In the area he studied, annual rainfall that enabled regeneration of tree species commenced from mid-November and lasted until the end of March or

the beginning of April. He noted that when the woodland was burnt later in the year (in October just before annual rainfall), there was a strong chance that it would be destroyed as regeneration of the dominant canopy tree species was affected and proliferation of a dominant grassy layer was allowed. Early burning (in May to June or June to July, just after the rainy season) allowed for maintained regeneration of the tree species present. In the control plot that enjoyed complete protection from fire, a dense thicket developed that probably consisted of woody species sensitive to the effect of fire (Geldenhuys 1977a).

A later account by Kasumba (1986) deals with the same situation in the Zambezi Teak Forests/Kalahari Sands. In that area, annual rainfall commenced in September and lasted through to the end of April (Huckabay 1986). Kasumba (1986) noted that in plots where the woodland was lightly burnt early in March or April, there were more individuals of the principal tree species (*Baikiaea plurijuga* Harms). He suggested that the practice be continued thus eliminating the need for the prevailing law that banned the presence of any fires in the area. This was also a law that caused great difficulty in extinguishing any fire that was started by accident or by unknown parties.

Geldenhuys (1977a) compared the effects of early, late, and no burning in two different areas (Rundu and Makambu in the Kavango in Namibia). In the area, annual rainfall commenced in November and lasted through to March. He noted that the availability of nutrients and the drainage ability of the soil had a dramatic effect on the ability of the soil to support the overlying vegetation, including the thicket that developed in the control area. He also noted that the regeneration of *P. angolensis* is significantly better when late burning of the woodland vegetation is undertaken in October or early November, just before the annual rainfall. The observation justifies Van Daalen's (1991) conclusion that seed germination is improved by burning to remove the grassy layer followed by rainfall (as discussed in 3. 4. 1.).

A similar study was later done by Geldenhuys (1977b) on the nearby Nakabunze Reserve in Eastern Caprivi in Namibia. He recommended that fires should be applied biannually in the early dry season just before the annual rainfall in areas where *P. angolensis* occurred. This required burning a limited area where the fire can be restricted until it extinguished itself so that it cannot affect the regeneration of any tree species present. The alternative of burning early just after the rainy season would most likely be a better option if the period of annual rainfall in the area was known. Patches of grass that occur would only be slightly burnt but very little fire control of the area would then be required.

5. 2. 2. Effect on seeds

Early beliefs held that seeds of *P. angolensis* that were slightly burnt were unable to germinate. Therefore only seeds that were not damaged by fire, had not suffered damage to the testa/seedcoat and originated from mature trees of 35 years were capable of germination (Bleys *et al*. 1982, Boaler 1966, Munyanziza & Oldeman 1996). However, later research by Van Daalen (1991) clarified the relationship between seed germination, fire and rainfall. His results indicated that seed collected directly from the pod and not from the ground had a higher degree of germination. This indicates that seed viability is very likely reduced when the seed is released from the pod and remains on the ground. Mould that developed on seed mechanically extracted seemed to have very little effect on the ability of the seed to germinate. Seeds were able to withstand "cold fires" of 450ºC lasting 30s but not "hot fires" of 450ºC lasting 90s (Van Daalen 1991). This was later confirmed by Munyanziza and Oldeman (1996) who mechanically scarified seeds with a hot wire to determine viability and thereby increased seed germination to over 90%. Thus, Van Daalen (1991) concluded that seed fall must coincide with a fire, that would remove the grassy layer allowing seeds to come into contact with the soil, and rain, that would allow the establishment of a seedling (Van Daalen 1991).

5. 2. 3. Effect on developing seedlings and saplings

Pterocarpus angolensis is known to have a shallow root system (Geldenhuys 1977a) and it has been proposed that this feature also provides protection for the developing seedlings and saplings from fires (Tuite & Gardiner 1990b). After a fire, the saplings can sometimes be seen in recently abandoned clearings where the annual fires, although short-lived, are much fiercer than normal due to the presence of dense grass and scrub growth (Von Breitenbach 1973). The saplings enjoy the resultant abundant light and space and respond by rapid growth in trunk diameter and crown size (Von Breitenbach 1973). Fires also prune side branches and multiple stems on the saplings thereby adding nutrients to the soil and contributing to the characteristic crown shape of trees (Boaler 1966). A lack of fire is signalled by several factors as listed by Von Breitenbach (1973) including the retention of several stems, reduced shoot length, die-back of a greater portion of the shoots and the production of "birds nests" or whorls of twigs at the end of shoots.

5. 2. 4. A model of the effects of fire on P. angolensis

Graz (1996) developed a model to investigate the effects of rainfall, fire, competition with other

plant species, and grazing by herbivores on *P. angolensis* trees:

- When woodland production or canopy closure is postponed, seed production increases i.e. a lack of competition from neighbouring tree species encourages development of *P. angolensis* trees.
- As the frequency of burning increases, there is a decrease in the intensity of fires that occur. As fire intensity decreases, the number of seeds that germinate increases. This is concurrent with what Van Daalen (1991) stated; germination only occurs in the presence of cold fires and not hot fires. In a woodland frame, the number of seeds produced decreases and thus the number of seeds that germinate also decreases.
- Suffrutex survival increases with fire frequency when the probability of the fire occurring is less than 50% (i.e. the result is a cold fire because there is little fuel load for the fire to sustain itself). Since fire decreases competition from neighbouring plants and, provided the suffrutex survives the fire, the suffrutex can take advantage of the positive situation. This justifies Von Breitenbach's (1973) observation as noted in growth and development under the influence of fire.
- The degree of sensitivity to the effects of fire is unknown although trees are known to be resistant. If trees possess very low sensitivity to fire, then they survive both hot and cold fires. However, if the fire is very intense then trees will die sooner (e.g. an intensity of above six induces mortality within five years). If trees possess very high sensitivity to fire, then survival begins to decrease when there is a 20% probability of a fire occurring (e.g. an intensity of above three induces mortality within three years). This is because fire promotes regeneration and the growth of competing plants by recycling nutrients that are stored in older dead or dying plant material.
- The degree of sensitivity to competition from neighbouring plant species is also unknown. Regardless of the degree of sensitivity and canopy closure, a 20% mortality in a population of *P. angolensis* trees will occur within a period of five years.

6. BIOTIC FACTORS THAT IMPACT ON *P. ANGOLENSIS* WITH AN EMPHASIS ON MUKWA DISEASE

Several biotic agents affect *P. angolensis* negatively. These include the presence of other plants competing for the same resources (as mentioned in 5. 2. 1.) as well as a mistletoe infection and various animals, insects and fungi that obtain nutrition from trees in some form or another. One fungus in particular, *Fusarium oxysporum* Schltdl., has received much attention and has been linked to mukwa disease of *P. angolensis*. It provides the core focus of this review.

6. 1. Plants

Only one plant species, a mistletoe in the genus *Loranthus* L., has occassionally been seen in the crown of *P. angolensis* (Boaler 1966, Orpen 1982, Vermeulen 1990). It has also been observed on trees dead or dying from mukwa die-back and incites galls and cankers where attached (Geary 1972). In a review of the Loranthaceae, Wiens and Tölken (1979) subdivided the genus further so that only one species, *L. europaeus* Jacq. is recognized. Based on their monograph and the phytogeography of mistletoes in Africa (Polhill & Wiens 1998), only one genus, *Erianthemum* Tieghem, has a distribution and a wide host range corresponding to reports of the mistletoe on *P. angolensis*. Of the 16 species discussed by Polhill and Wiens (1998), only three species, namely *E. dregei* (Eckl. & Zeyh.) Tieghem, *E. ngamicum* (Sprague) Danser and *E. virescens* (N. E. Br.) Wiens & Polh. have been reported from the same area as the *Loranthus* that was recorded on *P. angolensis*.

According to Browne (1968), species of *Loranthus* are widely distributed in the eastern hemisphere, particularly in the tropical regions. They occur in the crowns of dicotyledonous trees and rely on the host plant for water and nutrients from the soil. A heavy infestation severely weakens the host plant and can result in permanent stunting or death. Interestingly *Loranthus scurrula* L. (also known as *L. gracilifolius* Roxb. *ex* Schult. f. and now known as *Scurrula parasitica* L.) has been recorded on another species on *Pterocarpus* in India and Pakistan, *P. marsupium* (Browne 1968, Davidson 1945).

6. 2. Animals

Various mammals feed on different parts of *P. angolensis*. Monkeys, baboons, and the yellow-footed squirrel have been reported eating the pods (Palmer & Pitman 1972, Van Wyk 1972, Van Wyk & Van Wyk 1997, Vermeulen 1990, Von Breitenbach 1973) while rats are known to eat the seeds (Groome *et al*. 1957b). Warthogs feed on the succulent seedlings and fleshy taproots (Von Breitenbach 1973), while spring hares (*Pedetes capensis* Forster) can also damage the seedlings (Groome *et al*. 1957b). Porcupines make extensive use of the bark in times of drought and can severely ring bark trees, although trees manage to recover in the following rainy season (Krynauw 1998). Kudu and other large mammals browse the leaves (Van Wyk 1972, Von Breitenbach 1973).

Elephants play a huge role and, of all larger mammals, probably cause the most damage to *P. angolensis*. Above all others, they seem to prefer pushing over mature trees (Browne 1968, Van Wyk 1972). Damage done seems to occur in the drier months of the year (Schoeman 1982,

Vermeulen 1990) and this can be explained by their preference for woody plants on dry sandy soils as opposed to usually eating herbaceous plants on clayey soils (Trollope *et al*. 1998) probably to eat the unburnt leaves of the trees (Jacobs 2001). Elephants are also known to selectively debark trees. Although the damage done may be minimal, it often has a negative effect on the growth and form of the tree and can increase a tree's susceptibility to fire damage (Geldenhuys 1977b). Seedlings can be damaged by having the swollen part of their roots dug out, younger trees may be broken or pushed over and more mature and bigger trees are usually debarked (Geldenhuys 1977b). Von Breitenbach (1973) noted, however, that trees apparently damaged by elephants had, in fact, been blown over by wind, damaged by fire, or damaged by tribesmen stripping the bark or tapping for resin.

6. 3. Insects and fungi

Boaler (1966) devised a table that listed beetles found feeding on various parts of *P. angolensis* that is updated for the purposes of this review (Table 3). A complete list of fungi found associated with *P. angolensis* was compiled by Vermeulen (1990) and is also updated in this review (Table 4). The majority of the fungi isolated from *P. angolensis* are not known to be pathogens but are instead saprophytes, generalist rot fungi, brown rot fungi or white rot fungi (Vermeulen 1990). One fungus in particular, has received much attention due to its association with mukwa disease on *P. angolensis* and the remainder of this section is devoted to it.

6. 3. 1. History of mukwa disease

As early as 1957, Groome *et al*. (1957b) noted that *P. angolensis* trees were fairly free of attack from fungi. However, since then there have been several reports of *P. angolensis* trees dying from a disease where the principal symptom is blight or die-back (mukwa disease). Mukwa disease was first noticed in Zambia in 1958 or 1959 just west of Livingstone and was subsequently found in the Kalahari Sands or Zambezi Teak Forests in 1964 (Anon 1973). In March 1966, it was reported in Bambezi Forest Reserve, about 100km north-west of Bulawayo. Calvert (Calvert 1972, Piearce 1979) produced evidence that there had been earlier outbreaks of the disease in Botswana in the early 1930s, in Zambia in the late 1940s and early 1950s, and in Zimbabwe in the early 1930s, late 1940s and early 1950s (Fig. 4). All the earlier outbreaks occurred in the same area as subsequent reports of outbreaks i.e. close to the Victoria Falls and Livingstone (the Livingstone area). By July 1964, mukwa disease had only been reported close to Victoria Falls village in Zimbabwe, but spread widely thereafter (Geary 1972, Anon 1973). Attempts made to establish monocultures of trees in trial plots and plantations in its native areas resulted in catastrophic outbreaks of the disease (Gibson

& Jones 1977, Piearce 1979). Currently the disease can, therefore, only be considered as occurring on the border between Botswana, Zimbabwe and Zambia (Fig. 5) and as a form of stand-level die-back in the region (Van Wyk *et al*. 1993).

6. 3. 2. Causal agent

Several authors (Angus 1964, Piearce 1979, 1986) related the findings of W. R. Bainbridge (Bainbridge & Edmonds 1964, Piearce 1979) in his study as "Quick decline: cause undetermined". Angus however mentioned that his primary suspect as the responsible pathogen was *Armillaria mellea* (Vahl) P. Kumm. Piearce (1979) indicated that only *Fusarium oxysporum* has been consistently isolated from discoloured wood chips and his later reports (Piearce 1983, 1986) substantiate the association.

6. 3. 3. Symptoms

Symptoms described by the authors (Calvert 1972, Piearce 1979, 1986) include a generally unhealthy appearance of the crown foliage, wilt and chlorosis resulting in stag heads. Defoliation, desiccation, the common occurrence of epicormic buds on the healthy/undiscoloured side of trees with new leaves developing are common. The bark discolours from brown to grey and can be peeled off in large flaps, and the xylem and sap are stained. Xylem/sapwood streaks are a dull blue-grey or orange-red discolouroration that appear as a complete flecked ring or as discontinuous arcs and, in microtome sections, contain amorphous deposits, occlusions, and fungal hyphae. All the symptoms can be observed at the height of the rainy season in January and February. Bark lesions (either cracking or flaking) can also be found on some diseased trees. All trees in a patch are affected and die after two to three years or sooner. Vascular streaking appears to originate from several points in the root system or from a single root that is rotten. Van Wyk *et al*. (1993) noted that phloem streaking was often emphasized in affected trees and this characteristic was mentioned by Calvert (Calvert 1972, Piearce 1979, 1986) as being a distinguishing factor for affected trees.

6. 3. 4. Regeneration as an explanation

R. M. Moyo, the then District Manager for the Forestry Commission in Zimbabwe (Judge 1986) noted that the trees in Zimbabwe were killed faster by mukwa disease than they could be harvested by foresters. In contrast, Calvert (1986) argued that *P. angolensis* is not dying out, merely regenerating. This was supported by his observation (Calvert 1986) that trees followed a cyclic

growth pattern in response to the disease. In Zambia during 1983, Piearce (1983, 1986) noted that mukwa disease was finally beginning to wane. He based this view on the observation that the rate of new infections had decreased and recovery of some diseased trees had been observed. Most recently, Mushove (1996) confirmed that *P. angolensis* trees in the area were regenerating and concluded that mukwa disease must be episodic.

Van Wyk *et al*. (1993) also noted the apparent increase in saplings as mukwa disease progressed, indicating species regeneration. They postulated a similarity between stand-level die-back of the *P. angolensis* trees that they were considering in the Kalahari Sands or Zambezi Teak Forests and stand-level die-back of the 'ōhia'a (*Metrosideros polymorpha* Gaud.) (Myrtacae) trees in Hawaii. Both trees are pioneers on new substrates; *M. polymorpha* occurs on new lava flows (Mueller-Dombois 1987) while *P. angolensis* is able to survive fires (as discussed under 5. 2. Fire and rainfall). Two pathogens acting as secondary agents (the primary agents were considered abiotic stresses and disease factors) were suspected in *M. polymorpha* die-back. These are a root pathogen, *Phytophthora cinnamomi* Drech., and a cerambycid wood borer, *Plagithmysus bilineatus* Sharp (Mueller-Dombois 1986, Papp *et al*. 1979). Another root pathogen, *F. oxysporum* (Calvert 1972, Geary 1972, Piearce 1979, Anon 1973, Vermeulen 1990), has been implicated in mukwa disease on *P. angolensis* but, as yet, no beetles of the family Cerambycidae have been described on trees (Table 3). No major climatic events have been implicated in *M. polymorpha* die-back. Isolated events of major shifts in climate can have contributed in some cases but most trees in an *M. polymorpha* stand had already died (Mueller-Dombois 1986). In dramatic contrast, several authors have implicated drought as a causative factor in *P. angolensis* die-back. Nutrient availability has been considered as a factor but ultimately rejected for *M. polymorpha* die-back (Vitousek *et al*. 1983) and can also be rejected for *P. angolensis* as the species occurs in miombo woodland where soil nutrient availability is generally poor (Bell 1982, Campbell *et al*. 1996).

Jacobi *et al*. (1983) suggested that *M. polymorpha* die-back observed is a natural phenomenon that recurs and is related to primary succession of *M. polymorpha*. Establishment of seedlings and sapling maturation were apparently strongly related to the degree of opening in the forest canopy. This ties in with the cohort senscence theory which details the following. After a catastrophe or a decrease in the forest canopy, many large cohorts/groups of individuals of the same (woody) plant species are established non-uniformly (a patchy distribution) on the disturbed area. Eventually these cohorts begin to senscence/age from the effect of one or more environmental stresses that can be either abiotic or biotic (similarly cohorts occurring in areas exposed to the same environmental stress/stresses can begin to senscence at the same time). At this stage a fluctuating/variable

environmental site factor begins to contribute to stand-level die-back as its intensity increases over time. Due to the stress imposed on trees, they become susceptible to attack by other biotic agents that begin to cause rapid decline or die-back or, if the stands are able to temporarily recover following the environmental site factor, can cause a lingering decline or die-back (Mueller-Dombois 1983, 1986).

Other plant species in stands of *M. polymorpha* were also found either dying or dead. These included tree ferns (*Cibotium chamissoi* Klf.) in one area and canopy *Acacia koa* A. Gray trees in other areas. Similarly, in other stands of *M. polymorpha*, both it and *A. koa* were observed as vigorous (Mueller-Dombois 1986). In *P. angolensis* stands, other trees, namely *Burkea africana*, *Erythrophleum africanum* (Benth.) Harms, *Lannea stuhlmanni* (Engl.) Eyles, *Strychnos cocculoides* Baker and *Terminalia sericea* Burch *ex* D. C. (the most severely affected), are also seemingly affected (Anon 1973, Piearce 1979). These, like *P. angolensis*, are all shallow rooted species. *Baikiaea plurijuga* (Rhodesian teak) is a deep rooting species and does not appear to be affected (Anon 1973, Calvert 1972, Geary 1972, Piearce 1979, Vermeulen 1990).

6. 3. 5. Drought as a causative factor

The primary environmental factor associated with mukwa disease appears to be drought (Calvert 1972, Geary 1972, Anon 1973, Piearce 1979, 1986, Vermeulen 1990). This was especially true in Matabeleland in Zimbabwe that, in 1973, experienced 12 consecutive seasons of poor rainfall (Anon 1973). The rainfall data for the area from the two closest weather stations (Bulawayo in Zimbabwe and Livingstone in Zambia) based on the World Weather Records (vol. 5: Africa) for 1951 to 1980 (Anon 1967, 1979, 1987) is included in Table 5.

A correlation between annual rainfall in the area and mukwa disease seems evident. From 1958 to 1959, there was a drop at Livingstone from 1 410 mm to 620 mm. In general, rainfall hovered around this level at both weather stations until 1965 (apart from a total of 1 078 mm in 1962 at Livingstone). From 1966, the rainfall improved for two years at Livingstone but only began to improve from 1972. Calvert reported new outbreaks (Calvert 1972, Piearce 1979) in Zimbabwe in 1958/9, 1964, 1966, 1967 and 1968 and this is supported by the rainfall data (Table 5) from Bulawayo. From 1973 to 1977, the disease was studied at Livingstone by Piearce (1979) and this is also supported by the rainfall data including an exception of 1 280 mm in 1974 (Table 5).

6. 3. 6. Other factors

Van Wyk *et al*. (1993) concluded that a range of factors (Fig. 6) contribute to the development of mukwa disease. Amongst these, the presence of the mistletoe *Loranthus* sp., can exacerbate drought stress as mistletoes derive their water requirements from their host plant. Another factor that Van Wyk *et al*. (1993) considered was the effect of fire on affected trees. They argued that fire can potentially damage *P. angolensis* trees by burning xylem and phloem tissues already under stress due to pathogen attack. In addition, bark lesions can allow the fire access to the tissue beneath the bark which cannot provide adequate protection against fire usually observed.

7. CONSERVATION OF *P. ANGOLENSIS*

The use of *P. angolensis* as a source of timber is a major concern for its conservation. Despite its status in some areas as being protected and where harvesting is prosecutable by state law, it is still under threat. Although trees are protected under the law, people continue to inject them with poisonous substances to ensure that they are dead for harvesting (C. J. Geldenhuys pers. comm., Krynauw 1998).

In southern Malawi most of the trees that are accessible to fellers have been cut down and the furniture industry currently has to rely on supplies from Mozambique (Clarke *et al*. 1996, Lowore *et al*. 1994). Mozambique has an export industry that includes South Africa, Germany and Portugal (Clarke *et al*. 1996, Sitoe & Ribeiro 1995). This export industry is, however, small as most of the wood from *P. angolensis* is consumed domestically in the local building industry, particularly in the big cities (Brigham *et al*. 1996, Sitoe & Ribeiro 1995). In Zimbabwe, past exploitation of the species and current threat has led to a warning that it will soon, if not already, be extinct in the area (Bradley & Dewees 1993, Clarke *et al*. 1996, Mushove 1991). In Tanzania, up to 85% of all wood sawn and consumed by the furniture industry is made up of *P. angolensis* (Brigham *et al*. 1996) and although the use of alternative exotic softwoods (e.g. *Eucalyptus* L'Hér) has been considered, a decrease in revenue and inconvenience in wood preparation have been cited as major deterrents (Shackleton 1996). In each case, the law dictates that a tree has to have a certain minimum diameter at breast height (DBH) before it can be cut down so as to encourage the tree to produce sufficient saplings thereby aiding in its conservation. However, this provision may have been ignored and broken in some areas (Caro *et al*. 2005).

In South Africa the furniture industry does not exclusively use *P. angolensis*, however, it is nevertheless included as the first among a sample of five most commonly used trees (Shackleton *et al*. 1996). In a recent report on the woodcarving industry, Steenkamp (1999) noted that the species, present in the Limpopo Province, Mpumalanga Province and Kwazulu-Natal Province, was regularly encountered in the informal sector (very high appearance). Despite its medium distribution across three of the nine provinces, availability reported by woodcarvers was low to medium indicating that the species is on its way to becoming rare, if not extinct, here (Steenkamp 1999).

8. CONCLUSIONS

Pterocarpus angolensis is obviously well-adapted for the harsh habitat in which it exists. Despite its lack of dominance in miombo woodland, its ability to withstand the effects of fire allow it to be a primary colonizer of areas that have been burnt or cleared for agriculture. However there are numerous factors that impede or inhibit the recruitment of seedlings into saplings. A recent study (Caro *et al*. 2005) based in the Rukwa Region in Tanzania emphasizes this concern along with the worry that the species is being overexploited for its wood in neighbouring African countries. Simply put, unless the regulatory bodies are extremely strict it is very likely that *P. angolensis* will become an endangered species capable of becoming extinct.

However, personal observations as well as those of one of the co-supervisors (C. J. Geldenhuys) indicate that regeneration and recruitment do occur in the wild. It is likely that in some areas this regeneration is overlooked and goes unnoticed as the seedlings are close to the ground and that subsequent observation after a few months during the next growing season would indicate the growth of the new shoots (Geldenhuys 2005). Caro *et al*. (2005) suggested that regeneration is episodic and dependent on the surrounding environment and the conditions therein. Some of these conditions noted as significant include whether a canopy is present, the presence of competing plants, and whether there is a water source and what its accessibility to the plant is (Caro *et al*. 2005). A slow growth rate in trees (Therrell *et al*. 2007) may be an explanation for the slow recruitment observed by Caro *et al*. (2005). A recent study (Chisha-Kasumu *et al*. 2006) investigated the possibility of growing the seedlings artificially using micropropagation/tissue culture methods and the results were fairly positive. The resultant protocol may be used in the future to ensure sustained conservation of the species, especially in areas where re-establishment may be necessary.

Little is known about the fungal diversity on *P. angolensis*. As is evident, the majority of fungi

isolated from this tree are not known to be pathogens but are instead saprophytes, generalist rot fungi, brown rot fungi or white rot fungi (Vermeulen 1990). In South Africa, four potential pathogens on *P. angolensis* have been recorded. An unknown species of *Fusarium* Link has been isolated from the seed (Van der Riet *et al*. 1998). The taxonomy of the species warrants further investigation to determine whether it represents a distinct formae specialis of the *F. oxysporum* species complex that, in this case, can be associated specifically with mukwa disease. An unknown species of *Sphaeropsis* Sacc. has been isolated from the species. *Sphaeropsis* spp. are anamorphs of *Botryosphaeria* Ces. & De Not. that possess dark and opaque conidia (Denman *et al*. 2000) and represents one of the several genera of Botryosphaeriaceae. The Botryosphaeriaceae are known pathogens of both herbaceous and woody plants (Arx & Müller 1954, Barr 1979) and often induce symptom expression in their host plant when the plant has been stressed (Smith *et al*. 1996). Finally two pathogens have been associated with leaf spots on *P. angolensis*. *Phomopsis pterocarpi* S. Hughes produces amphigenous leaf spots with dark brown colonies (Van der Westhuizen 1982, Vermeulen 1990) and *Phyllachora pterocarpi* Syd. & P. Syd. produces numerous raised black spots causing "tar-spot" disease (Doidge 1942, 1950). The taxonomy of both *Pho. pterocarpi* and *Phy. pterocarpi* warrants re-evaluation. For example, a taxonomic reassessment of the fungus *Phomopsis proteae* Wakef. occurring on native Proteaceae in South Africa revealed that it was actually a species of *Botryosphaeria*, *B. proteae* (Wakef.) Denman & Crous (Denman, Crous & Wingfield 1999).

The aim of the subsequent chapters in this thesis is to contribute to the knowledge of the fungal diversity present on *P. angolensis*. This study arose largely due to die-back and death of the tree being observed in the Mawewe Nature Reserve/Cattle Game Project by Krynauw (1998, 2000). These symptoms had been observed by the local communities. Some people in these communities had therefore started cutting down trees that were diseased, but still living. These people were subsequently arrested and successfully prosecuted with a fine, after which a legal representative acting on their behalf had approached the Mpumalanga Parks Board with the request to legally harvest *P. angolensis* on the reserve. A study was therefore conducted by Krynauw (1998, 2000) in the reserve to determine the availability of *P. angolensis* for harvesting as well as potential factors influencing *P. angolensis*. Krynauw noted that trees within the reserve appeared to have suffered from more fire damage than trees outside the reserve. Similarly, the collection of bark for medicinal purposes appeared prominent. Trees inside the reserve also had a lower fruiting percentage than trees both outside the reserve and in the control area. Finally a high presence of pathogens in general was also noted. However none of these pathogens were properly identified due to the financial constraints of the study. Krynauw (1998, 2000) suggested that before harvesting of any kind can

occur on the reserve, these pathogens had to be identified.

The subsequent chapters therefore provide an investigation into whether cases of die-back of *P. angolensis* in South Africa are the result of a biotic agent (in our case, a fungus) or due to the environment. This will be achieved by firstly identifying potential fungal pathogens and then characterizing them based on morphology and DNA sequence data (i.e. phylogenetic analysis) to discriminate between individual species. New species found will also be described. Finally pathogenicity of selected isolates will be accessed by inoculation into branches of kiaat (*P. angolensis*) in the field. Finally the results will be summarized and a plan for management in the affected area (and any other potential areas) will be outlined and discussed.

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Table 1. Species of the genus *Pterocarpus* Jacq. and the regions where they occur. Any further taxonomic divisions are noted next to the species concerned (Rojo 1972, Von Breitenbach 1973).

Species	Region	Notes
P. acapulcensis Rose	Tropical South America	
P. amazonum (Benth.) Amshoff	Tropical South America	
P. amphymenium D. C.	Tropical South America	
P. angolensis[b]	Tropical Africa	
P. brenanii[b] Barb. & Torre	Tropical Africa	
P. dalbergioides D. C.	South Eastern Asia, specifically Indo Malesia and the Pacific Region	
P. erinaceus[a] Poir	Tropical Africa	
P. indicus Willd.	South Eastern Asia, specifically Indo Malesia and the Pacific Region	Both formae of *P. indicus*, namely *indicus* Willd. and *echinatus* (Pers.) Rojo occur.
P. lucens Lepr.	Tropical Africa	Two subspecies of *P. lucens* occur, being *antunesii*[b] (Taub.) Rojo and *lucens*[a] Lepr.
P. macrocarpus Kurz	South Eastern Asia, specifically Indo Malesia and the Pacific Region	
P. marsupium Roxb.	South Eastern Asia, specifically Indo Malesia and the Pacific Region	
P. mildbraedii[a,b] Harms	Tropical Africa	

Species	Region	Notes
P. officinalis Jacq.	Tropical Africa and Tropical South America	Two subspecies of *P. officinalis* occur, being *officinalis* Jacq. that occurs only in tropical South America and *gilletii*[b] (De Wild.) Rojo that occurs only in tropical Africa.
P. osun[a] Craib	Tropical Africa	
P. rohrii Vahl.	Tropical South America	
P. rotundifolius[b] (Sonder) Druce	Tropical Africa	
P. santilinoides[a] D. C.	Tropical Africa and Tropical South America	
P. santalinus L.	South Eastern Asia, specifically Indo Malesia and the Pacific Region	
P. soyauxii Taub.	Tropical Africa	
P. tinctorius[b] Welw.	Tropical Africa	

All the species occurring in tropical Africa can be grouped into two main geographical regions with the exception of *P. soyauxii* which traverses into both regions (Fig. 2):

[a] These five species occur to the north of the equitorial rainforest belt.

[b] These seven species occur to the south of the equitorial rainforest belt.

Table 2. The position of the beak or style can be used to distinguish between different species (Von Breitenbach 1973).

Position of beak/style	Species with the characteristic
Terminally beaked	One South American species: *P. acapulcensis*. One African species: *P. brenanii*.
Laterally beaked	Five Indo-Malesian species: *P. dalbergioides*, *P. indicus*, *P. macrocarpus*, *P. marsupium*, and *P. santalinus*. Two American species: *P. amphymenium* and *P. rohrii*. Two African species: *P. erinaceus* and *P. mildbraedii*.
Basally beaked[a]	*P. angolensis*, *P. osun*, *P. soyauxii* and *P. tinctorius*.

[a]Only African species are basally beaked.

Table 3. A revised table of insects found feeding on various parts of *P. angolensis*.

Order	Family	Species	Notes	Plant part where found	Reference
Coleoptera	Buprestidae (jewel beetles, flat-headed borers)	Unknown	Larvae bore into dying wood. Short-lived adults feed on foliage (Holm & Bellamy 1996, Picker, Griffiths & Weaving 2002)	Leaves	Boaler (1966)
		Chrysobothris dorsata Fabricius	Larvae second most common of beetle larvae found in soft dying wood (Holm & Bellamy 1996)	Green logs	Selander (1986)
	Chrysomelidae (leaf beetles)	*Melosoma* sp.	Adults feed on flowers and foliage while larvae feed on or bore into leaves, stems or roots (Picker *et al*. 2002).	Young leaves	Boaler (1966)
	Coccinelidae (ladybirds, ladybugs)	Unknown	Subfamily Epilachninae is herbivorous; the adults feed on the upper leaf surface while the larvae feed on the underside of the leaf (Fürsch 1996).	Coppices leaves	Boaler (1966)

Order	Family	Species	Notes	Plant part where found	Reference
Coleoptera	Curculionidae (weevils, snout beetles)	Unknown	Both adults and larvae are herbivorous. Females bore into fruit, seeds or stems to lay their eggs (Picker *et al*. 2002).	Bark of young twigs	Boaler (1966)
		Unknown		Leaves	Boaler (1966)
		Systates sp.		Young leaves	Boaler (1966)
	Curculionidae – Scolytinae	Unknown		Bark and sapwood	Boaler (1966)
	Curculionidae – Platypodinae	*Doliopygus praeclarus* Schedl.		Occasionally attacks fresh trap logs.	Beaver & Löyttyniemi (1991)
					Löyttyniemi (1980)
		Systates crenatipennis Fairmaire	Also known as *S. pollinosus* Gerstaecker. Adult weevils eat the foliage of many trees and have caused severe defoliation of *Acacia mearnsii* De Wild., *A. melanoxylon* R.Br. and *Eucalyptus camaldulensis* Dehnh. (Gardner 1957, Browne 1968).	Bark of young twigs	Boaler (1966)
	Lamellicornae	Unknown	None	Young twigs	Boaler (1966)
	Meloidae (blister, oil, CMR beetles)	Unknown	Secrete cantharidin that can blister human skin. Larvae feed on eggs or on stores of pollen and nectar in bee cells. Adults feed on flowers, foliage or nectar (Picker *et al*. 2002).	Young leaves	Boaler (1966)

Order	Family	Species	Notes	Plant part where found	Reference
Coleoptera	Noctuidae (owlet moths)	Unknown – larvae	Adults attracted to overripe fruit. Larvae feed on plants, some bore into stems (Picker *et al*. 2002).	Young leaves	Boaler (1966)
	Plataspidae (pill bugs)	Unknown	Suck juices from various species of the wild peas. Thought they may feed on fungal hyphae just beneath the bark (Picker *et al*. 2002).	Suck young twigs	Boaler (1966)
	Scarabeidae - Melolonthinae (leaf chafers, white grubs)	*Melolontha melolontha* L. (cockchafer beetle)	Young adults feed on tender young leaves of various dicotyledonous tree species (Browne 1968).	Coppices leaves	Boaler (1966)

Order	Family	Species	Notes	Plant part where found	Reference
Homoptera	Cercopidae – Aphrophorinae (spittle bugs, froghoppers)	*Ptyelus flavescens* Fabricius (rain insect)	Nymphs and adults feed on the sap from the aerial parts of many plants including *Acacia* L. spp., *Albizia chinensis* Merr. and *Melia azadarach* L. Infestation of saplings can reduce vigour and malform growth (Forsyth 1966, Browne 1968).	Bark of young twigs	Van Wyk (1972)
		Ptyelus grossus Fabricius (rain insect)	As above. Hosts include *Eucalyptus* L'Hér, *Podocarpus* Labill. (Gardner 1957), *Acacia mearnsii*, *Ficus* L. and *Morus* L. (Le Pelley 1959)	Bark of young twigs	Van Wyk (1972)
Orthoptera	Acrididae (short-horned grasshoppers, locusts)	*Anacridium melanorhodon* Walker (Tree locust)	Also recorded on *Acacia nilotica* (L.) Delile and *Zizyphus mauritiana* Lambk. Shortly after hatching, hoppers move into crowns of trees and often cause complete defoliation (Pearson 1958, Browne 1968)	Leaves. Can cause complete defoliation.	Browne (1968)

Table 4. A revised list of fungi found growing on various parts of *P. angolensis*.

Fungus	Found where	Note	Reference
Armillaria mellea (Vahl) P. Kumm (honey fungus)	Malawi Tanzania Zambia	Saprophytic in soil, it becomes virulent once established on dead wood (Browne 1968).Secondary invader of mukwa die-back. Found on dead and dying root collars. Described as forming a white mycelial mat and black zone lines in the roots showing incipient decay (Geary 1972).	Peregrine & Siddiqi (1972) Browne (1968) Piearce (1982) Boaler (1966) Geary (1972)
Aspergillus Mich *ex* Fr. sp.	Zambia Zimbabwe	Saprophytic worldwide, occurring in warm climates (Domsch, Gams & Anderson 1993). Associated with phloem streaking in mukwa disease.	Piearce (1979) Van Wyk *et al*. (1993)
Aspergillus aculeatus Iizuka	Zambia		Piearce (1979) *ex* Ivory (unpubl.) Piearce (1982)
Aspergillus niger van Tiegh.	South Africa	Currently known as *A. niger* var. *niger*. Found on the seed surface after sterilization with sodium hypochloride.	Van der Riet *et al*. (1998)
Aureobasidium pullulans (de Bary) G. Arnaud	Zambia	Saprophytic but is believed to be weakly parasitic. Hosts include *Acer saccharum* Marsh. and *Picea glauca* Hort *ex* Beisnn. Infection causes adventitious shoot formation and forked shoot growth (Browne 1968).	Piearce (1979) *ex* Ivory (unpubl.) Piearce (1982)
Coniophora cerebella Alb. & Schwein.	Zambia	Mainly saprophytic in damp conditions but also attacks via wounds and causes brown cubical rot. Reported on *Cupressus lusitanica* Mill. in Kenya and Tanzania as a result of waterlogged soil (Browne 1968). Treatment of wood with organic solvents results in susceptibility to the fungus.	Anon (1966)

Fungus	Found where	Note	Reference
Coriolopsis floccosa (Jungh.) Ryvarden *Coriolopsis polyzona* (Pers.) Ryvarden	Zambia	White rot fungi found on dead logs, stumps and branchwood.	Piearce (1986)
Flavodon flavus (Klotzsch) Ryvarden ["*fulvus*"]	Zambia	White rot fungi found on dead logs, stumps and branchwood.	Piearce (1986)
Fomes (Fr.) Fr. sp.	Tanzania	Brown rot fungus found on the bole of living trees.	Boaler (1966)
Fomes hornodermus (Mont.) Cooke	South Africa (Venda)	Currently known as *Perenniporia martii* (Berk.) Ryvarden.	Van der Westhuizen (1982) Vermeulen (1990)
Funalia protea (Berk.) D. A. Reid	Zimbabwe	White rot fungus currently known as *Coriolopsis floccosa*.	Sharp (1982) Vermeulen (1990)
Fusarium Link sp.	South Africa	Found on seed surface sterilized with sodium hypochloride.	Van der Riet *et al*. (1998)
	Zambia	Associated with incipient heart rot.	Piearce (1979) *ex* Ivory (unpubl.)
Fusarium oxysporum Schltdl.	Zambia	Primary pathogen associated with mukwa disease. Saprophytic but becomes parasitic on roots and root collars causing root rot, vascular wilt and damping off. Hosts include *Albizia julibrissin* Durazz. and *Olea europaea* L. (Browne 1968).	Piearce (1982)
Ganoderma P. Karst. sp.	Zimbabwe	White rot fungus found on dead logs, stumps and branchwood.	Calvert (1972) Piearce (1986)
Ganoderma lucidum (Curtis) P. Karst.	Zimbabwe	Wound pathogen causing root and butt rot. It can also attack the roots when a tree is stressed. Hosts include *Acacia mearnsii*, *A. melanoxylon*, *Pterocarpus indicus* and *P. marsupium* (Browne 1968).	Sharp (1982) Vermeulen (1990)

Fungus	Found where	Note	Reference
Hexagonia Poll *ex* Fr. sp.	Zambia	Secondary invader of mukwa disease. Found on dead conks.	Geary (1972) Piearce (1979)
Hexagonia hirta (P. Beauv.) Fr.	Tanzania	Found on dead twigs. Recorded as *H. hystrix* and *Trametes hystrix* Cooke.	Boaler (1966)
	Zambia	Found on dead logs, stumps and branchwood.	Piearce (1979, 1982, 1986)
Hexagonia hydnoides (Sw.) M. Fidalgo	Zambia	Found on dead logs, stumps and branchwood.	Piearce (1986)
Hexagonia pobeguinii Har.	South Africa	None	Doidge (1950) Van der Westhuizen (1982) Vermeulen (1990)
	Tanzania	Found on dead twigs and on the boles of dying trees.	Boaler (1966)
	Zambia	Found on dead logs, stumps and branchwood.	Piearce (1982, 1986)
Hexagonia tenuis (Hook) Fr.	Zimbabwe	Found on dead logs, stumps and branchwood. A pathogen that infects through large wounds to cause stem rot (Browne 1968). *Grevillea robusta* A. Cunn *ex* R. Br. is a known host (Spaulding 1961).	Calvert (1972) Piearce (1986)
Hexagonia umbrinella Fr.	Zambia	Found on dead logs, stumps and branchwood.	Piearce (1986)
Hexagonia zambesiana Torrend			
Hymenochaete gigaspora D. A. Reid	Zambia	White rot fungus found on dead logs, stumps and branchwood.	Piearce (1982, 1986)
Lentinus strigosus (Schwein.) Fr.	Zambia	Brown rot fungus found on dead logs, stumps and branchwood.	Piearce (1986)

Fungus	Found where	Note	Reference
Lenzites striata (Sw.) Fr.	Zambia	Brown rot fungus currently known as *Gloeophyllum striatum* (Sw.) Murrill. Treatment of wood with organic solvents results in susceptibility to the fungus.	Anon (1966)
Nigrospora Zimm. sp.	Zimbabwe	Saprophytic.	Van Wyk *et al*. (1993)
Penicillium Link *ex* Fr. sp.	Zambia Zimbabwe	Global saprophytes occurring in more temperate regions (Domsch *et al*. 1993).	Piearce (1979) *ex* Ivory (unpubl.) Van Wyk *et al*. (1993)
Perenniporia ochroleuca (Berk.) Ryvarden	Zambia	White rot fungus found on dead logs, stumps and branchwood.	Piearce (1986)
Phellinus rimosus (Berk.) Pil.	Zimbabwe	Associated with heart and white rot.	Masuka & Ryvarden (1993)
Phomopsis pterocarpi S. Hughes	South Africa Zambia	Produces leaf spots with dark brown colonies that are amphigenous. Reported on *Pterospermum acerifolium* Willd., *Aloe vera* (L.) Burm. f. (Firdousi & Vyas 1990), *Ougenia dalbergioides* Benth. and *Jatropha curcas* L. (Sharma 1975).	Van der Westhuizen (1982) Vermeulen (1990) Angus (1964) Piearce (1979, 1982)
Phyllachora pterocarpi Syd. & P. Syd.	South Africa Tanzania Zambia	Found on the leaves of both seedlings and trees. Causes “tar-spot” consisting of numerous raised, black spots. Severe infection causes defoliation in young plants.	Doidge (1942, 1950) Boaler (1966) Browne (1968) Gibson (1962) Angus (1964) Piearce (1979)
Polystictus fulvo-cinereus D. A. Reid	Tanzania	Found on dead twigs and on the boles of dying trees rotting the wood. Treatment of wood with organic solvents results in susceptibility to the fungus. Currently known as *Coriolopsis caperata* (Berk.) Murrill.	Anon (1966) Boaler (1966)

Fungus	Found where	Note	Reference
Pycnoporus sanguineus L. (Murrill)	Zambia	White rot fungus found on dead logs, stumps and branchwood.	Piearce (1986)
Sphaeropsis Sacc. sp.	South Africa	Anamorphs of *Botryosphaeria* Ces. & De Not. that possess dark and opaque conidia (Denman *et al*. 2000).	Van der Westhuizen (1982) Vermeulen (1990)
Trametes Fr. sp.	Zambia Zimbabwe	Brown rot or white rot fungus found on dead conks. Secondary invader of mukwa die-back.	Geary (1972) Sharp (1982) Vermeulen (1990)
Trametes cingulata Berk.	Zambia	Found on dead logs, stumps and branchwood.	Piearce (1986)
Trametes scabrosa (Pers.) G. Cunn.	Zambia	Currently known as *Earliella scabrosa* (Pers.) Gilb. & Ryvarden.	Piearce (1982)
Vanderbylia ungulata D. A. Reid	Tanzania Zambia	Records of the white-rot fungus in Zambia indicate it can be a wound pathogen (Piearce 1986). Also reported on *Pericopsis angolensis* (Baker) van Meeuwen [=*Afrormosia angolensis* Harms.] (Decock & Masuka 2003, Reid 1973).	Reid (1973) Vermeulen (1990)

Table 5. Rainfall data for the weather stations in Bulawayo (Zimbabwe) and Livingstone (Zambia). Data available for the years 1951 to 1980 is provided by the World Weather Records (Anon 1967, 1979, 1987). The average for both stations is also included.

Year	Annual Rainfall for Bulawayo (mm)	Annual Rainfall for Livingstone (mm)	Average Rainfall between Bulawayo and Livingstone (mm)
1951	782	872	827
1952	829	1059	944
1953	627	876	751.5
1954	810	787	798.5
1955	865	823	844
1956	616	840	728
1957	571	801	686
1958	673	1410	1041.5
1959	469	620	544.5
1960	490	571	530.5
1961	737.7	675	706.35
1962	650.6	1078	864.3
1963	486.2	682	584.1
1964	491.5	621	556.25
1965	197.2	372	284.6
1966	683.5	819	751.25
1967	437.5	739	588.25
1968	468	589	528.5
1969	551.6	891	721.3
1970	344.9	519	431.95
1971	519	622	570.5
1972	656	823	739.5
1973	604	875	739.5
1974	794	1280	1037
1975	795	612	703.5
1976	746	760	753
1977	869	813	841
1978	1014	947	980.5
1979	579	721	650
1980	513	530	521.5

Figure 1. A distribution map of the genus *Pterocarpus*. Numbers at the top of each fraction represent the total number of species occurring in the area. Numbers at the bottom of each fraction represent the non-endemic/introduced species in the area. The map was copied from Rojo (1972).

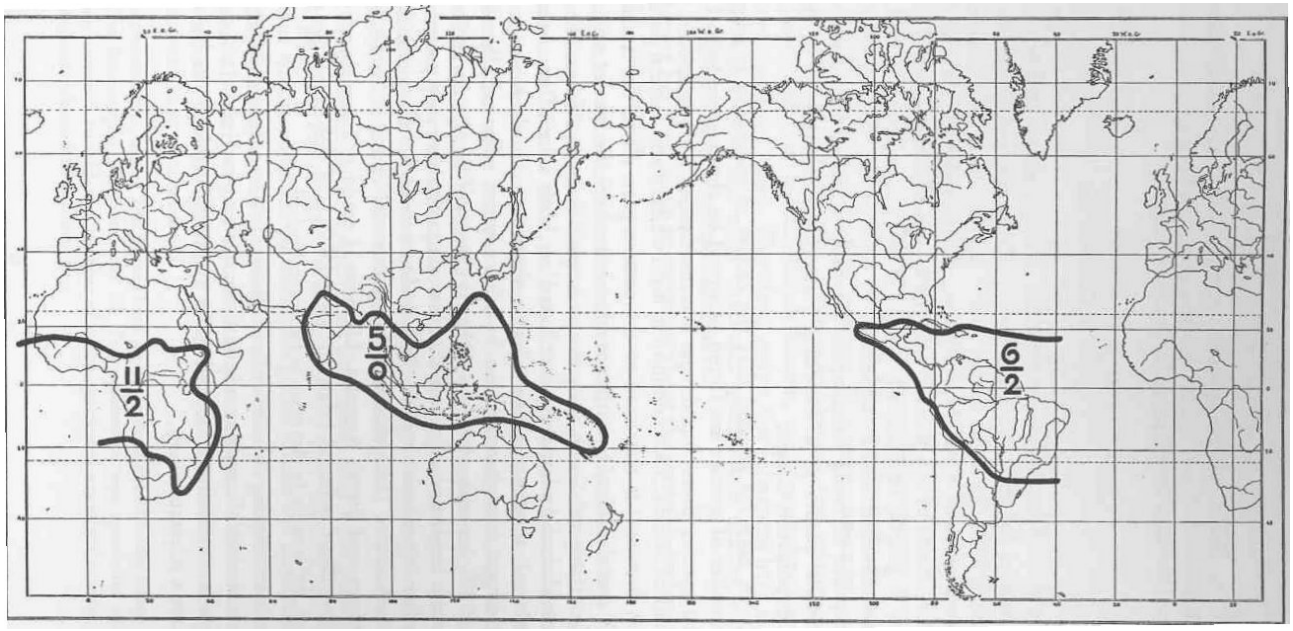


Figure 2. A rough map showing the distribution of African species of *Pterocarpus*. As can be seen, species can be considered as occurring either north or south of the equitorial rain forest belt. Copied from Von Breitenbach (1973).

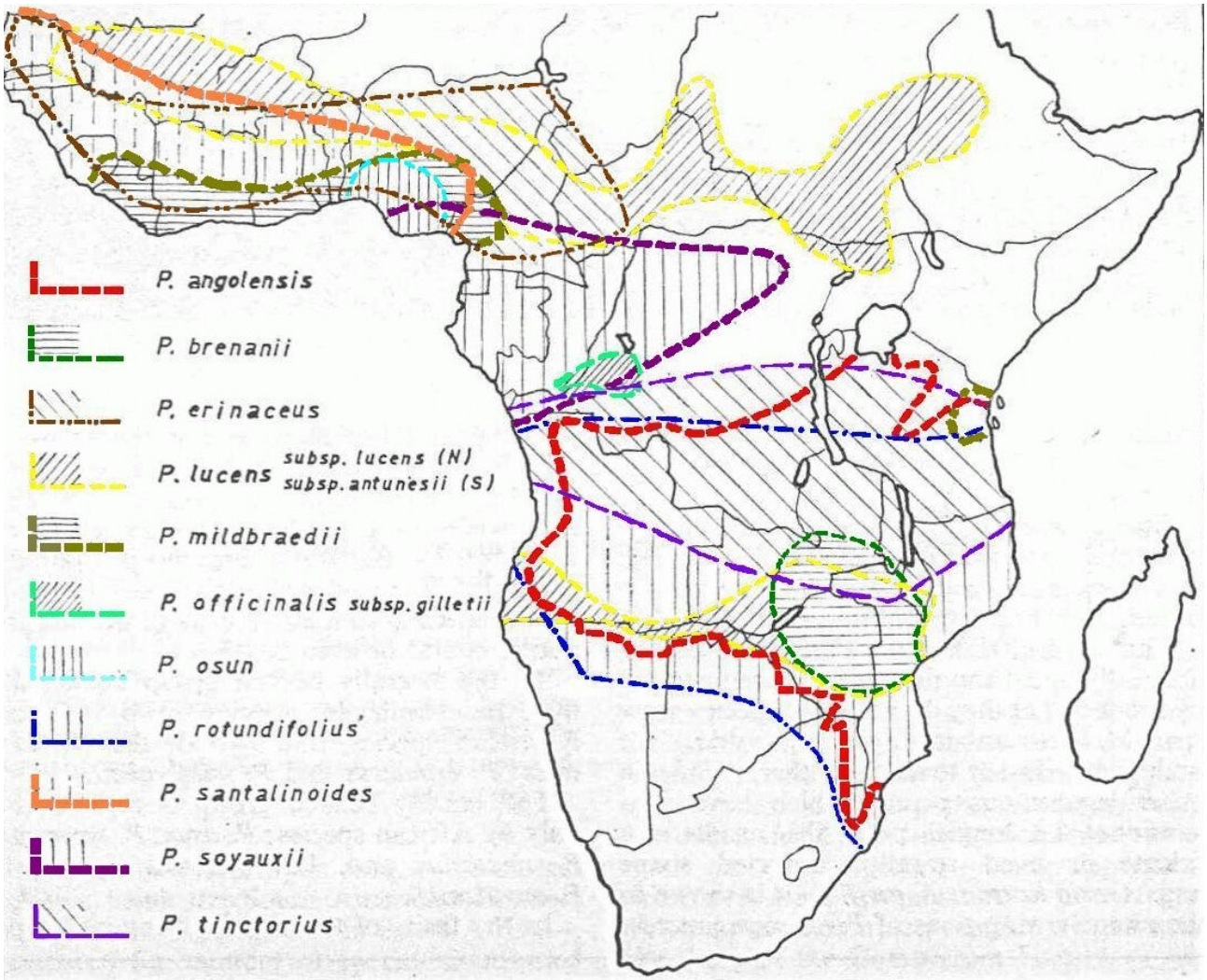
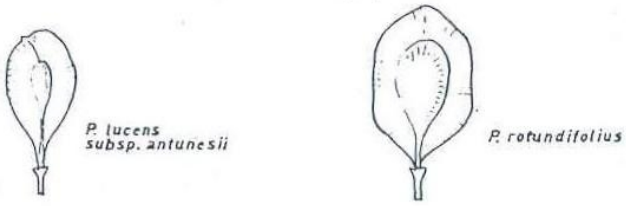

P. angolensis
P. brenanii
P. erinaceus
P. lucens subsp. lucens (N) subsp. antunesii (S)
P. mildbraedii
P. officinalis subsp. gilletii
P. osun
P. rotundifolius
P. santalinoides
P. soyauxii
P. tinctorius

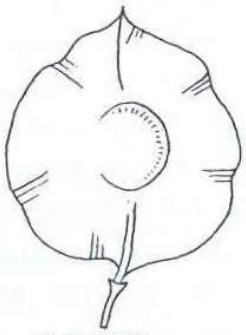
Figure 3. A diagram of the different pod types and the different positions of the beak or style. Copied from Von Breitenbach (1973).

Elliptic wing-pods

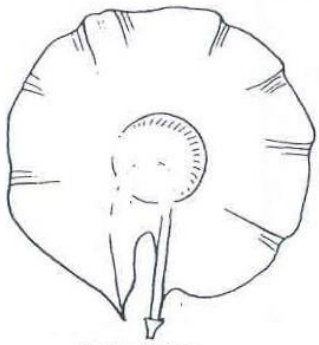


Circular wing-pods

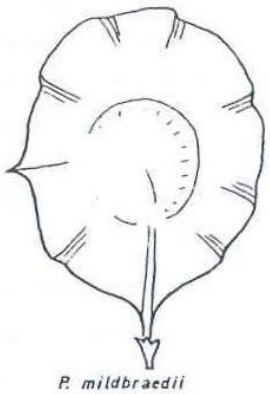
with terminal beak



with basal beak



with lateral beak



with basal beak
and bristly centre

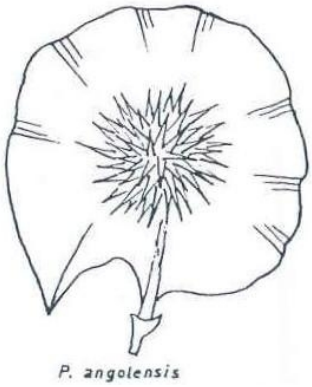


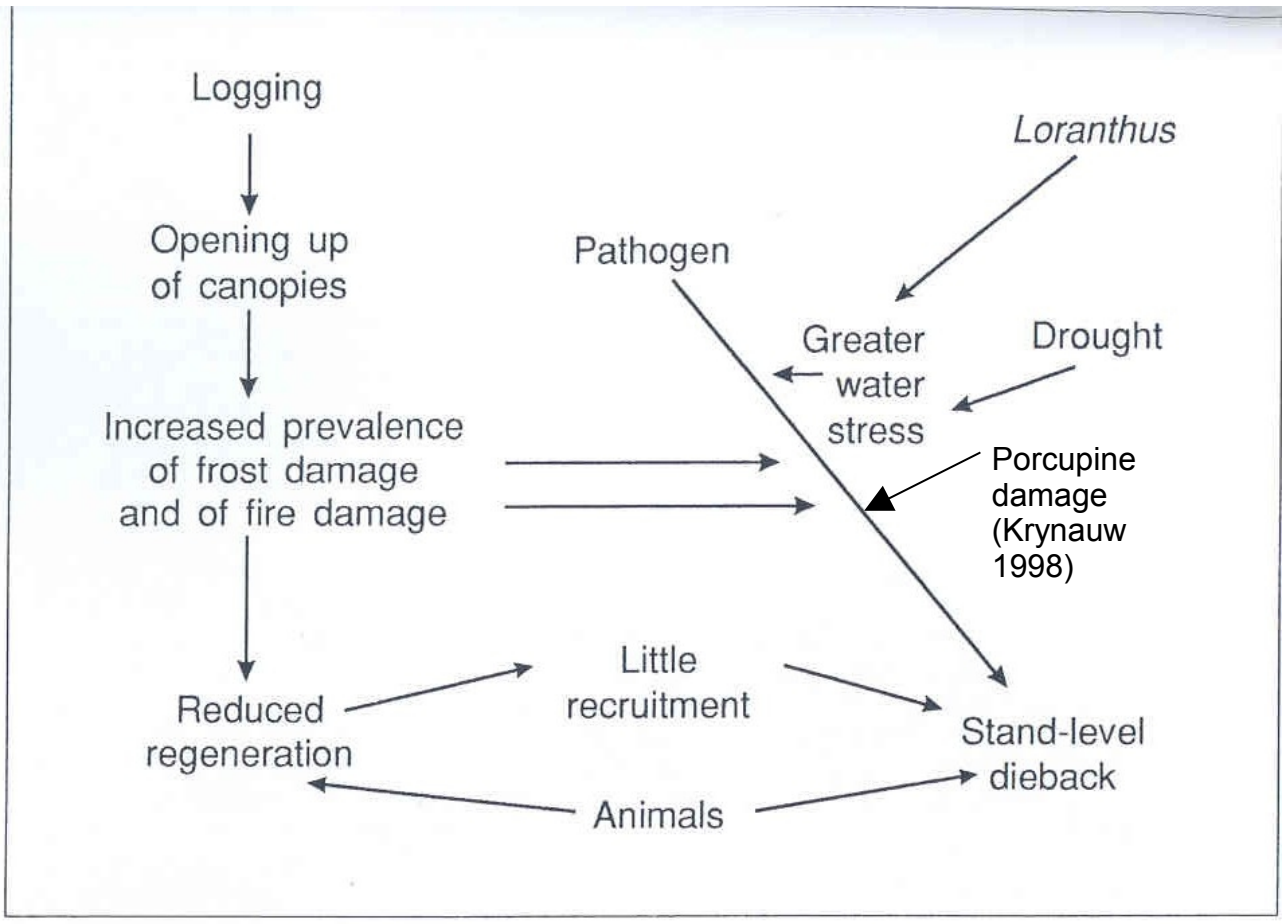
Figure 4. Earlier outbreaks of mukwa die-back in Zambia and Namibia as documented by Calvert (1972) and based on the original map in Piearce (1979). Outbreaks 1 and 2 were reported by Rainford in the late 1940s and early 1950s respectively, outbreak 3 by Martyn in 1958/9, outbreak 4 by Bainbridge in 1963/4, outbreaks 5 and 6 by Geary in 1965/7, outbreak 7 by Katambora from 1973 to 1977 and outbreak 8 by Dambwa from 1974 to 1978. Light blue lines indicate rivers and streams. Dark blue lines indicate roads. Purple lines indicate railways to and from villages. Yellow lines indicate international borders. Map generated using the software package Google Earth.


Zambia
Namibia
Botswana
Zimbabwe
Livingstone, Zimbabwe
1
2
3
4
5
6
7
8
Image © 2005 EarthSat
©2005 Google
Pointer 17°30'38.19" S 25°18'24.29" E elev 953 m
Streaming 100%
Eye alt 207.60 km

Figure 5. Map of the area where mukwa disease has been reported. Weather stations are positioned at Bulawayo and Livingstone (see Table 2). Mukwa disease was first reported just west of Livingstone in 1959 (Anon 1973), in the Victoria Falls village in July 1964 (Geary 1972), and in March 1966 in Bambezi Forest Reserve (about 100km north-west of Bulawayo) (Anon 1973). Purple lines indicate railways to and from the villages. Yellow lines indicate international borders. Map generated using the software package Google Earth.


Zambia
Namibia
Livingstone, Zimbabwe
Victoria Falls, Zimbabwe
Zimbabwe
Bambezi Forest Reserve
Botswana
Bulawayo
Image © 2005 EarthSat
Google
Pointer 18°45'52.05" S 26°57'19.59" E elev 1060 m
Streaming 100%
Eye alt 475.77 km

Figure 6. An outline of factors implicated in the development of mukwa die-back (Van Wyk *et al*. 1993).



CHAPTER 2

BOTRYOSPHAERIACEAE ASSOCIATED WITH *PTEROCARPUS ANGOLENSIS* (KIAAT) IN SOUTH AFRICA

This chapter has been accepted for publication as: Mehl JWM, Slippers B, Roux J, Wingfield MJ, 2010. Botryosphaeriaceae associated with *Pterocarpus angolensis* (kiaat) in South Africa. *Mycologia*

ABSTRACT

Pterocarpus angolensis (kiaat) is a well-known tree species in South Africa, valued for its use in traditional medicine and as a source of timber for woodcarving and furniture. In recent years, there have been several reports of *P. angolensis* trees dying in South Africa, Zambia and Zimbabwe. A survey of material from diseased *P. angolensis* trees in South Africa yielded isolates of the Botryosphaeriaceae, an important fungal family known to cause a number of tree diseases. The aim of this study was to identify these Botryosphaeriaceae and to determine their pathogenicity to *P. angolensis* using branch inoculations. Seven species of the Botryosphaeriaceae were identified based on a combination of morphological characteristics and sequences from the ITS and EF-1α gene regions. Four of these represent undescribed taxa for which the names *Pseudofusicoccum violaceum*, *P. olivaceum*, *Diplodia alatafructa* and *Fusicoccum atrovirens* are provided. The remaining three species collected include *Lasiodiplodia theobromae*, *L. pseudotheobromae*, and *L. crassispora*. Inoculation trials on tree branches showed that *L. pseudotheobromae* and one isolate of *D. alatafructa* differed significantly from control inoculations. The high levels of virulence and common occurrence of *L. pseudotheobromae* suggests that this species could play a role in tree die-back and death.

1. INTRODUCTION

Pterocarpus angolensis (kiaat) is a well-known native Southern African tree species prized for its use in traditional medicine (Coates Palgrave 1977) and as a source of timber in the woodcarving and furniture industries of several African countries (Lowore 1993). The heartwood of the tree is both durable and attractive, resulting in the species becoming a target for exploitation. This has raised concerns regarding the regeneration and health of the species (Caro *et al*. 2005).

In recent years, there have been a number of reports of disease and death of *P. angolensis* trees in South Africa (Krynauw 1998, 2000), Zambia and Zimbabwe (van Wyk *et al*. 1993). In Zambia and Zimbabwe, a disease known as mukwa, referring to the local name of the species in these areas, has been reported. Mukwa disease is characterized by defoliation, wilt, die-back, bark discoloration, vascular and phloem streaking and the production of epicormic shoots (Calvert 1972, Piearce 1979, 1986, van Wyk *et al*. 1993). In South Africa, the disease has been characterized by branch die-back, heart rot and death of mature trees (Krynauw 1998, 2000).

During a recent survey of diseased *P. angolensis* trees in the Mpumalanga Province of South Africa (Mehl unpublished), isolates resembling the Botryosphaeriaceae were isolated from diseased trees. Species of this well-known and widely distributed family of Ascomycete plant pathogens (Schoch *et al*. 2010) occur as endophytes in both gymnosperms and angiosperms, and woody and herbaceous plants (Arx & Müller 1954, Barr 1972). Diseases caused by species of the Botryosphaeriaceae include fruit rots, leaf spots, seedling damping-off and collar rot, cankers on stems and branches (including twigs) and roots, blight of shoots and seedlings, gummosis, blue-stain of the sapwood, die-back and tree death (Slippers *et al*. 2007, Slippers & Wingfield 2007). Stress due to drought and physical damage such as hail has been linked to disease expression (Slippers & Wingfield 2007), although a number of other predisposing factors may also favour the onset of disease.

The aim of this study was to identify species of the Botryosphaeriaceae associated with *P. angolensis* in South Africa. Species collected were characterized based on their morphology and comparisons of DNA sequence data. Pathogenicity of the species isolated was also evaluated by means of branch inoculations to assess their possible involvement with die-back of *P. angolensis*.

2. MATERIALS AND METHODS

2. 1. Sample collection and isolation

Branches and twigs showing symptoms of die-back as well as healthy specimens were collected from *P. angolensis* trees from five locations in the Mpumalanga Province of South Africa. These locations were selected based on reports of tree mortality in the areas, as well as to cover a broad range of sites representing the natural distribution of *P. angolensis*. Areas sampled included Mawewe Nature Reserve/Cattle Game Project, Buffelskloof Nature Reserve, Bushbuckridge settlement, the Sudwala Caves area and Pretoriuskop in the Kruger National Park.

Branches, both symptomatic and asymptomatic, were collected from four trees at Pretoriuskop, twenty trees in the Sudwala Caves area, seven dying trees in Mawewe Nature Reserve, fourteen trees alongside the road at Bushbuckridge settlement and from twenty trees in Buffelskloof Nature Reserve. Material was also collected from stem wounds on trees in the Sudwala Caves area and Mawewe Nature Reserve. Samples included those collected from dead and dying trees in Mawewe Nature Reserve and Buffelskloof Nature Reserve.

All samples were kept in a walk-in refrigerator and isolations were made after two weeks and four weeks. A pilot trial had been undertaken on the samples from Pretoriuskop to establish the optimal time for isolations to be made. In this case, isolations were done after one week, two weeks, four weeks and eight weeks. After two weeks and four weeks, the largest number of isolates of Botryosphaeriaceae was recorded (results not shown). Isolations were made as outlined by Pavlic *et al*. (2004).

2. 2. Culture characteristics and morphology

Cultures were transferred to 2 % water agar (Biolab, South Africa) overlaid with sterilized pine needles and small sections from branches of *P. angolensis*. Sporulation was induced following the methods described by Mohali *et al*. (2006). Fruiting structures that emerged were sectioned by hand and released conidia were mounted in 85 % lactic acid on glass microscope slides. Digital microscopic photographs of conidia were taken using a HRc Axiocam digital camera and the accompanying Axiovision 3.1 software (Carl Zeiss Ltd., München, Germany). Thirty measurements were made of the length and breadth of the conidia. Culture colours were determined using the colour charts of Rayner (1970).

Single conidial cultures were made by spreading a spore mass onto water agar using a sterile inoculation loop. Plates were incubated at room temperature for 12-24 hours and germinating conidia transferred to malt extract agar (1.5 % malt extract, 2 % agar) amended with 0.005 g streptomycin sulphate (MEA+S). For non-sporulating isolates, single hyphal tips were transferred to MEA+S. Cultures were incubated at 25 ºC and are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa. Dried cultures were deposited in the National Collection of Fungi (PREM) and representative strains with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Culture morphology was captured using a mounted Sony Digital Still Camera DSC85 Cybershot 4.1 MP. Isolates were then grouped based on culture and conidial morphology.

For new species of the Botryosphaeriaceae identified in this study, twenty measurements were made of the conidiomata, fifty measurements were made of the locules (where present), fifty measurements were made of the conidiogenous cells (except for one species where 40 measurements were made, as noted in the description) and fifty measurements were made of the conidia.

2. 3. Growth studies

Mycelial plugs (5 mm in diameter) of isolates representing new species of the Botryosphaeriaceae identified in this study were placed at the centres of 90 mm MEA plates, with the plug orientated so that the mycelium faced the agar surface. For each isolate, five plates each were incubated at temperatures ranging from 10 ºC to 35 ºC, at five degree intervals and the experiment was repeated once.

2. 4. DNA extraction and PCR amplification

Three to four isolates of each different morphological group of isolates were selected for DNA sequence comparisons (Table 1). Isolates chosen were also selected so that at least two different sampling sites were represented. DNA was extracted from cultures as described by van Wyk *et al*. (2006) except that the nucleic acid pellets were resuspended in 50 µl TE buffer (100 mM Tris-HCl, 10 mM EDTA, adjusted to pH 8.0) and digested with 5 µl RNase A (1 mg/ml) at 60 ºC for 1 hour. DNA concentrations were quantified using the NanoDrop® ND-1000 and accompanying software (NanoDrop Technologies, DuPont Agricultural Genomics Laboratories, Delaware, USA).

The ITS rDNA locus, including the ITS1, 5.8S gene and ITS2 were amplified using the primer pair ITS1 and ITS4 (White *et al*. 1990). A portion of the elongation factor 1α (EF1α) gene region was also amplified to verify the results from the ITS phylogeny, using the primer pairs EF1-728F and EF1-986R (Carbone & Kohn 1999), and EF1F and EF2R (Jacobs *et al*. 2004). Both gene regions were sequenced for isolates representing all the different morphological groups. For isolates that did not produce fruiting structures on pine needles or branches and that consequently could not be grouped based on culture characteristics, only the ITS rDNA region was sequenced. In addition, the ITS rDNA locus was amplified and sequenced for isolates of the Botryosphaeriaceae with pigmented conidia that could not be distinguished based on morphology.

Polymerase chain reactions (PCRs) consisted of ~10 ng template DNA, 0.2 mM of each primer, 2.5 mM each dNTP, 1.5 X PCR buffer, 25 mM $MgCl_2$, and 0.5 U *Taq* polymerase. Reaction volumes were adjusted to 25 µl by adding sterile Sabax water. Amplification reactions were performed on a Bio-Rad iCycler Thermal Cycler. Cycling conditions included an initial denaturation step of 96 ºC for 1 minute followed by 35 cycles of denaturation at 94 C for 30 seconds, annealing at 54 C for 1 minute, and extension at 72 C for 90 seconds. A final extension at 72 C for 10 minutes was also performed. PCR products were separated on 2 % agarose-ethidium bromide gels run on a TAE buffer system (Maniatis *et al*. 1982) and visualized under ultraviolet light. Product sizes were estimated using a Lambda DNA/*Eco*RI+*Hin*dIII marker 3 (Fermentas Life Sciences, USA).

2. 5. DNA sequencing and phylogenetic analysis

PCR products were purified using 6 % Sephadex columns (Sigma, Steinheim, Germany) as per the manufacturer's instructions. Sequencing PCRs were made using the same primers as in the original PCR and also purified using the Sephadex columns. The PCR amplicons were sequenced in both directions using the ABI PRISM(TM) Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) following the manufacturer's instructions on an ABI PRISM 3100(TM) automated sequencer.

Sequences were analyzed and edited using MEGA4 (Tamura *et al*. 2007). Additional sequences for phylogenetic analysis were obtained from nucleotide blast comparisons (blastn) with sequences in GenBank. Sequences were aligned with MAFFT 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh & Toh 2008) using a manual strategy based on the G-INS-i algorithm.

Sequence datasets were analyzed using PAUP 4.0b10 (Swofford 2002). Analyses were done using the heuristic search option with 100 random addition sequence replications on both gene datasets as well as the combined dataset. In all cases, tree-bisection-reconnection (TBR) branch swapping was applied and maxtrees was unlimited. Uninformative characters were excluded and the remaining gaps in the sequence alignment treated as a fifth base (NEWSTATE). A partition homogeneity test (Swofford 2002) was done to determine whether the relationships generated from both gene regions were statistically congruent and that the two gene datasets could be combined. For the partition homogeneity test, the heuristic search option was selected and 1 000 replications were done. A bootstrap analysis (50 % majority rule, 1 000 replications) (Felsenstein 1995) was done on the individual datasets to determine the confidence levels of the tree branching points. Trees were rooted to two isolates of *Guignardia* sp. Viala & Ravaz (Pavlic *et al*. 2008) as outgroup taxa.

Bayesian analysis was done on the individual datasets to determine the posterior probability/stringency of the branch nodes using Monte Carlo Markov Chain (MCMC) algorithms (Larget & Simon 1999). No characters were excluded (D. Posada, pers. comm.). JModelTest 0.1.1 (Posada 2008), with the corrected Akaike Information Criterion (AICc) (Sigiura 1978) selected, was used to determine the best nucleotide substitution model for the two individual datasets. MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) was used for the Bayesian analysis.

Two independent runs were done, both of 5 M generations. Four chains were used and trees were sampled every 100 generations, resulting in 50000 trees. Burnin was set at 15000 generations (150 trees), after likelihood values had converged to stationery, providing 49850 trees for sampling.

2. 6. Pathogenicity tests

Twenty trees in the Sudwala Caves area were selected for two branch inoculation trials in March and September 2007. As each tree was genetically unique, trees were assigned labels for both inoculation trials when lesion lengths were measured. Branch diameter was measured for the second inoculation trial. Branches were inoculated with two representative isolates of each species (Fig. 10). In the second inoculation trial, CMW22674 and CMW22682, were included.

Branches were randomly selected on each tree for inoculation so that one branch per isolate and one branch per tree for the negative control (a sterile MEA plug) was inoculated. A cork borer (9 mm diam.) was used to remove a disc of bark from the branches to expose the cambium. The cork borer

was sterilized between inoculations in 70 % ethanol followed by flaming. Mycelial plugs (9 mm diam.) taken from 7-day old cultures were placed in the wounds, with mycelium facing the cambium, using a sterile scalpel. Wounds were sealed with Parafilm to prevent desiccation. Lesion lengths were measured six weeks after the branches had been inoculated and re-isolations were done from all the inoculated branches on every fifth tree to ascertain that the lesions were associated with the fungi inoculated.

Data generated from the inoculation trials were subjected to a two-way analysis of variance (ANOVA) using the General Linear Model Procedure from SAS, Type III Sum of Squares, F-test of SAS, and Fisher's Pairwise Test (SAS Institute 2004). For the data from the first trial, the model included the tree, the species of fungus, and the isolate nested within the species as predictors of lesion length where trees were considered as blocks. For the data from the second trial, branch diameter was added as a covariable. When results were significantly different ($P \leq 0.05$), the Least Squares Means were generated, and in conjunction with Fisher's Pairwise Test, the differences amongst the means were evaluated for statistical significance.

3. RESULTS

3. 1. Isolate collection and morphology

Isolates produced anamorph structures, either on pine needles or small branches of host tissue, or both concurrently. Structures yielded either dark *Diplodia*-like conidia (35 isolates) or hyaline *Fusicoccum*-like conidia (67 isolates) (total 102 isolates). The latter group with hyaline conidia could be broadly separated into two groups, based on conidial size and shape in combination with their culture morphology. The first of these groups produced conidia that measured, on average, 15-40 x 5-15 µm (39 isolates) and the second group produced conidia that had an average size of 20-55 x 9-15 µm (28 isolates).

3. 2. DNA sequencing and phylogenetic analyses

PCR amplification of the ITS and EF-1α gene regions yielded fragments of ~560 bp and 700-750 bp, respectively. Sequences generated for the phylogenetic analysis in this study were deposited in GenBank (Table 1). Identities of isolates together with culture and accession numbers, majority consensus Bootstrap trees generated from the two datasets (Table 2) as well as the trees resulting from the Bayesian analysis were deposited in TreeBASE

(http://www.treebase.org/treebase/index.html) under accession number SN4677. Based on the results from jModelTest 0.1.1., 3-parameter models were applied to both the EF-1α (TPM3uf) and ITS (TPM1uf) datasets. Gamma (G) and proportion of invariable site (I) parameters were applied to both models to accommodate variable rates across sites.

There were minor differences in the topologies of the trees emerging from analysis of sequences of the two gene regions but these differences were only observed within genera amongst some species. For example, some of the *Pseudofusicoccum* spp. could not be delineated based on the ITS sequences alone, but were clearly resolved based on the EF-1α sequence data. Sequence data from the ITS and EF-1 gene regions have been combined in previous studies and consequently those for this study were combined into a single dataset (Table 2, Fig. 1).

The isolates from *P. angolensis* grouped in four genera representing *Pseudofusicoccum* Mohali, Slippers & M. J. Wingf. (Fig 1. - Clade 1), *Diplodia* Fr. (Fig. 1 - Clade 2), *Lasiodiplodia* Ellis & Everh. (Fig. 1 - Clade 3), and *Fusicoccum* Corda (Fig. 1 - Clade 4, Fig. 2). Based on the phylogenetic analyses and spore measurements, seven species of the Botryosphaeriaceae could be resolved (Figs. 1, 9). Three of these taxa represent known species, namely *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *L. crassispora* T.I. Burgess & Barber and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous. The remaining four taxa represent undescribed species that are described below.

3. 3. Taxonomy

Pseudofusicoccum olivaceum J. W. M. Mehl & B. Slippers sp. nov. Figs. 3a, 4. MB513501
Etym.: Name refers to the olivaceous colour formed in culture.
Teleomorph: not observed, but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Conidiomata pycnidialia, subcuticula, unilocularis, atrobrunnea, pro parte maxima solitaria, applanata, mycelio tecta. *Ostiolum* centralis, rotundum et papillatum. *Cellulae conidiogenae* hyalinae, holoblasticae, glabrae, cylindricae, guttulatae, rotundae, percurrenter cum 1-2 proliferationibus obscuris prolificentes, vel in plano eodem periclinaliter incrassatae, paraphyses visae. *Conidia* hyalina, parietibus tenuis, unicellularia, eseptata, interdum contento granulari, guttulata, circumdata strato mucido persistente, apicibus basibusque obtusis ad rotunda late, bacilliforma.

Typus: South Africa: Mpumalanga Province: Kruger National Park: Pretoriuskop, from an asymptomatic branch of *P. angolensis*, Sep. 2005, *J. Roux* (PREM60328, fruiting structures induced on needles of *Pinus* sp. and branches of *P. angolensis* on WA, culture ex-type CBS124939 = CMW20881).

Conidiomata on both pine needles and host material pycnidial, subcuticular, unilocular, dark brown, mostly solitary, applanate, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown *textura angularis*; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, (480.4-)531.9-646.2(-688.3) µm (average of 50 conidiomata 589.0 µm). *Ostiole* central, circular and papillate. *Conidiogenous cells* hyaline, holoblastic, smooth, cylindrical, guttulate, circular, proliferating percurrently to form one or two indistinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses present, (2.8-)4.7-8.6(-12.7) x (1.7-)2.9-4.5(-6.3) µm (average of 50 conidiogenous cells 6.6 x 3.7 µm). *Conidia* hyaline, thin-walled, unicellular, aseptate, occasionally granular, guttulate, surrounded by a persistent mucoid sheath, both apex and base blunt to broadly rounded, bacilliform, (17.9-)19.9-25.7(-30.4) x (5.9-)6.3-7.7(-8.9) µm (average of 50 conidia 22.8 x 7.0 µm).

Cultural characteristics: Mycelium fluffy, initially white to amber (21'b) on the edges and olivaceous (23k) at the edges, becoming white to olivaceous with age. Optimum temperature for growth 25 ºC.

Host: Pterocarpus angolensis.

Additional specimens examined: South Africa, Mpumalanga Province: Sudwala Caves area, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60329; culture CBS124940 = CMW22637); South Africa, Mpumalanga Province: Sudwala Caves area, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60331; culture CBS124941 = CMW22643); South Africa, Mpumalanga Province: Kruger National Park, Pretoriuskop, *P. angolensis*, Sep. 2005, *J. Roux* (PREM60332 = CMW20442); South Africa, Mpumalanga Province: Sudwala Caves area, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60330 = CMW22639).

Notes: BLAST results for the ITS sequences revealed an identity of 98 % with sequences of *Ps. kimberleyense* (GenBank accession EU144059; 505 of 512 bases), *Ps. ardesiacum* (GenBank accession EU144062; 504 of 512 bases), *Ps. adansoniae* (GenBank accession EF585532; 502 of 512 bases), and *Ps. stromaticum* (GenBank accession DQ436935; 502 of 512 bases) while BLAST

results for the elongation factor 1α sequences revealed an identity of 97 % with sequences of *Ps. stromaticum* (GenBank accession DQ436936; 292 of 298 bases), *Ps. ardesiacum* (GenBank accession EU144077; 291 of 298 bases), and *Ps. kimberleyense* (GenBank accession EU144074; 291 of 298 bases).

Pseudofusicoccum violaceum J. W. M. Mehl & B. Slippers sp. nov. Figs. 3b, 5. MB513500
Etym.: Name refers to the distinct violet/purple colour often formed in culture.
Teleomorph: not observed, but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Conidiomata pycnidialia, superficialia, unilocularis, atrobrunnea, pro parte maxima solitaria, fere globosa, mycelio tecta. *Ostiolum* centralis, rotundum et papillatum. *Cellulae conidiogenae* hyalinae, holoblasticae, glabrae, cylindricae, percurrenter cum 1-2 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae, paraphyses non visae. *Conidia* hyalina, parietibus tenuis, unicellularia, eseptata, contento granulari, guttulata, circumdata strato mucido persistente, apicibus basibusque obtusis ad rotunda late, bacilliforma.

Typus: South Africa: Mpumalanga Province: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60333, fruiting structures induced on needles of *Pinus* sp. and branches of *P. angolensis* on WA - *holotypus*, culture ex-type CBS124936 = CMW22679).

Conidiomata on both pine needles and host material pycnidial, superficial, unilocular, dark brown, mostly solitary, more or less globose/circular, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown *textura angularis*; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, diameter (470.4-)498.2-615.9(-659.4) µm (average of 50 cells 557.1 µm). *Ostiole* central, circular and papillate. *Conidiogenous cells* hyaline, holoblastic, smooth, cylindrical, proliferating percurrently to form one or two distinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses not observed, (5.9-)6.1-11.2(-17.0) x (2.7-)3.6-5.0(-6.3) µm (average of 50 conidiogenous cells 8.6 x 4.3 µm). *Conidia* hyaline, thin-walled, unicellular, aseptate, granular, guttulate, surrounded by a persistent mucoid sheath, both apex and base blunt to broadly rounded, bacilliform, (26.5-)29.8-36.1(-39.6) x (8.0-)8.7-10.3(-11.6) µm (average of 50 conidia 33.0 x 9.5 µm).

Cultural characteristics: Mycelium fluffy, initially white to amber (21'b) in the centre and

violet (59'i) on the edges, turning olivaceous (23k) to greenish black (31""k) in the centre and becoming olivaceous to greenish black with age. Optimum temperature for growth 30 ºC.

Host: Pterocarpus angolensis.

Additional specimens examined: South Africa, Mpumalanga Province: Mawewe Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60334; culture CBS124938 = CMW22671); South Africa, Mpumalanga Province: Mawewe Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60335 = CMW22675); South Africa, Mpumalanga Province: Mawewe Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60336 = CMW22683).

Notes: BLAST results for the ITS sequences revealed an identity of 99 % with sequences of *Ps. kimberleyense* (GenBank accession EU144059; 511 of 513 bases) and *Ps. ardesiacum* (GenBank accession EU144062; 510 of 513 bases) while BLAST results for the elongation factor 1α sequences revealed an identity of 98 % with sequences of *Ps. ardesiacum* (GenBank accession EU144077; 292 of 297 bases) and *Ps. kimberleyense* (GenBank accession EU144074; 292 of 297 bases).

Diplodia alatafructa J. W. M. Mehl & B. Slippers sp. nov. Figs. 3c, 6. MB513498
Etym.: Name refers to the Latinized form of the host genus from which it was isolated. *Pterocarpus* (L.) Jacq. is Greek for “winged fruit”, hence the equivalent Latin “alatafructa”.
Teleomorph: not observed, but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Conidiomata pycnidialia, superficialia, unilocularis, atrobrunnea vel nigra, pro parte maxima solitaria, fere globosa, mycelio tecta. *Ostiolum* centrale et cylindrice, histogene. *Cellulae conidiogenae* holoblasticae, hyalinae, discretae, cylindricae, percurrenter cum 2-3 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae. *Conidia* primo hyalina, cum maturitate pigmenta et atrobrunnea, unicellularia, raro uniseptata aut biseptata, raro striata, ellipsoidea vel obovoidea, parietibus crassis, contento granulari, apice rotundata, sine guttulis, glabris.

Typus: South Africa: Mpumalanga Province: Sudwala Caves area, from a bark wound on *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60337, fruiting structures induced on needles of *Pinus* sp. and branches of *P. angolensis* on WA – *holotypus*; culture ex-type CBS124931 = CMW22627).

Conidiomata on both pine needles and host material pycnidial, superficial, unilocular, dark brown to black, mostly solitary, more or less globose/circular, covered with mycelium/hyphae, wall composed of three layers: an outer thick-walled dark brown *textura angularis*; a middle layer of light brown to reddish brown thin-walled cells; and an inner layer of hyaline thin-walled cells, diameter (114.0-)129.0-154.0(-160.0) µm (average of 50 cells 141.4 µm). *Ostiole* central and circular. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, discrete, spherical to cylindrical, proliferating percurrently to form two or three distinct annellations, or proliferating at same level giving rise to periclinal thickenings, (10.0-)12.6-18.2(-23.2) x (8.1-)10.8-14.2(-15.6) µm (average of 40 conidiogenous cells 15.4 x 12.5 µm). *Conidia* initially hyaline becoming pigmented and dark brown with age, unicellular, rarely septate or biseptate, rarely striate, ellipsoid to obovoid, thick-walled, granular, rounded at apices, eguttulate, smooth, (22.4-)24.6-29.2(-32.9) x (9.3-)11.0-13.8(-15.8) µm (average of 50 conidia 26.9 x 12.4 µm).

Cultural characteristics: Mycelium fluffy, initially white to amber (21'b) in the centre turning dark amber within 7 d and becoming white to dark amber (19'b), almost olivaceous (23k) with age. Submerged mycelia (reverse) same except becoming white to dark amber, almost olivaceous, on the edges, and olivaceous in the centre with age. Optimum temperature for growth 25 ºC.

Host: Pterocarpus angolensis

Additional specimens examined: South Africa, Mpumalanga Province: Sudwala Caves area, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60338, culture CBS124932 = CMW22635); South Africa, Mpumalanga Province: Buffelskloof Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60339, culture CBS124933 = CMW22721); South Africa, Mpumalanga Province: Buffelskloof Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60340 = CMW22703).

Notes: BLAST results for the ITS sequences revealed an identity of 99 % with sequences of *D. seriata* (GenBank accession EU080933; 501 of 503 bases) while BLAST results for the elongation factor 1α sequences revealed an identity of 95 % with sequences of both *D. seriata* (GenBank accession EU392282; 250 of 259 bases) and *D. pinea* (GenBank accession EU392263; 249 of 259 bases).

Fusicoccum atrovirens J. W. M. Mehl & B. Slippers sp. nov. Figs. 3d, 7. MB513499

Etym.: Name refers to the dark green colour the fungus forms in culture.

Teleomorph: not observed, but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Conidiomata pycnidialia superficialia, multilocularis, atrobrunnea vel nigra, eustromatica, multipleces, effusa, globosa, mycelio tecta. *Loculus* defixus sine ostiolis visis, histogenis. *Cellulae conidiogenae* hyalinae, holoblasticae, glabrae, discretae, cylindricae, percurrenter cum 1-2 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae. *Conidia* hyalina, parietibus tenuis, unicellularia, eseptata, contento granulari, ellipsoidea vel obovoidea.

Typus: South Africa: Mpumalanga Province: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60341, fruiting structures induced on needles of *Pinus* sp. and branches of *P. angolensis* on WA - *holotypus*, culture ex-type CBS124934 = CMW22674).

Conidiomata on both pine needles and host material pycnidial, superficial, multilocular, dark brown to black, eustromatic, complex, effuse, globose, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown *textura angularis*; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, diameter (179.8-)212.3-273.4(-285.8) µm (average of 50 cells 242.8 µm). *Locule* embedded without visible ostioles, diameter (36.9-)42.8-60.4(-68.2) µm (average of 50 locules 51.6 µm). *Coniophores* absent. *Conidiogenous cells* hyaline, holoblastic, smooth, discrete, cylindrical, proliferating percurrently to form one or two distinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses present, (10.5-)13.7-19(-21.9) x (2.1-)3.3-4.4(-5.4) µm (average of 50 coniodogenous cells 16.3 x 3.8 µm). *Conidia* hyaline, thin-walled, unicellular, aseptate, rarely becoming septate upon germination, granular, ellipsoid to obovoid, (27.1-)30.9-36.0(-40.3) x (5.7-)7.1-9.9(-11.8) µm (average of 50 conidia 33.5 x 8.5 µm).

Cultural characteristics: Mycelium fluffy, initially white to olivaceous (23k) in the centre, edges becoming olivaceous to greenish black (31""k) with age. Submerged mycelia (reverse) initially white to dark amber (19'b) on the edges to olivaceous in the centre, becoming olivaceous to greenish black with age. Optimum temperature for growth 30 ºC.

Host: Pterocarpus angolensis.

Additional specimens examined: South Africa, Mpumalanga Province: Mawewe Nature Reserve, *P. angolensis*, Dec. 2005, *J. W. M. Mehl* (PREM60342, culture CBS124935 = CMW22682).

Notes: BLAST results of the ITS sequences revealed an identity of 91 % with sequences of *B. mamane* (GenBank accession EF118052; 455 of 501 bases) while BLAST results of the elongation factor 1α sequences revealed an identity of 87 % with sequences of *B. corticis* (GenBank accession EU673291; 231 of 265 bases) and 100 % homology with sequences of *B. mamane* generated in this study. However, the ITS phylogeny and several morphological characters support the delineation of *F. atrovirens* as a distinct species from *B. mamane*.

3. 4. Species distribution

The most common species isolated in this study were the two *Pseudofusicoccum* spp., *Ps. olivaceum* (38 isolates, 37.26 %) and *Ps. violaceum* (28 isolates, 27.45 %). Both of these were isolated from asymptomatic branches, suggesting that they are endophytes of *P. angolensis*. *Lasiodiplodia pseudotheobromae* was the third most common species isolated (22 isolates, 21.57 %), mostly obtained from stem wounds. Only five or fewer isolates were obtained for the remaining species identified in this study. In terms of locations sampled; 35 isolates (34.31 %) representing five of the species obtained in this study originated from Mawewe Nature Reserve, 25 isolates (24.51 %) representing two species were obtained from Bushbuckridge Settlement, 19 isolates (18.63 %) representing three species were obtained from the Sudwala Caves area, 15 isolates representing two species were obtained from Pretoriuskop, and 3 isolates representing two species were obtained from Buffelskloof Nature Reserve.

3. 5. Pathogenicity tests

Inoculations resulted in lesions (Fig. 8) within six weeks for both inoculation trials for all isolates and the controls (Fig. 10). In the first trial, isolate ($F = 3.11$, $P = 0.0037$) and fungal species ($F = 5.02$, $P < 0.0001$) were significant predictors of lesion length. Isolates of *L. pseudotheobromae* (CMW22629, $P = 0.0049$, and CMW22656, $P = 0.0001$), *L. crassispora* (CMW22653, $P = 0.0184$) and *D. alatafructa* (CMW22703, $P = 0.0004$) differed significantly from those associated with the control inoculations. In the second trial, only fungal species ($F = 15.63$, $P < 0.0001$) was a significant predictor of lesion length. Neither isolate ($F = 1.51$, $P = 0.1531$) nor branch diameter ($F = 3.78$, $P = 0.0529$) were significant predictors of lesion length. Isolates of *L. pseudotheobromae*

(CMW22629, P < 0.0001; and CMW22656, P < 0.0001) and *D. alatafructa* (CMW22703, P < 0.0001) differed significantly from those associated with the control inoculations. In all cases inoculated fungi were re-isolated from branches and no Botryosphaeriaceae were isolated from the branches inoculated as controls.

4. DISCUSSION

In this study, seven species of the Botryosphaeriaceae were isolated from *P. angolensis* trees in South Africa. Of these fungi, four represent new species described here as *Pseudofusicoccum olivaceum*, *Ps. violaceum*, *Diplodia alatafructa* and *Fusicoccum atrovirens*. The remaining species identified represent the known taxa, *Lasiodiplodia theobromae*, *L. crassispora* and *L. pseudotheobromae*. Prior to this study, only an unknown species of *Sphaeropsis* Sacc., a genus taxonomically related to species in the Botryosphaeriaceae (Denman *et al*. 2000, Phillips *et al*. 2008), had been known from the tree (Vermeulen 1990).

The description of *Ps. olivaceum* and *Ps. violaceum* from *P. angolensis* expands the host and geographic range of *Pseudofusicoccum* spp. This recently described genus accommodates species of the Botryosphaeriaceae producing *Fusicoccum*-like conidia with persistent mucoid sheaths (Crous *et al*. 2006). At the time of its description, *Pseudofusicoccum* was monotypic for *Ps. stromaticum*, isolated from hybrid *Eucalyptus* (Myrtaceae) trees in the Cojedes State (Mohali *et al*. 2006) and *Acacia mangium* in the Portuguesa State, both in Venezuela (Mohali *et al*. 2007). Subsequently, Pavlic *et al*. (2008) identified three new species; *Ps. adansoniae*, *Ps. kimberleyense*, and *Ps. ardesiacum*, from some native Australian trees; a *Eucalyptus* sp., *Ficus opposita* (Moraceae), *Acacia synchronica* (Fabaceae: Mimosidae) and *Adansonia gibbosa* (Bombacaceae), in north Western Australia. Each species was isolated from at least two of these tree species. These results suggest that species of *Pseudofusicoccum* occur mostly on native trees, have a broad host range and are tropical in distribution. All species have optimal growth temperatures of 25-30 ºC and all but one (*Ps. stromaticum* isolated ~10º north of the equator) are found in the southern hemisphere. Recently, Begoude (2009) isolated *Ps. olivaceum* from native *Terminalia sericea* trees, also in the Sudwala Caves area, strengthening the view that this fungus is a common endophyte on other native tree species in South Africa and that species in the genus are plurivorous, rather than host-specific.

Morphologically, both *Pseudofusicoccum* species identified in this study are distinct from other species in the genus. The violet/purple colour formed in culture is distinctive of *Ps. violaceum* and has not been observed in any other *Pseudofusicoccum* species. Conidiomata of *Ps. violaceum* on

both pine needles and branches of *P. angolensis* are superficial and conidiomata of *Ps. olivaceum* sub-cuticular, while conidiomata of *Ps. adansoniae*, *Ps. ardesiacum* and *Ps. kimberleyense* are semi-immersed (Pavlic *et al*. 2008) and conidiomata of *Ps. stromaticum* superficial (Mohali *et al*. 2006). Conidia of *Ps. violaceum* are larger than those of *Ps. stromaticum*, *Ps. adansoniae*, and *Ps. ardesiacum*, only slightly larger than *Ps. kimberleyense*, and remain aseptate post-germination (Mohali *et al*. 2006, Pavlic *et al*. 2008). Conidia of *Ps. olivaceum* are smaller than those of *Ps. kimberleyense* and wider than those of *Ps. adansoniae* (Pavlic *et al*. 2008). Conidiogenous cells of *Ps. violaceum* and *Ps. olivaceum* are wider than those of other *Pseudofusicoccum* species while conidiogenous cells of *Ps. olivaceum* are wider than only *Ps. stromaticum* (Mohali *et al*. 2006, Pavlic *et al*. 2008). Both conidia and coniodogenous cells are larger in *Ps. violaceum* than those of *Ps. olivaceum* and this feature helps to distinguish between the two species.

Both isolates of *F. atrovirens* obtained in this study were from a single tree in Mawewe Nature Reserve. Its limited occurrence, despite extensive sampling in this and the other areas, suggests that the species possibly originated from another plant host in the area. The remaining species in the genus *Botryosphaeria* (anamorph: *Fusicoccum*) (*B. dothidea*, *B. corticis*, *B. mamane*, and *F. ramosum*) have also been reported on multiple plant hosts, with the exception of *F. ramosum* that was isolated as an endophyte of *E. camaldulensis* in Western Australia (Pavlic *et al*. 2008). *Botryosphaeria dothidea*, the type species of the genus, has a cosmopolitan distribution and a broad host range, including angiosperms and gymnosperms (de Wet *et al*. 2007). The fungus has been isolated from asymptomatic *Eucalyptus* hybrids (Mohali *et al*. 2007) and is the cause of shoot blight and canker formation on pistachio in the USA (Ma & Michailides 2002). *Botryosphaeria corticis* has only been reported from *Vaccinium* spp. in the USA associated with stem cankers (Phillips *et al*. 2006).

Fusicoccum atrovirens is most closely related to *B. mamane*. *Botryosphaeria mamane* was recently reported from hybrid *E. urophylla* x *E. grandis* clones and *A. mangium* in Venezuela, associated with die-back symptoms, but also isolated as an endophyte (Mohali *et al*. 2007). Prior to this, the fungus was reported associated with witches'-broom on *Sophora chrysophylla* in Hawaii (Gardner 1997) but there were no authentic cultures and attempts to reisolate the species were unsuccessful (Crous *et al*. 2006). Although *F. atrovirens* and *B. mamane* share identical EF-1α gene sequences, the two are distinct species based on both the ITS phylogeny as well as several distinct morphological characters (Gardner 1997). Such differences include: ostiolate locules with a diameter of 100-200 µm in *B. mamane*, whereas locules in *F. atrovirens* are smaller (diameter of 35-70 µm) and seemingly do not possess ostioles. Furthermore, microconidia have been observed in

B. mamane but not in *F. atrovirens*, paraphyses have been reported only in *F. atrovirens*, and conidia can be one- or two-sepatate in *B. mamane* (Mohali *et al*. 2007) but remain aseptate until germination in *F. atrovirens*. Phylogenetically and morphologically *F. atrovirens* is distinct from the remaining species in the genus. The conidiomata on both pine needles and *P. angolensis* branch tissue are superficial, in contrast to *B. dothidea*, *B. corticis* and *F. ramosum* where conidiomata are semi-immersed/embedded in host tissue and both conidiogenous cells and conidia are larger than those of *F. ramosum*. Conidia are also larger than those of *B. dothidea* and *B. corticis*.

Although the BLAST results indicate close homology to *D. seriata*, *D. alatafructa* is a distinct species based on both morphology and phylogenetic inference. Morphologically, *D. seriata* has conidiogenous cells shorter (5.5 µm) than those of *D. alatafructa* (~10.0-23.0 µm). In the description of this species, it was noted that *D. seriata* has immersed conidiomata while *D. alatafructa* produces superficial conidiomata, but further studies are needed to determine whether this is a consistent character. Both the multiple gene genealogy and single gene phylogenies support the delineation of *D. alatafructa* as a distinct species in *Diplodia*.

The four isolates of *D. alatafructa* obtained in this study originate from two locations and were isolated both as endophytes from healthy as well as diseased plant tissue. It is likely that the species originated from other plant hosts in these areas, especially considering that Begoude (2009) reported it from *T. sericea* trees in the Sudwala Caves area, where it was collected together with *Ps. olivaceum*. Despite the diversity of species in the genus, only six species of *Diplodia*, confirmed based on sequence data, are known to occur in the Southern Hemisphere, including *D. pinea* A morphotype, *D. seriata*, *D. cupressi*, *D. africana*, *D. rosulata* and *D. porosum* (Alves *et al*. 2006, Damm *et al*. 2007, de Wet *et al*. 2000, Gure *et al*. 2005, Phillips *et al*. 2007, van Niekerk *et al*. 2004). Of these six species, *D. pinea* and *D. cupressi* have been reported from only gymnosperms, *D. seriata* has been reported from both gymnosperms and angiosperms, and *D. africana*, *D. rosulata* and *D. porosum*, like *D. alatafructa*, are known only from angiosperm hosts (de Wet *et al*. 2007).

Three *Lasiodiplodia* spp.; *L. crassispora*, *L. pseudotheobromae* and *L. theobromae*, were isolated from *P. angolensis* in this study. This was not surprising as *Lasiodiplodia* spp. are known to occur in tropical and subtropical regions, regions where *P. angolensis* also occurs. All three species are known to be plurivorous with *L. theobromae* reported from both gymnosperms and angiosperms and the remaining species known from angiosperm hosts. Both *L. pseudotheobromae* and *L. theobromae* have a cosmopolitan distribution and have been reported from the tropics in both the

northern and southern hemispheres (Alves *et al*. 2008). However, *L. crassispora* appears restricted to the southern hemisphere (Begoude 2009, Burgess *et al*. 2006) and results of this study add weight to that view. It is thus likely that many, if not most, woody plant species in tropical regions are hosts of these common and often pathogenic *Lasiodiplodia* spp.

In the pathogenicity trials, both isolates of *L. pseudotheobromae* and one isolate of *D. alatafructa* differed significantly from the control inoculations. In the isolations, *L. pseudotheobromae* was the only species for which isolates were obtained from both external stem wounds, as well as from internal asymptomatic tissue. The other species obtained were either all isolated externally from stem wounds (*L. theobromae*, *L. crassispora*) and diseased tissue (*D. alatafructa*), or internally from asymptomatic branches (*F. atrovirens*, *P. olivaceum*, *P. violaceum*). The virulence of the isolates of *L. pseudotheobromae*, along with results of other similar studies (Begoude 2009, Begoude *et al*. 2009) suggests that the species can exist as both a latent pathogen within *P. angolensis* and could play a role in increasing the likelihood of tree die-back and death when trees are stressed. The single isolate of *D. alatafructa* with a high level of virulence in both trials, was obtained from diseased tissue, specifically from the stem of a dying tree, while the other isolate used in the trials was from a stem wound. The isolates of *D. alatafructa* were obtained from geographically distinct areas and genotypic differences were observed in the sequences obtained, clearly indicating that the two are different strains with different levels of virulence. This is not surprising as it is well known that different isolates of Botryosphaeriaceae can differ in their pathogenicity (Mohali *et al*. 2009, Old *et al*. 1986, Pavlic *et al*. 2007, Stanosz *et al*. 2007).

This study is the first to consider the role of the Botryosphaeriaceae in the decline and die-back of *P. angolensis* trees. Some of the seven species of the Botryosphaeriaceae identified in this study are clearly endophytes in this tree. These include species with both hyaline (*F. atrovirens*, *P. olivaceum*, *P. violaceum*) and pigmented (*L. pseudotheobromae*) conidia. Isolates of only two species were pathogenic in field trials. Nevertheless, isolation of the species identified in this study from dead and dying trees and from stem wounds suggests that they could contribute to the overall decline of *P. angolensis* trees in South Africa, but they are probably not primary agents of disease. It is likely, given that the Botryosphaeriaceae are associated with trees under stress (Slippers & Wingfield 2007), that the decline affecting trees observed in South Africa is the result of environmental stresses. The diversity of the Botryosphaeriaceae associated with *P. angolensis* trees in neighbouring countries and the association of this group of fungi with Mukwa disease in Zambia and Zimbabwe, merit further study.

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Table 1. Isolates used in the phylogenetic analysis. Culture numbers in bold indicate ex-type cultures. Accession numbers in italics were obtained from GenBank.

Culture no.	Other collection no.	Identity	Host	Location*	Collector	GenBank Accession no.	
						ITS	EF-1α
CBS119047		*Botryosphaeria corticis*	*Vaccinium corymbosum*	Hammonton, New Jersey, USA	PV Oudemans	*DQ299245*	*EU017539*
ATCC22927		*B. corticis*	*Vaccinium* sp.	North Carolina, USA	RD Millholland	*DQ299247*	*EF614931*
CMW8000	CBS115476	*B. dothidea*	*Prunus* sp.	Crocifisso, Ticino, Switzerland	B Slippers	*AY236949*	*AY236898*
CMW7780	BOT1636	*B. dothidea*	*Fraxinus excelsior*	Molinizza, Ticino, Switzerland	B Slippers	*AY236947*	*AY236896*
CMW13425	CBS117445	*B. mamane*	*A. mangium*	Portuguesa state, Venezuela	S Mohali	*EF118046*	GU134939
CMW13429	CBS117446	*B. mamane*	*Eucalyptus* hybrid	Cojedes state, Venezuela	S Mohali	*EF118048*	GU134940
CBS418.64		*"Botryosphaeria" tsugae*	*Tsuga heterophylla*	British Columbia, Lake Cowichan, Canada	A. Funk	*DQ458888*	*DQ458873*
CMW15198	WAC12398	*Dichomera eucalypti*	*E. diversicolor*	Warne, WA	TI Burgess	*AY744371*	*DQ093214*
CMW15953	BOT10	*Dic. eucalypti*	*E. diversicolor*	Denmark, WA	TI Burgess	*DQ093195*	*DQ093216*
CBS120835	STE-U5908	*Diplodia africana*	*Prunus persica*	Paarl, Western Cape, SA	U Damm	*EF445343*	*EF445382*
CBS121104	STE-U5946	*D. africana*	*P. persica*	Paarl, Western Cape, SA	U Damm	*EF445344*	*EF445383*
CMW22627	CBS124931	*D. alatafructa*	*P. angolensis*	Road servitude, Sudwala Caves area, SA	J Mehl & J Roux	FJ888460	FJ888444
CMW22635	CBS124932	*D. alatafructa*	*P. angolensis*	Road servitude, Sudwala Caves area, SA	J Mehl & J Roux	FJ888461	FJ888445
CMW22721	CBS124933	*D. alatafructa*	*P. angolensis*	Buffelskloof Nature Reserve, SA	J Mehl & J Roux	FJ888478	FJ888446
CBS168.87		*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z Solel	*DQ458893*	*DQ458861*
CBS261.85		*D. cupressi*	*C. sempervirens*	Bet Dagan, Israel	Z Solel	*DQ458894*	*DQ458862*
CMW7060	CBS431.82	*D. mutila*	*Fraxinus excelsior*	Kleine Plas, Maarseveen, Netherlands	HA van der Aa	*AY236955*	*AY236904*
CBS112553		*D. mutila* ("*B. stevensii*")	*Vitis vinifera*	Montemor-o-Novo, Portugal	AJL Phillips	*AY259093*	*AY573219*
CBS121887		*D. olivarum*	*Olea europaea*	Scorrano, Bosco Belvedere, Lecce, Puglia, Italy	S Frisullo	*EU392302*	*EU392279*
CBS121886		*D. olivarum*	*O. europaea*	San Pietro Vernotico, Brindisi, Puglia, Italy	S Frisullo	*EU392301*	*EU392278*
CMW1185	CBS109727	*D. pinea* A morphotype	*P. radiata*	Jonkershoek, Stellenbosch, SA	WJ Swart	*DQ458897*	*DQ458882*
CMW4881	CBS109725	*D. pinea* C morphotype	*P. patula*	Plantation 3, Habinsaran, Sumatra, Indonesia	MJ Wingfield	*DQ458896*	*DQ458881*
CBS116470	Pr3	*D. rosulata*	*P. africana*	Gambo, Ethiopia	A Gure	*EU430265*	*EU430267*
CBS116472	Pr5	*D. rosulata*	*P. africana*	Gambo, Ethiopia	A Gure	*EU430266*	*EU430268*
CMW189	CBS118110	*D. scrobiculata*	*Pinus resinosa*	USA	MA Palmer	*AY253292*	*AY624253*
CMW7775	BOT1642	*D. seriata* ("*B. obtusa*")	*Ribes* sp.	New York, USA	B Slippers	*AY236954*	*AY236903*

Culture no.	Other collection no.	Identity	Host	Location*	Collector	GenBank Accession no.	
						ITS	EF-1α
CBS112555		*D. seriata*	*Vitis vinifera*	Portugal, Montemor-o-Novo	AJL Phillips	*AY259094*	*AY573220*
CBS115035		*Dothiorella iberica*	*Quercus ilex*	Monzón, Aragón, Spain	N Ibarra	*AY573213*	*AY573228*
CBS115041		*Do. iberica*	*Q. ilex*	Aragón, Spain,	AJL Phillips	*AY573202*	*AY573222*
IMI 63581b		*D. sarmentorum* (*Otthia spiraeae*)	*Ulmus sp.*	Warwickshire, England	EA Ellis	*AY573212*	*AY573235*
CBS115038		*Do. sarmentorum*	*Malus pumila*	Delft, Netherlands	AJL Phillips	*AY573206*	*AY573223*
CMW22674	CBS124934	*Fusicoccum atrovirens*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888473	FJ888456
CMW22682	CBS124935	*F. atrovirens*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888476	FJ888457
CMW26167	CBS122069	*F. ramosum*	*E. camaldulensis*	Bell Gorge, WA	TI Burgess	*EU144055*	*EU144070*
MUCC684		*Guignardia* sp.	*Agonis flexuosa*	Yalgorup, WA	TI Burgess	*EU675682*	*EU686573*
MUCC685		*Guignardia* sp.	*A. flexuosa*	Yalgorup, WA	TI Burgess	*EU675681*	*EU686572*
CMW14691	WAC12533	*Lasiodiplodia crassispora*	*Santalum album*	Ord River, Kununurra, WA	TI Burgess	*DQ103550*	*DQ103557*
CMW14688	WAC12534	*L. crassispora*	*S. album*	Ord River, Kununurra, WA	TI Burgess	*DQ103551*	*DQ103558*
CMW22653		*L. crassispora*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888465	FJ888452
CMW22654		*L. crassispora*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888466	FJ888453
CMW22655		*L. crassispora*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888467	FJ888454
CMW22697		*L. crassispora*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888477	FJ888455
CMW14077	CBS115812	*L. gonubiensis*	*Syzygium cordatum*	Gonubie, Eastern Cape, SA	D Pavlic	*AY639595*	*DQ103566*
CMW14078	CBS116355	*L. gonubiensis*	*S. cordatum*	Gonubie, Eastern Cape, SA	D Pavlic	*AY639594*	*DQ103567*
CMW26162	CBS122519	*L. margaritacea*	*A. gibbosa*	Tunnel Creek Gorge, WA	TI Burgess	*EU144050*	*EU144065*
CMW26163	CBS122065	*L. margaritacea*	*A. gibbosa*	Tunnel Creek Gorge, WA	TI Burgess	*EU144051*	*EU144066*
CBS456.78		*L. parva*	Cassava-field soil	Dep. Meta, Vilavicencio, Colombia	O Rangel	*EF622083*	*EF622063*
CBS356.59		*L. parva*	*Theobroma cacao*	Agalawatta, Sri Lanka	A Riggenbach	*EF622082*	*EF622062*
CBS120832	STE-U5803	*L. plurivora*	*P. salicina*	Stellenbosch, Western Cape, SA	U Damm	*EF445362*	*EF445395*
CBS121103	STE-U4583	*L. plurivora*	*V. vinifera*	SA	F Halleen	*AY343482*	*EF445396*
CBS116459		*L. pseudotheobromae*	*Gmelina arborea*	San Carlos, Costa Rica	J Carranza-Velásquez	*EF622077*	*EF622057*
CBS116460		*L. pseudotheobromae*	*A. mangium*	San Carlos, Costa Rica	J Carranza-Velásquez	*EF622078*	*EF622058*
CMW22650		*L. pseudotheobromae*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888464	FJ888447
CMW22666		*L. pseudotheobromae*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888470	FJ888448

Culture no.	Other collection no.	Identity	Host	Location*	Collector	GenBank Accession no.	
						ITS	EF-1α
CMW22667		*L. pseudotheobromae*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888471	FJ888449
CBS111530		*L. theobromae*	Unknown	Unknown	Unknown	*EF622074*	*EF622054*
CMW18420	BOT979	*L. theobromae*	*Casuarina cunninghamii*	Mbale, Uganda	J Roux	*DQ103534*	*DQ103564*
CMW22663		*L. theobromae*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888468	FJ888450
CMW22664		*L. theobromae*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888469	FJ888451
CMW13455	CBS117453	*Neofusicoccum andinum*	*Eucalyptus* sp.	Mountain Range, Mérida state, Venezuela	S. Mohali	*AY693976*	*AY693977*
CMW13446	CBS117452	*N. andinum*	*Eucalyptus* sp.	Mountain Range, Mérida state, Venezuela	S. Mohali	*DQ306263*	*DQ306264*
CBS110299		*N. luteum*	*V. vinifera*	Quinta do Marquês, Oeiras, Portugal	AJL Phillips	*AY259091*	*AY573217*
CMW9076	BOT2482	*N. luteum*	*Malus X domestica*	Kemeu, New Zealand	SR Pennycook	*AY236946*	*AY236893*
CMW9080	BOT2486	*N. parvum*	*Populus nigra*	TePuke/BP, New Zealand	GJ Samuels	*AY236942*	*AY236887*
CMW15950		*N. parvum*	*E. globulus*	Western Australia	TI Burgess	*DQ093193*	*DQ093213*
CMW26147	CBS122055	*Pseudofusicoccum adansoniae*	*Adansonia gibbosa*	Derby, WA	TI Burgess	*EF585523*	*EF585571*
CMW26148	CBS122056	*P. adansoniae*	*Ficus opposita*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EF585524*	*EF295489*
CMW26159	CBS122062	*P. ardesiacum*	*A. gibbosa*	Mount Hardman, Great North Highway, WA	TI Burgess	*EU144060*	*EU144075*
CMW26155	CBS122063	*P. ardesiacum*	*A. gibbosa*	Derby, WA	TI Burgess	*EU144061*	*EU144076*
CMW26156	CBS122058	*P. kimberleyense*	*Acacia synchronica*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EU144057*	*EU144072*
CMW26158	CBS122060	*P. kimberleyense*	*A. gibbosa*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EU144058*	*EU144073*
CMW20881	CBS124939	*P. olivaceum*	*Pterocarpus angolensis*	Pretoriuskop, KNP, SA	J Roux	FJ888459	FJ888437
CMW22637	CBS124940	*P. olivaceum*	*P. angolensis*	Road servitude, Sudwala Caves area, SA	J Mehl & J Roux	FJ888462	FJ888438
CMW22639		*P. olivaceum*	*P. angolensis*	Road servitude, Sudwala Caves area, SA	J Mehl & J Roux	FJ888463	FJ888439
CMW13434	CBS117448	*P. stromaticum*	*Eucalyptus* hybrid	San Carlos/Cojedes state, Venezuela	S Mohali	*AY693974*	*AY693975*
CMW13435	CBS117449	*P. stromaticum*	*Eucalyptus* hybrid	San Carlos/Cojedes state, Venezuela	S Mohali	*DQ436935*	*DQ436936*
CMW22679	CBS124936	*P. violaceum*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888474	FJ888442
CMW20436	CBS124937	*P. violaceum*	*P. angolensis*	Pretoriuskop, KNP, SA	J Roux	FJ888458	FJ888440
CMW22671	CBS124938	*P. violaceum*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888472	FJ888441
CMW22681		*P. violaceum*	*P. angolensis*	Mawewe Nature Reserve, SA	J Mehl & J Roux	FJ888475	FJ888443

*Abbreviations: KNP=Kruger National Park, SA=South Africa, WA=Western Australia, USA=United States of America

Table 2. Statistics for sequence datasets generated in this study and information on maximum parsimony trees for each set of analyses.

Clade considered		**All**		**Fusicoccum**	
Gene region	**EF-1α**	**ITS**	**Combined**	**EF-1α**	**ITS**
Total characters in dataset	826	701	1527	742	599
- variable characters	592	473	386	699	540
- parsimony informative	234	228	1141	43	59
Excluded characters	624	504	945	699	559
Characters considered for analysis	202	197	582	43	40
No. of most parsimonious trees	Unlimited	Unlimited	416	4	2
Tree Length	620	497	1602	49	45
Consistency Index (CI)	0.5855	0.5775	0.6398	0.9592	0.9556
Retention Index (RI)	0.9186	0.9141	0.9333	0.9836	0.9688
Rescaled Consistency Index (RC)	0.5378	0.5278	0.5972	0.9435	0.9257
Figure			Fig. 1	Fig. 2a	Fig. 2b

Figure 1. One of 416 most parsimonious trees of 1602 steps obtained for the combined dataset of the ITS and EF-1α gene regions, after exclusion of uninformative characters. The tree was rooted to two isolates of *Guignardia* sp. Posterior probability values from the Bayesian analysis are provided in bold above the branches, followed by Bootstrap values (1000 replicates – values lower than 50% not shown) in italics either below or above the branches. Genera defined by Crous *et al*. (2006) and Phillips *et al*. (2008) are indicated by encircled numbers (1 = *Pseudofusicoccum*, 2 = *Diplodia*, 3 = *Lasiodiplodia*, 4 = *Dothiorella*, 5 = *Botryosphaeria*, 6 = *Neofusicoccum*).

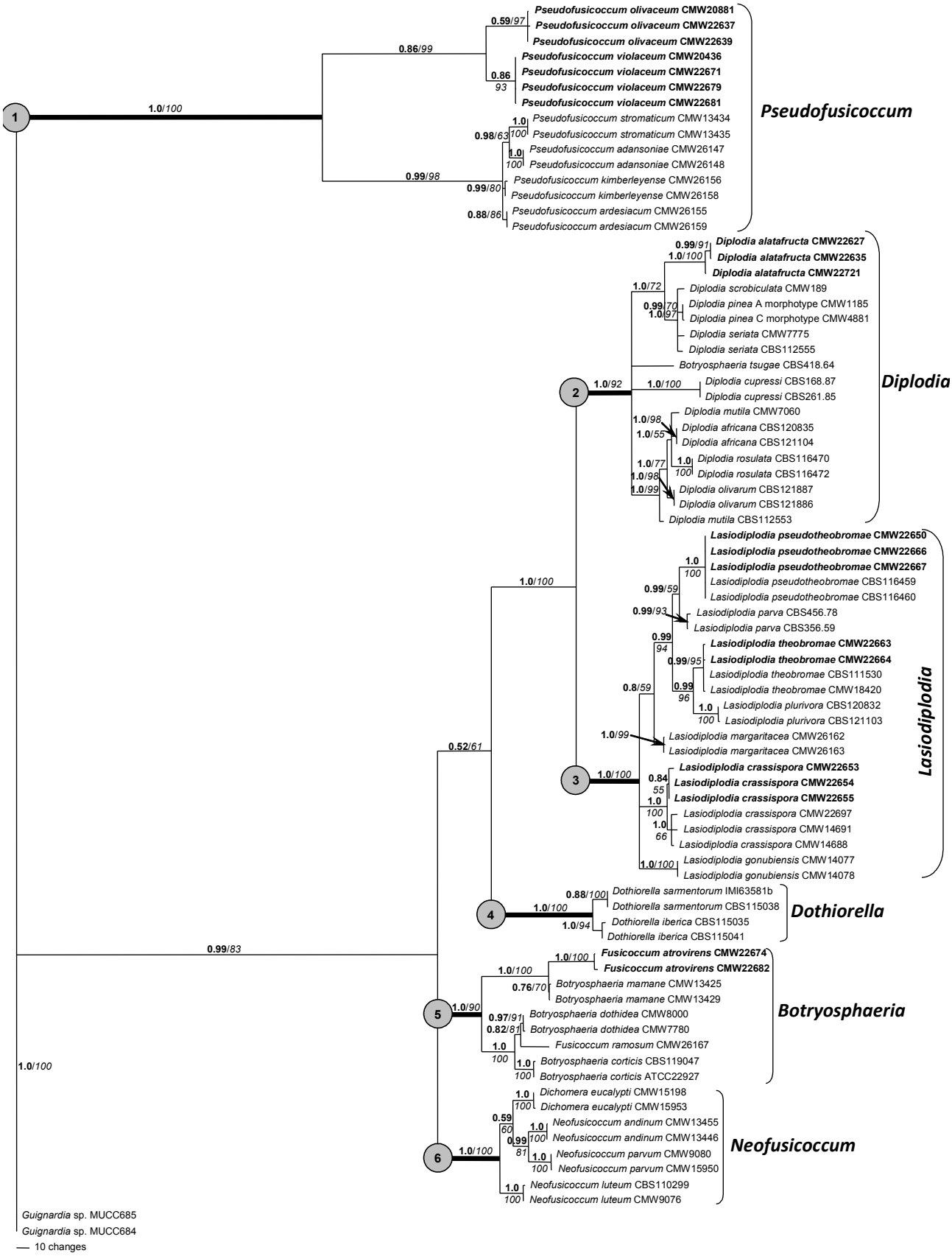
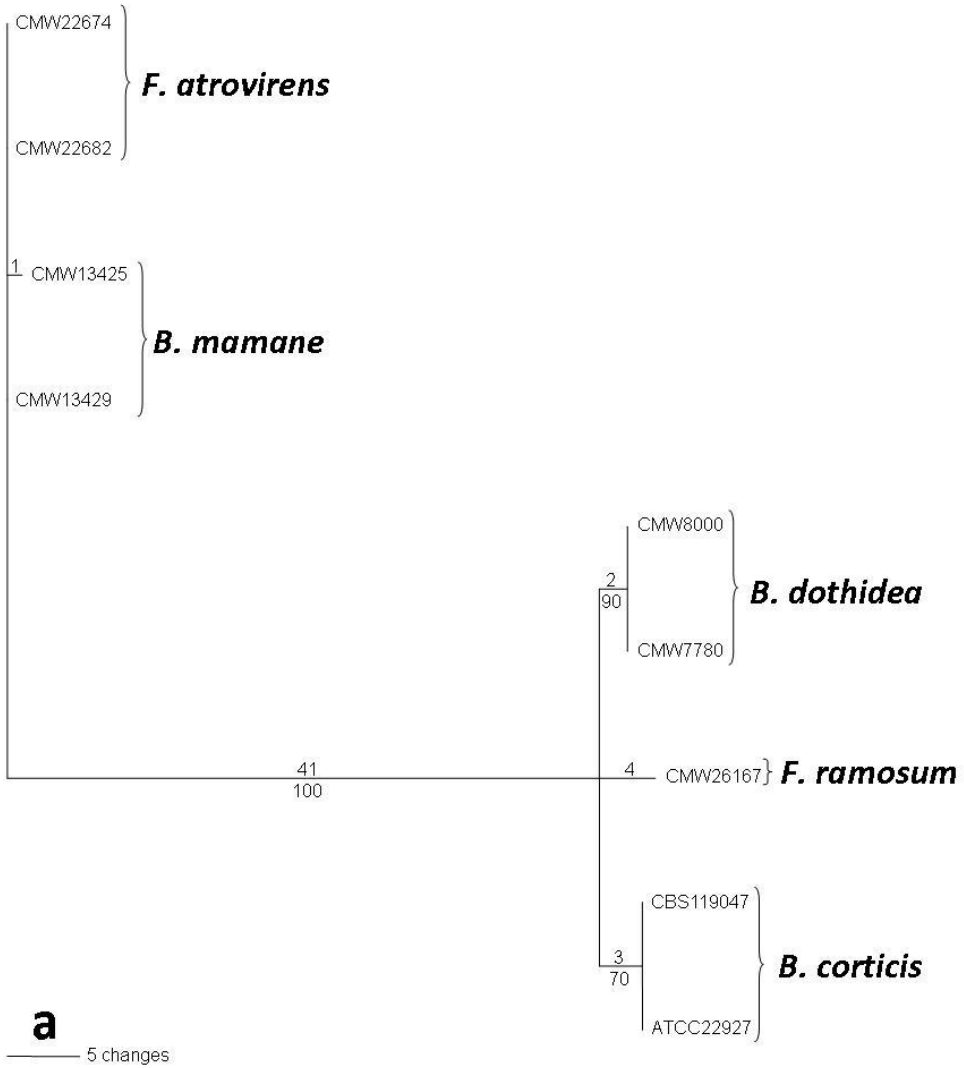
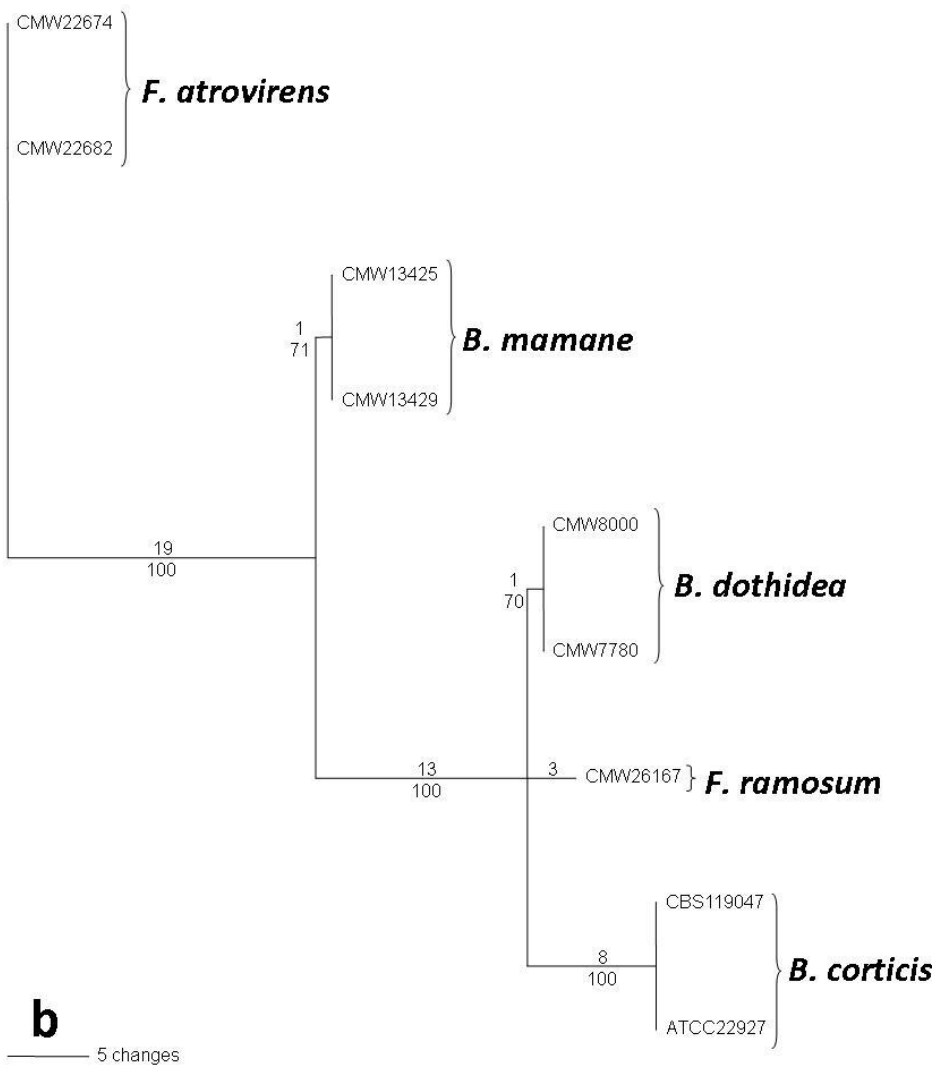

Pseudofusicoccum olivaceum CMW20881
Pseudofusicoccum olivaceum CMW22637
Pseudofusicoccum olivaceum CMW22639
Pseudofusicoccum violaceum CMW20436
Pseudofusicoccum violaceum CMW22671
Pseudofusicoccum violaceum CMW22679
Pseudofusicoccum violaceum CMW22681
Pseudofusicoccum stromaticum CMW13434
Pseudofusicoccum stromaticum CMW13435
Pseudofusicoccum adansoniae CMW26147
Pseudofusicoccum adansoniae CMW26148
Pseudofusicoccum kimberleyense CMW26156
Pseudofusicoccum kimberleyense CMW26158
Pseudofusicoccum ardesiacum CMW26155
Pseudofusicoccum ardesiacum CMW26159
Pseudofusicoccum
Diplodia alatafructa CMW22627
Diplodia alatafructa CMW22635
Diplodia alatafructa CMW22721
Diplodia scrobiculata CMW189
Diplodia pinea A morphotype CMW1185
Diplodia pinea C morphotype CMW4881
Diplodia seriata CMW7775
Diplodia seriata CBS112555
Botryosphaeria tsugae CBS418.64
Diplodia cupressi CBS168.87
Diplodia cupressi CBS261.85
Diplodia mutila CMW7060
Diplodia africana CBS120835
Diplodia africana CBS121104
Diplodia rosulata CBS116470
Diplodia rosulata CBS116472
Diplodia olivarum CBS121887
Diplodia olivarum CBS121886
Diplodia mutila CBS112553
Diplodia
Lasiodiplodia pseudotheobromae CMW22650
Lasiodiplodia pseudotheobromae CMW22666
Lasiodiplodia pseudotheobromae CMW22667
Lasiodiplodia pseudotheobromae CBS116459
Lasiodiplodia pseudotheobromae CBS116460
Lasiodiplodia parva CBS456.78
Lasiodiplodia parva CBS356.59
Lasiodiplodia theobromae CMW22663
Lasiodiplodia theobromae CMW22664
Lasiodiplodia theobromae CBS111530
Lasiodiplodia theobromae CMW18420
Lasiodiplodia plurivora CBS120832
Lasiodiplodia plurivora CBS121103
Lasiodiplodia margaritacea CMW26162
Lasiodiplodia margaritacea CMW26163
Lasiodiplodia crassispora CMW22653
Lasiodiplodia crassispora CMW22654
Lasiodiplodia crassispora CMW22655
Lasiodiplodia crassispora CMW22697
Lasiodiplodia crassispora CMW14691
Lasiodiplodia crassispora CMW14688
Lasiodiplodia gonubiensis CMW14077
Lasiodiplodia gonubiensis CMW14078
Lasiodiplodia
Dothiorella sarmentorum IMI63581b
Dothiorella sarmentorum CBS115038
Dothiorella iberica CBS115035
Dothiorella iberica CBS115041
Dothiorella
Fusicoccum atrovirens CMW22674
Fusicoccum atrovirens CMW22682
Botryosphaeria mamane CMW13425
Botryosphaeria mamane CMW13429
Botryosphaeria dothidea CMW8000
Botryosphaeria dothidea CMW7780
Fusicoccum ramosum CMW26167
Botryosphaeria corticis CBS119047
Botryosphaeria corticis ATCC22927
Botryosphaeria
Dichomera eucalypti CMW15198
Dichomera eucalypti CMW15953
Neofusicoccum andinum CMW13455
Neofusicoccum andinum CMW13446
Neofusicoccum parvum CMW9080
Neofusicoccum parvum CMW15950
Neofusicoccum luteum CBS110299
Neofusicoccum luteum CMW9076
Neofusicoccum
Guignardia sp. MUCC685
Guignardia sp. MUCC684
10 changes

Figure 2. Unrooted majority consensus phylograms of the a) EF-1α and b) ITS gene regions for species in the *Fusicoccum* clade, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above.


CMW22674
CMW22682
F. atrovirens
1
CMW13425
CMW13429
B. mamane
CMW8000
CMW7780
2
90
B. dothidea
41
100
4
CMW26167
F. ramosum
CBS119047
ATCC22927
3
70
B. corticis
a
5 changes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CMW22682
F. atrovirens
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CMW13429
1
71
B. mamane
19
100
CMW8000
CMW7780
1
70
B. dothidea
13
100
3
CMW26167
F. ramosum
CBS119047
ATCC22927
8
100
B. corticis
b
5 changes

Figure 3. Appearance of ten day old cultures on MEA in 90 mm Petri dishes. Mature 10-day old culture morphologies on MEA in 90 mm diam. Petri dishes. a. *Pseudofusicoccum olivaceum*. b. *P. violaceum*. c. *Diplodia alatafructa*. d. *Fusicoccum atrovirens*.

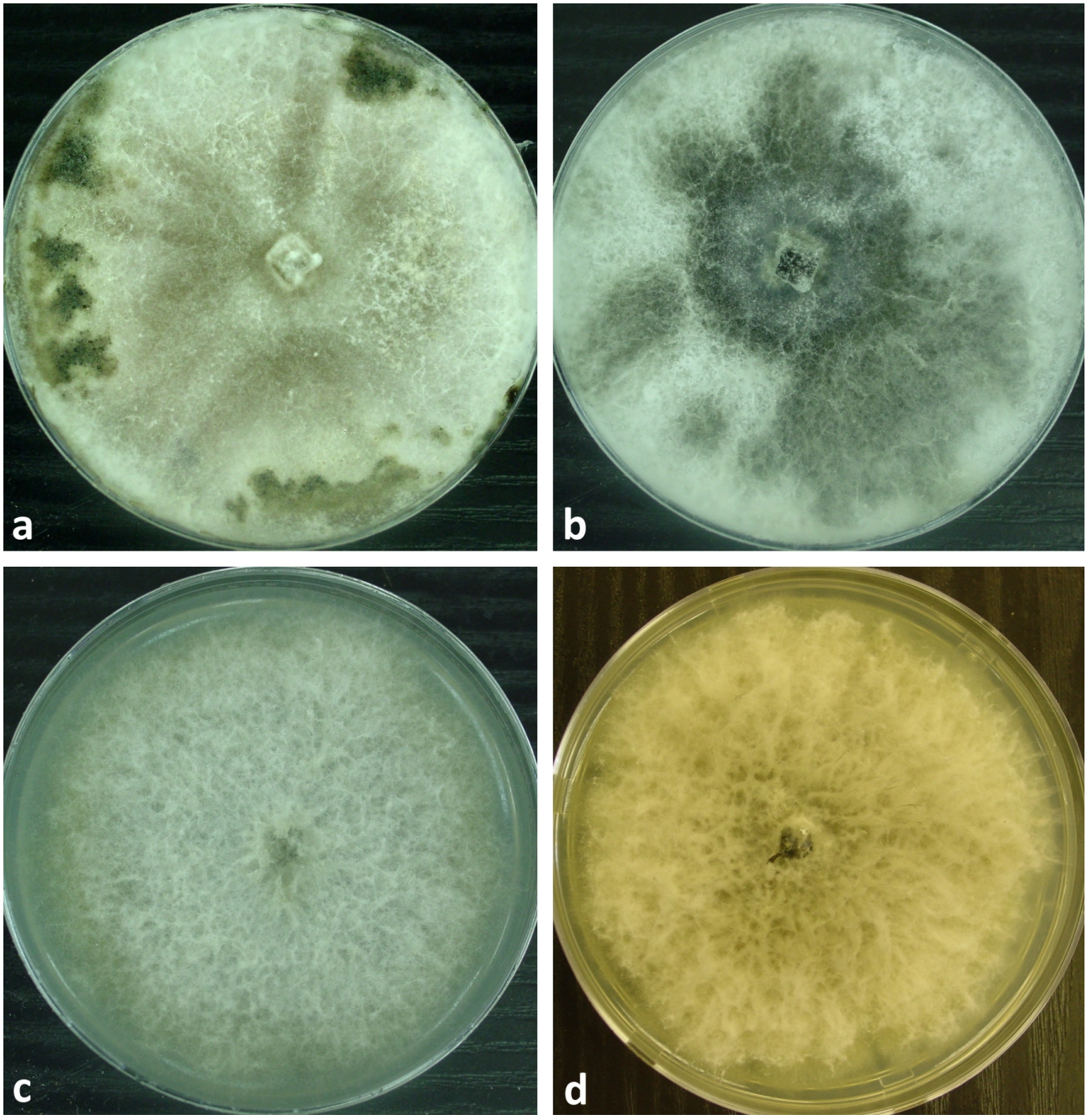

a
b
c
d

Figure 4. *Pseudofusicoccum olivaceum*. a) Pycnidia sporulating on a branch of the host (*Pterocarpus angolensis*). b-c) Conidiogenous cells with immature, developing conidia. d-e) Mature conidia. White arrows indicate annellations while black arrows indicate paraphyses. Scale bars: a) = 500 µm, b-e) = 10 µm.

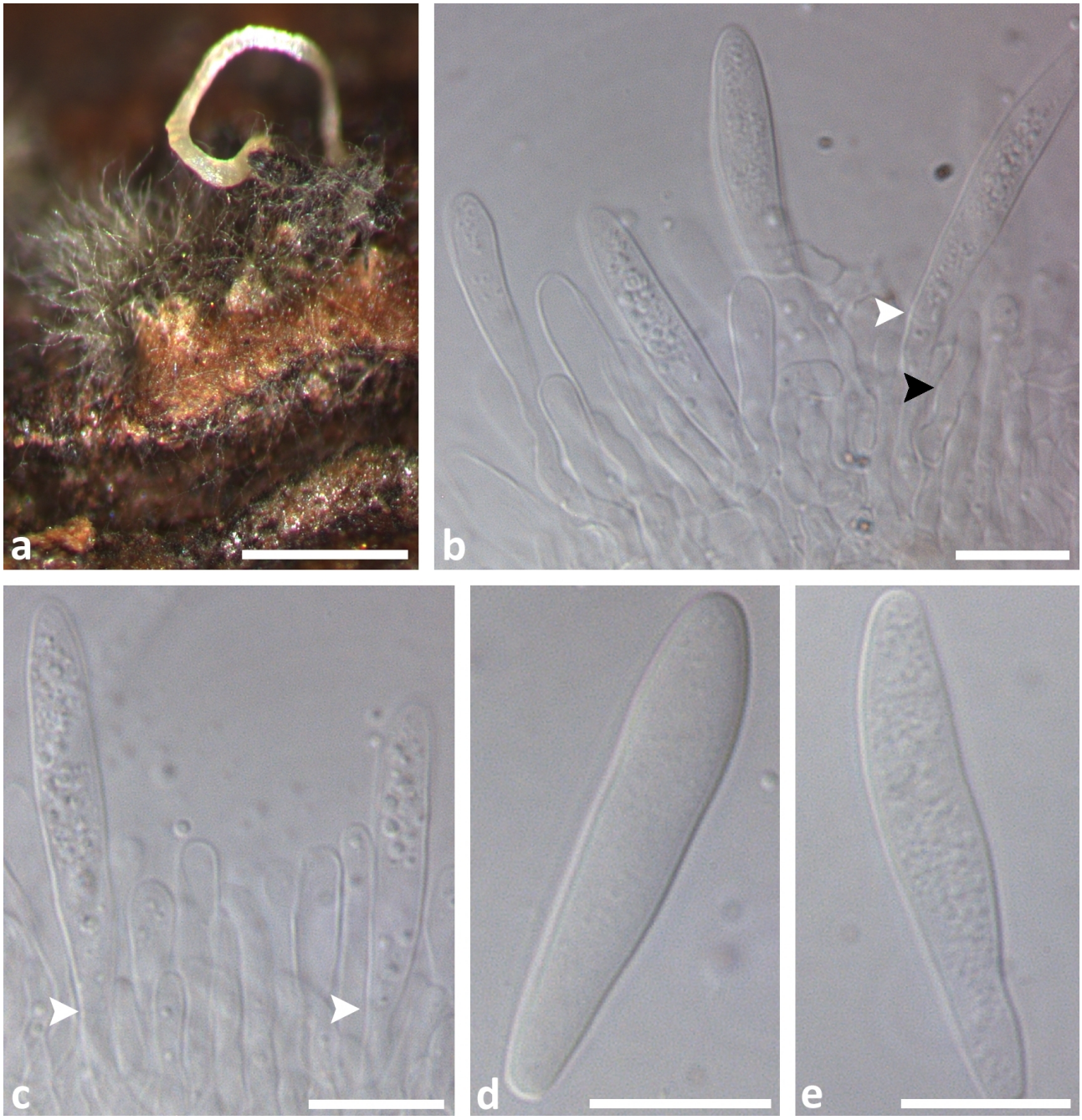


Figure 5. *Pseudofusicoccum violaceum*. a) Pycnidia sporulating on a branch of the host (*P. angolensis*) b-c) Conidiogenous cells and immature, developing conidia. d) Mature conidia. White arrows indicate annellations. Scale bars: a) 200 µm, b-d) = 10 µm.

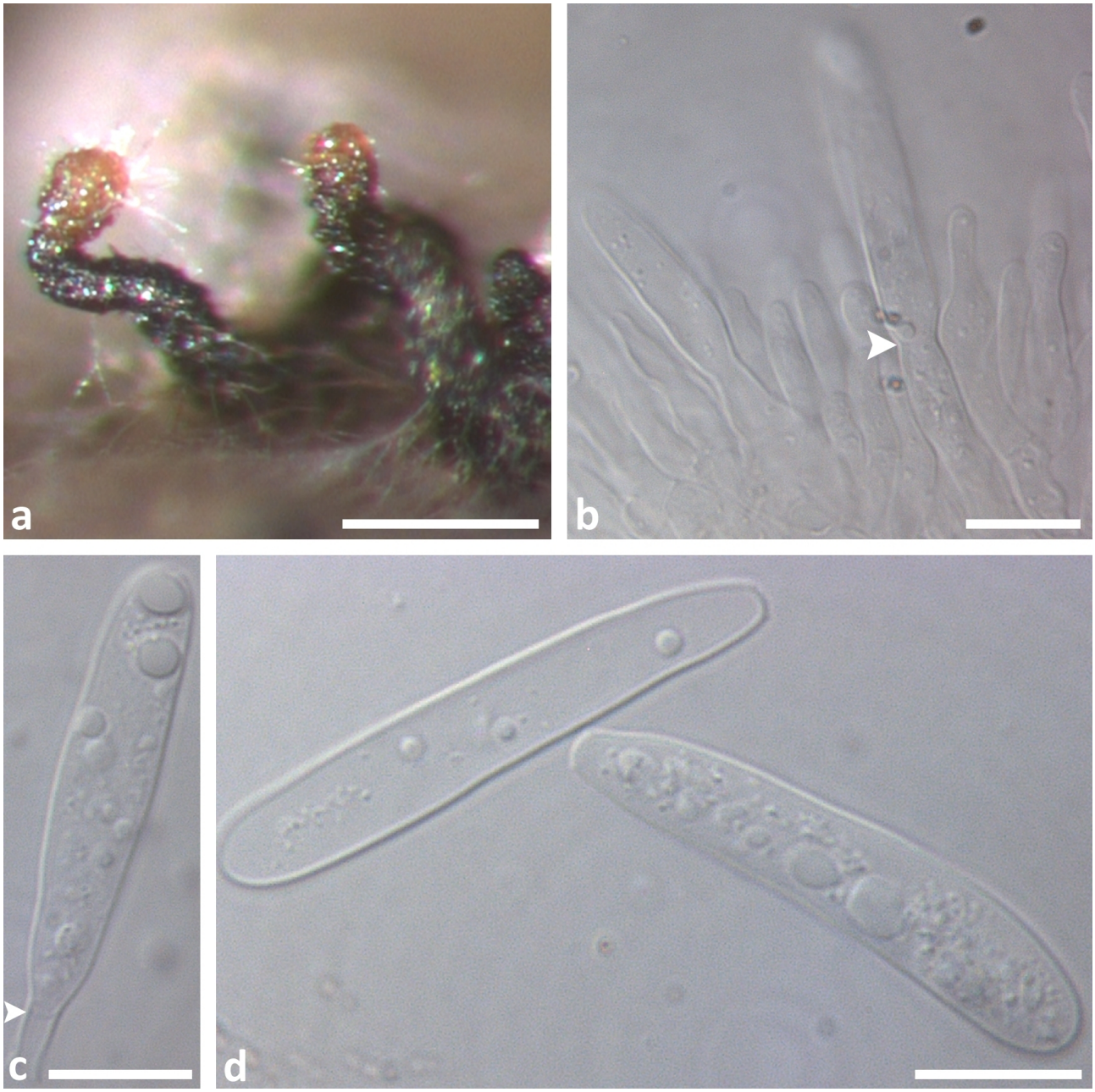

a
b
c
d

Figure 6. *Diplodia alatafructa*. a) Pycnidia sporulating on a branch of the host (*P. angolensis*) b-c) Conidiogenous cells and immature, developing conidia. d-e) Mature conidia. White arrows indicate annellations. Scale bars: a) = 1000 µm, b-e) = 10 µm.

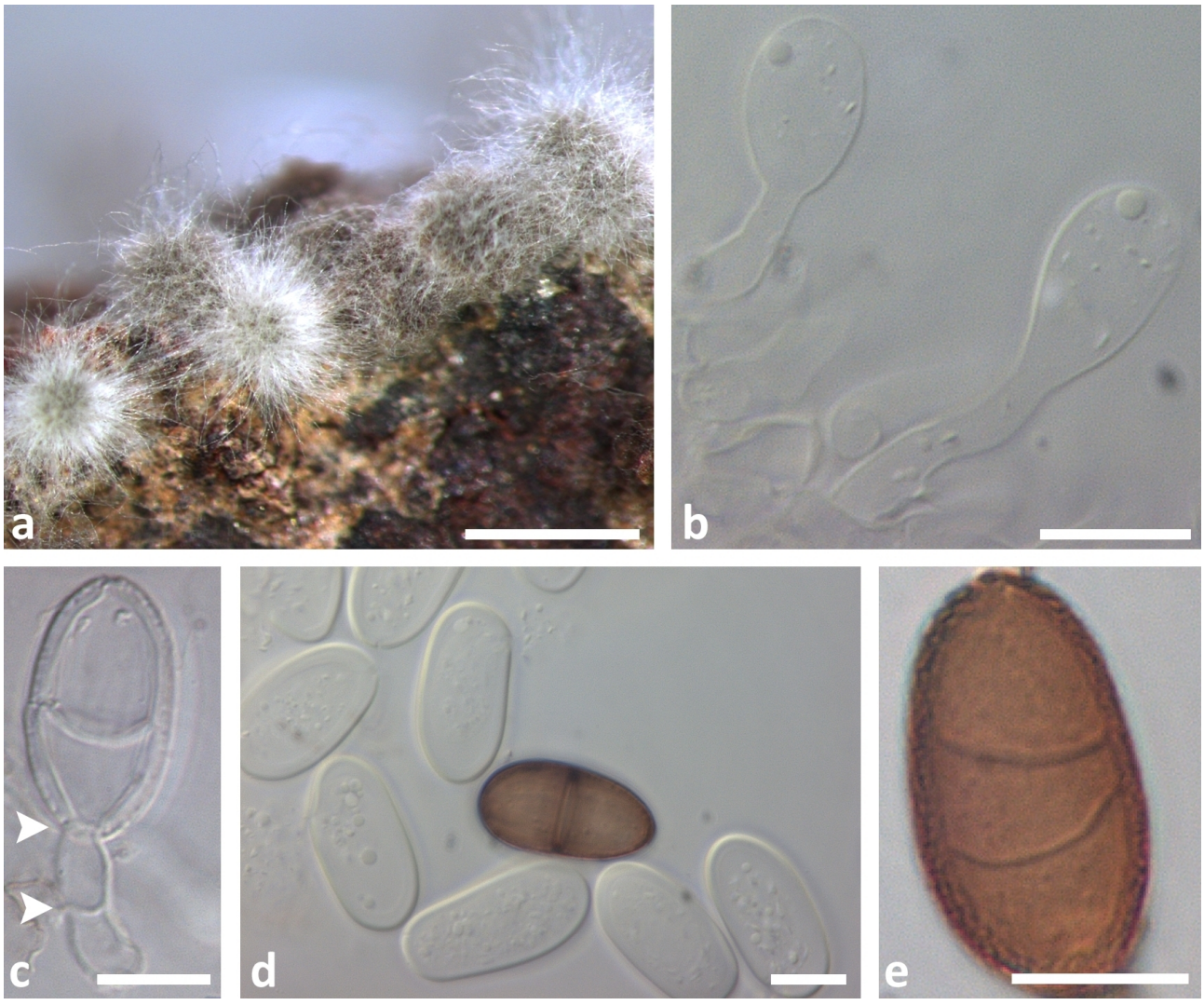

a
b
c
d
e

Figure 7. *Fusicoccum atrovirens*. a) Pycnidia sporulating on a branch of the host (*P. angolensis*). b-c) Conidiogenous cells and immature, developing conidia. d) Mature germinating conidium. e) Mature conidia. White arrows indicate annellations while black arrows indicate paraphyses. Scale bars: a) = 1 000 µm, b,c,e) = 10 µm., d) = 5 µm.

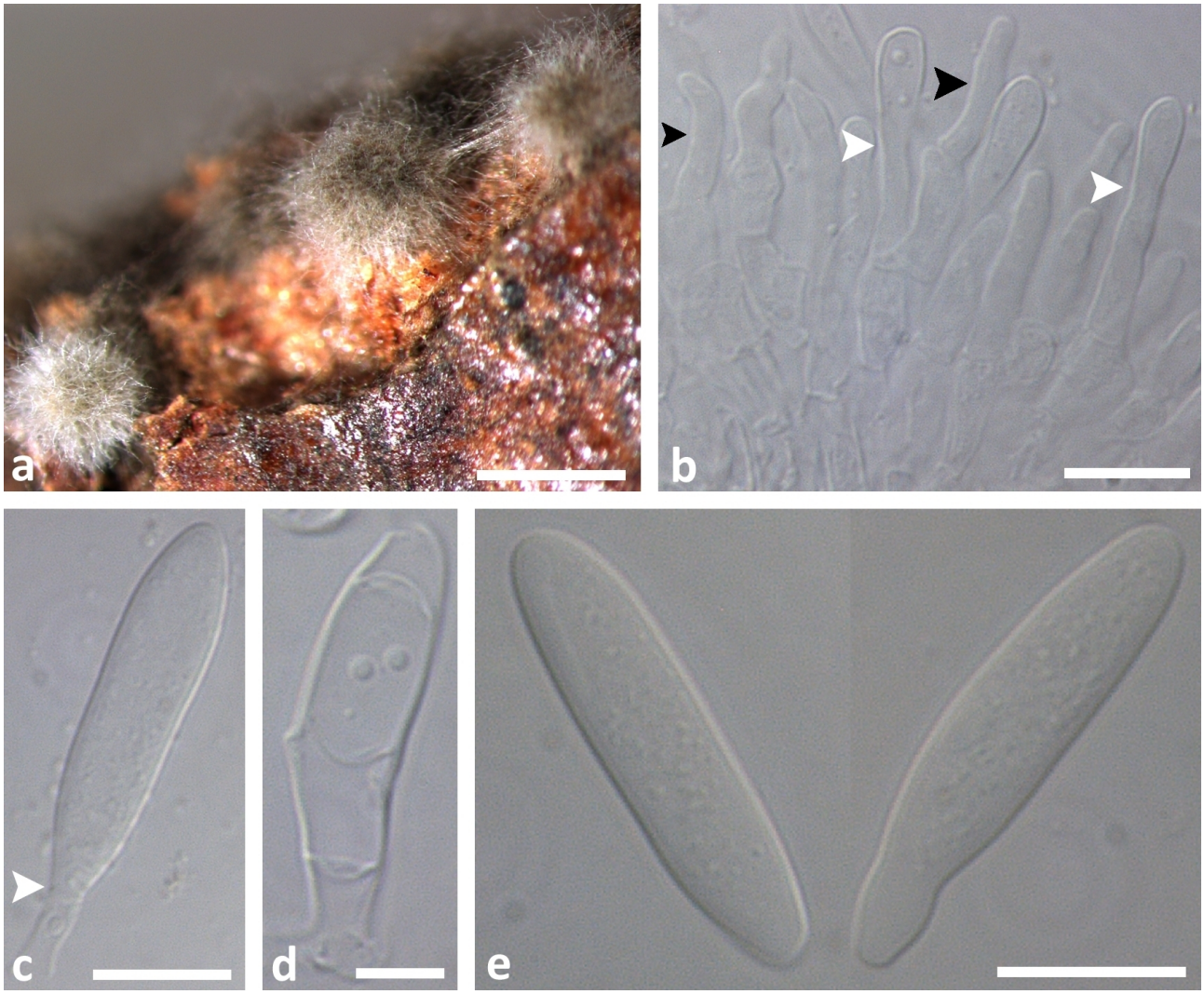

a
b
c
d
e

Figure 8. Lesions produced on inoculated *P. angolensis* branches in the field inoculations. From left to right: Control inoculation, *Pseudofusicoccum violaceum*, *P. olivaceum*, *Diplodia alatafructa*, *Lasiodiplodia pseudotheobromae*, *L. theobromae*, and *L. crassispora*. Incisions in the branch mark the edge of visible lesions and are indicated by arrows .

Figure 9. Map of the sites sampled. The greyed in area on the South African map indicates the Mpumalanga Province where samples were collected from. Sampling sites on the larger map of Mpumalanga Province are denoted by a grey dot with sample sizes (n), fungal species, and distribution fungal distributions obtained, indicated (South Africa map source: http://upload.wikimedia.org/wikipedia/commons/thumb/b/bc/Map_of_South_Africa_with_Mpumalanga_highlighted.svg/763px-Map_of_South_Africa_with_Mpumalanga_highlighted.svg.png, accessed 27/7/2009, Mpumalanga Province map source: http://www.stayinsa.co.za/southafrica/mpumalanga_hotels.html, accessed 27/7/2009)

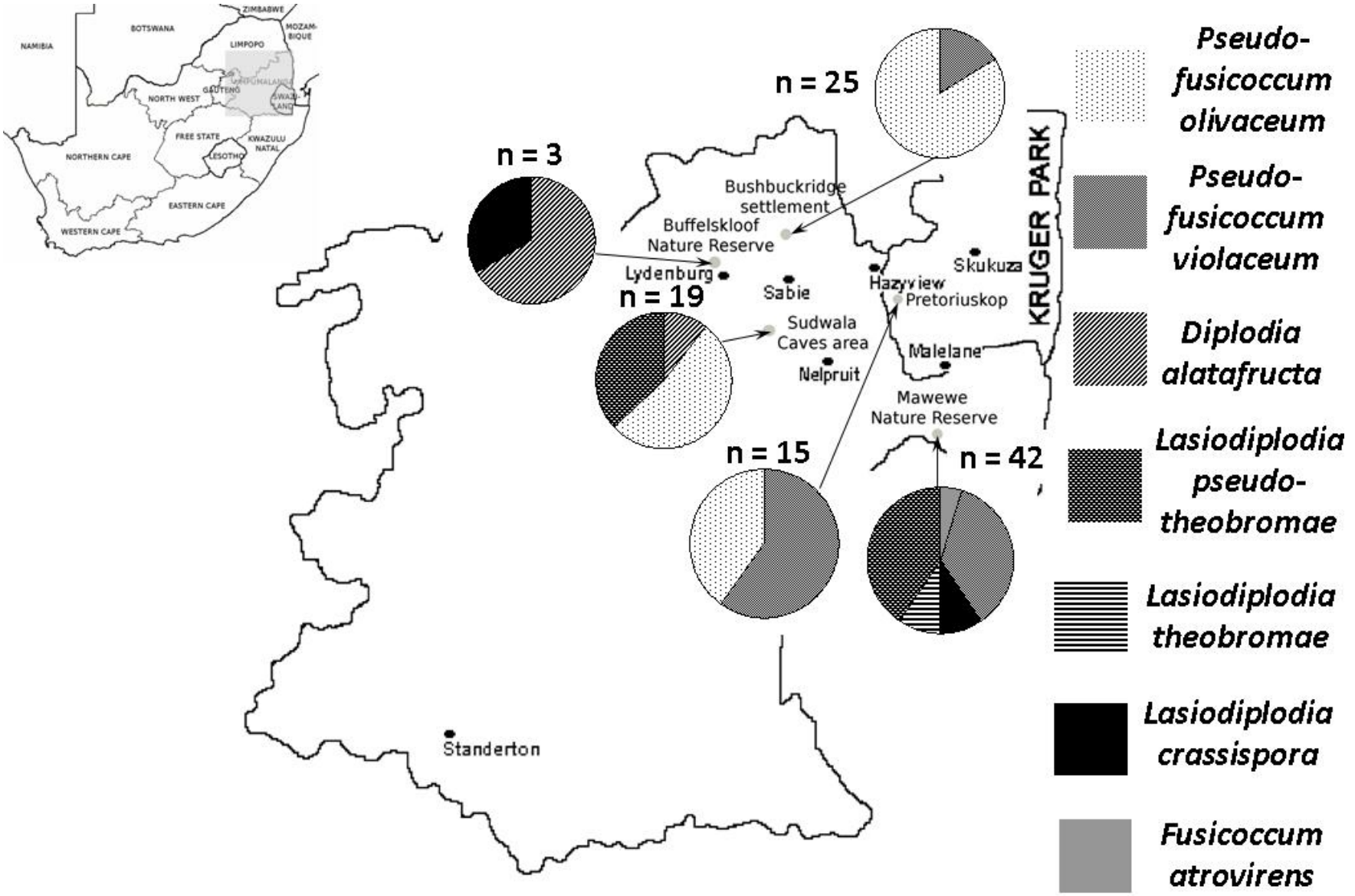
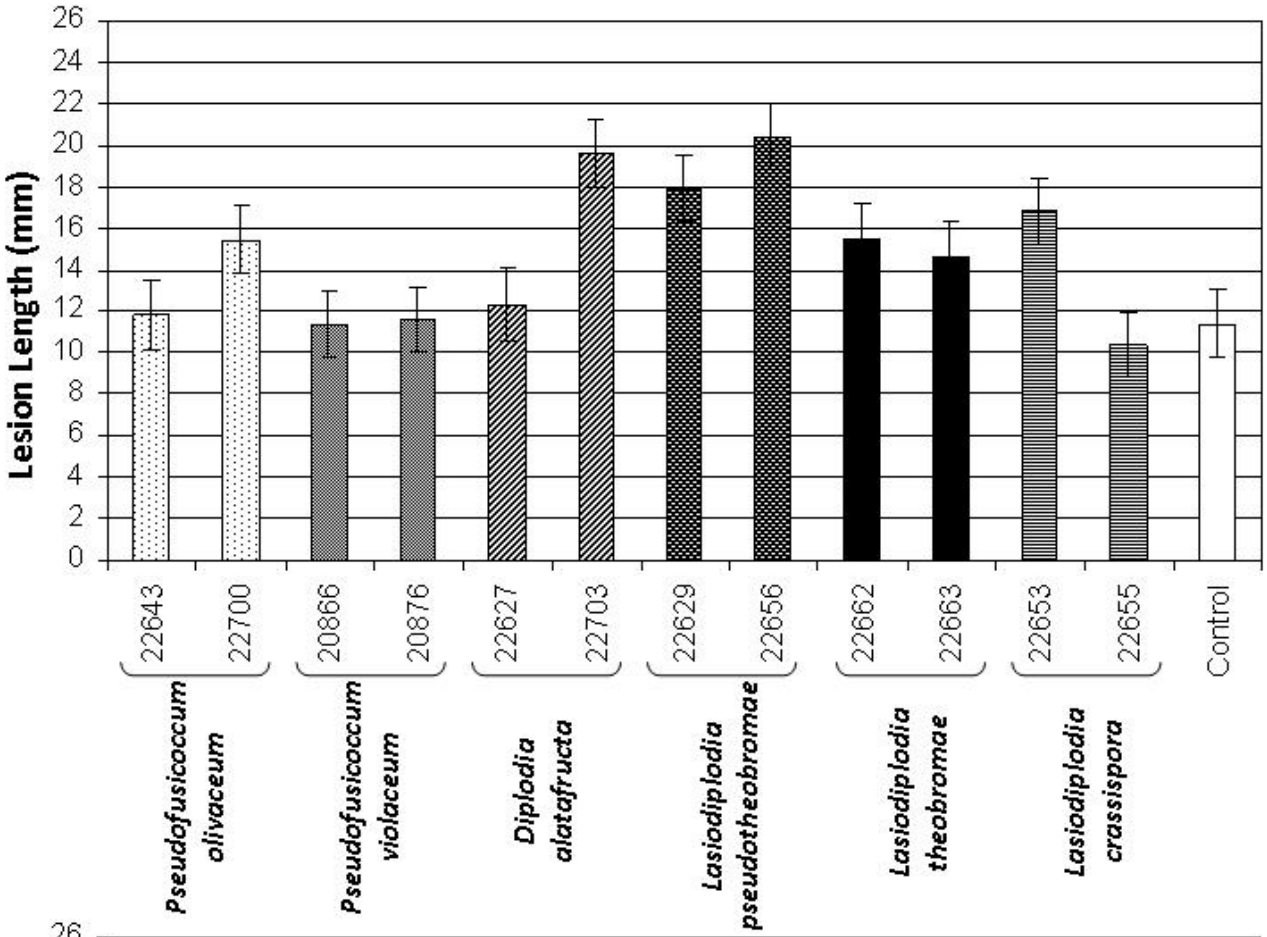
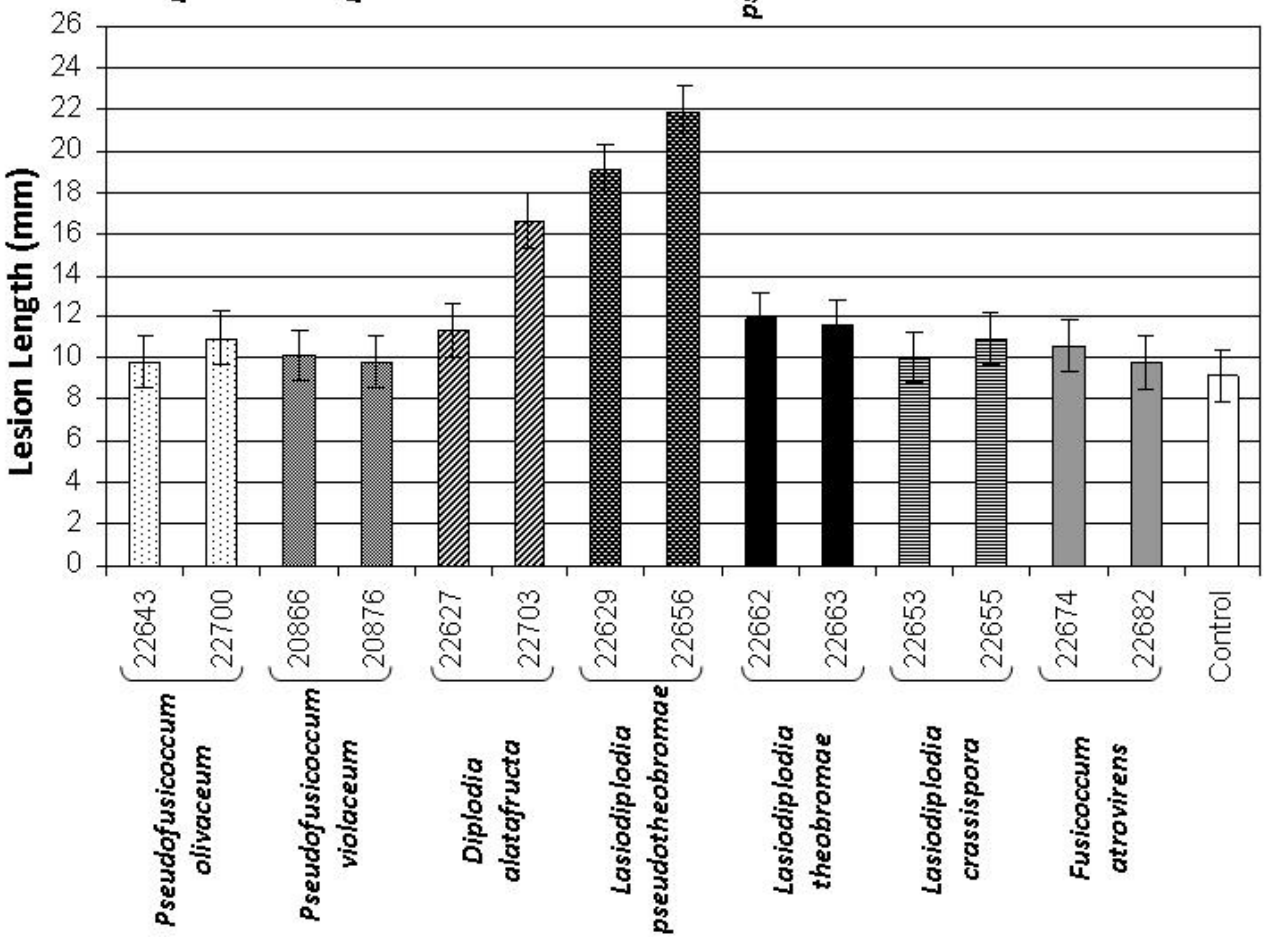

ZIMBABWE
BOTSWANA
MOZAM-BIQUE
NAMIBIA
LIMPOPO
MPUMALANGA
NORTH WEST
GAUTENG
SWAZI-LAND
FREE STATE
KWAZULU NATAL
NORTHERN CAPE
LESOTHO
EASTERN CAPE
WESTERN CAPE
n = 25
n = 3
n = 19
n = 15
n = 42
Bushbuckridge settlement
Buffelskloof Nature Reserve
Lydenburg
Sabie
Sudwala Caves area
Nelpruit
Hazyview
Skukuza
Pretoriuskop
Malelane
Mawewe Nature Reserve
KRUGER PARK
Standerton
Pseudo-fusicoccum olivaceum
Pseudo-fusicoccum violaceum
Diplodia alatafructa
Lasiodiplodia pseudo-theobromae
Lasiodiplodia theobromae
Lasiodiplodia crassispora
Fusicoccum atrovirens

Figure 10. Mean lesion lengths (mm) resulting from inoculation of *P. angolensis* branches with two isolates of each species of the Botryosphaeriaceae identified in this study and a control after six weeks. Bars represent 95 % confidence limits for each isolate.


Lesion Length (mm)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
22643
22700
20866
20876
22627
22703
22629
22656
22662
22663
22653
22655
Control
Pseudofusicoccum olivaceum
Pseudofusicoccum violaceum
Diplodia alatafructa
Lasiodiplodia pseudotheobromae
Lasiodiplodia theobromae
Lasiodiplodia crassispora


Lesion Length (mm)
0
2
4
6
8
10
12
14
16
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24
26
22643
22700
20866
20876
22627
22703
22629
22656
22662
22663
22653
22655
22674
22682
Control
Pseudofusicoccum olivaceum
Pseudofusicoccum violaceum
Diplodia alatafructa
Lasiodiplodia pseudotheobromae
Lasiodiplodia theobromae
Lasiodiplodia crassispora
Fusicoccum atrovirens

CHAPTER 3

CERATOCYSTIS SAVANNAE ASSOCIATED WITH STEM WOUNDS ON *PTEROCARPUS ANGOLENSIS* (KIAAT) IN SOUTH AFRICA

ABSTRACT

Pterocarpus angolensis (kiaat) is a well-known native Southern African tree used in a variety of traditional medicinal applications and it is also a source of timber. Bark harvesting for medicinal use exposes the underlying tissue to infection by wound-infecting pathogens, including species of *Ceratocystis*. The aim of this study was to identify *Ceratocystis* spp. isolated from stem wounds on *P. angolensis* in South Africa and evaluate their pathogenicity to these trees. Isolates of *C. savannae* were collected and their identity confirmed by application of the phylogenetic, morphological and biological species concepts. Comparison of sequence data for the ITS, BT and EF-1α gene regions showed that the *P. angolensis* isolates grouped closely to known isolates of *C. savannae* and *C. oblonga*. Only sequence data from the EF-1α gene region were able to delineate between these species, grouping *P. angolensis* isolates with *C. savannae*. Morphological data comparing the *P. angolensis* isolates and isolates of *C. savannae* and *C. oblonga* showed that all isolates were morphologically indistinguishable and that morphology alone cannot be used to delineate between species. Mating crosses between strains derived from the *P. angolensis* isolates and from the ex-type isolates of *C. savannae* and *C. oblonga* showed that C. oblonga and *C. savannae* are distinct biological species and that the *P. angolensis* isolates represent *C. savannae*. Inoculation of tree branches showed that none of the isolates collected in this study were pathogenic. This study shows that *C. savannae* and *C. oblonga* are cryptic species and it is the first to apply the biological species concept to members of the *C. moniliformis sensu lato* species complex.

1. INTRODUCTION

Pterocarpus angolensis (kiaat) is a well-known native Southern African tree species and it is a source of timber in several African countries (Lowore 1993). In addition, parts of the species, including the bark, roots and sap, are used in a variety of traditional medical applications such as in the treatment of diarrhea, gonnorhea, malaria and wounds, amongst many other ailments (Coates Palgrave 1977). Consequently the species is subject to exploitation involving harvesting of the bark and sap.

Bark harvesting has gained increasing prominence in recent years, due to its commercialization resulting from increased urbanization and subsequent demand for traditional medicines (Geldenhuys 2004). Bark removal and pruning, whether intentional or not, exposes the underlying wood tissue to microbial infection. In this regard, the ophiostomatoid fungi, including species of *Ceratocystis* (Kile 1993) and *Ophiostoma* (Harrington 1993) are commonly found on tree wounds. Species of these fungi that infect wounds include important pathogens such as *C. fimbriata* (Kile 1993), *C. fagacearum* (Juzwik *et al*. 2008), *C. albifundus* (Morris *et al*. 1993, Roux & Wingfield 2009) and the Dutch elm disease fungi *O. ulmi* and *O. novo-ulmi* (Brasier 1988, 1991). Others are apparent saprophytes or of unknown aetiology (Harrington 1993, Seifert 1993).

Ophiostomatoid fungi are well-known associates of insects (Malloch & Blackwell 1993). Those that are found on wounds are carried to these niches either directly by bark beetles (Coleoptera: Curculionidae: Scolytinae) and sap-feeders (Nitulidae, Drosophilidae and others) (Kile 1993) or indirectly by ambrosia beetles (Coleoptera: Curculionidae: Platypodinae) (Beaver 1989, Mueller *et al*. 2005), the latter producing frass contaminated with conidia or hyphal fragments that are dispersed by wind or water (Kile 1993).

Recent studies on wounded trees in Southern Africa have frequently resulted in the collection of either new species of ophiostomatoid fungi (Heath *et al*. 2009b, Kamgan *et al*. 2008a) or expanded the host and/or geographic ranges of one or more taxa of these fungi (Heath *et al*. 2009b, Kamgan *et al*. 2008a, b, Roux *et al*. 2004, 2007). The focus of these studies has either been on exotic commercially grown *Eucalyptus* spp. and *Acacia mearnsii* trees (Heath *et al*. 2009b, Kamgan *et al*. 2008b, Roux *et al*. 2004) or indigenous trees (Kamgan *et al*. 2008a, Roux *et al*. 2007). Ceratocystis spp. have commonly been isolated in the majority of these studies (Heath *et al*. 2009b, Kamgan *et al*. 2008a, Roux *et al*. 2004, 2007). This genus (recently reviewed by Roux & Wingfield 2009) harbours several well-known plant pathogens, including *C. fimbriata*, *C. pirilliformis* (Roux *et al*.

2004) and *C. albifundus* (Heath *et al*. 2009b, Kamgan *et al*. 2008a, Roux & Wingfield 2009, Roux *et al*. 2007). Both *C. fimbriata* (van Wyk *et al*. 2006) and *C. pirilliformis* (Kamgan *et al*. 2009) have a low genetic diversity and have not been reported on native tree species in South Africa, implying that they have probably been introduced into the country. In contrast, *C. albifundus* has a high genetic diversity (Barnes *et al*. 2005) and is known to occur on both native and exotic tree species in Southern Africa (Heath *et al*. 2009b, Kamgan *et al*. 2008a, Roux & Wingfield 2009, Roux *et al*. 2007) and is thus believed to be an African fungus.

The aim of this study was to identify a collection of *Ceratocystis* isolates obtained from artificial wounds made to the stems of *P. angolensis* trees (Mehl unpublished). The identity of these isolates was established by applying the phylogenetic, morphological and biological species concepts. Pathogenicity was also tested by means of branch inoculations.

2. MATERIALS AND METHODS

2. 1. Sample collection and isolation

Wounds were made on the main stems of ten *P. angolensis* trees following the technique of Barnes *et al*. (2003) using a chisel and a 4-pound hammer. The wounded trees were situated at the Sudwala Caves area and the Bushbuckridge settlement. Slices of wood displaying blue/brown discoloration were removed for isolations on a weekly basis.

Two methods were used to induce sporulation of *Ceratocystis* spp. when they were observed on the wood surface and also to stimulate ascomatal production when these structures were not present. The first method involved incubating samples in moist chambers at room temperature (20 – 25 ºC) and examining the wood surfaces daily for the production of ascomata. In the case of the second method, chips of discolored wood were wrapped between carrot slices (Moller & DeVay 1968) that had been soaked in water containing 0.05 g streptomycin sulphate (Sigma, Steinheim, Germany) to inhibit bacterial growth. Carrot baits were incubated at 25 °C for 7 – 10 days and then examined for the presence of ascomata.

Isolations were made from fruiting structures by transferring spore masses to malt extract agar (Biolab, Midrand, South Africa) amended with streptomycin sulphate (MEA+S) or, in some cases, transferring entire non-sporulating structures to the media. All resultant isolates were purified and subsequently single-spored or single hyphal-tipped. All cultures are maintained in the culture

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

2. 2. DNA extraction and PCR amplification

DNA was extracted from all isolates obtained in this study using the technique of van Wyk *et al*. (2006) except that the nucleic acid pellets were resuspended in 50 µl TE buffer (100 mM Tris-HCl, 10 mM EDTA, adjusted to pH 8.0) and digested with 5 µl RNase A (1 mg/ml) at 60 ºC for 1 hour. DNA concentrations were quantified using the NanoDrop® ND-1000 and accompanying software (NanoDrop Technologies, DuPont Agricultural Genomics Laboratories, Delaware, USA).

The ITS rDNA locus (including the ITS1, 5.8S gene and ITS2 regions), and portions of the β-tubulin (BT) and elongation factor 1α (EF-1α) gene regions were amplified using the primer pairs ITS1, ITS4 (White *et al*. 1990), Bt1a, Bt1b (Glass & Donaldson 1995), EF1F and EF2R (Jacobs *et al*. 2004). PCR reactions consisted of 8 ng template DNA, 0.2 µM of each primer, 16 µM each dNTP, 1 X FastStart *Taq* Reaction Buffer (supplied with the enzyme) with added $MgCl_2$, and 0.1 U/µl (5 U) FastStart *Taq* polymerase (Roche Molecular Biochemicals, Mannheim, Germany). Reaction volumes were adjusted to 50 µl by adding sterile Sabax water.

Amplification reactions were performed on a Bio-Rad iCycler Thermal Cycler. Cycling conditions were the same as those of van Wyk *et al*. (2006). PCR products were separated on 2 % agarose-ethidium bromide gels run on a TAE buffer system (Maniatis *et al*. 1982) and visualized under ultraviolet light. Product sizes were estimated using a Lambda DNA/*Eco*RI+*Hin*dIII marker 3 (Fermentas Life Sciences, USA).

2. 3. DNA sequencing and phylogenetic analysis

PCR products were purified using 6 % Sephadex columns (Sigma, Steinheim, Germany) following the manufacturer's instructions. Sequencing PCRs were done using the same primers as in the original PCR and also purified using the Sephadex columns. The PCR amplicons were sequenced in both directions using the ABI PRISM(TM) Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) following the manufacturer's instructions on an ABI PRISM 3100(TM) automated sequencer.

Sequences were analyzed and edited using MEGA4 (Tamura *et al*. 2007). Additional sequences for phylogenetic analysis were obtained from nucleotide blasting against sequences in GenBank (using blastn). Sequences were aligned with MAFFT 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh & Toh 2008) using a manual strategy based on the G-INS-i algorithm.

Sequence data were analyzed using PAUP 4.0b10 (Swofford 2002). Analyses were done using the heuristic search option with 100 random addition sequence replications on the three gene datasets as well as combined datasets. In all cases, tree-bisection-reconnection (TBR) branch swapping was applied. Uninformative characters were excluded and remaining gaps in the sequence alignment treated as a fifth base (NEWSTATE). Partition homogeneity tests (Swofford 2002) were done on all possible combinations of gene datasets to determine whether there was statistical congruence. In all cases, the heuristic search option was selected and 1000 replications done. Bootstrap analyses (50% majority rule, 1000 replications) (Felsenstein 1995) were done on both the individual and combined datasets to determine the confidence levels of the tree branch points. Two isolates of *C. virescens* were used as outgroup taxa (Heath *et al*. 2009b) and were rooted as a monophyletic sister group.

2. 4. Morphological comparisons

Fifty measurements were made for 23 taxonomically informative structures (Table 1) for each isolate obtained in this study. Ascomata, ascospores, and hyphal filaments were mounted in 85 % lactic acid and measurements taken using a HRc Axiocam digital camera and the accompanying Axiovision 3.1 software (Carl Zeiss Ltd., München, Germany). For comparative purposes, measurements were also made of characteristic taxonomic structures for authentic isolates of *C. savannae* Kamgan & Jol. Roux (Kamgan *et al*. 2008a) and *C. oblonga* R. N. Heath & Jol. Roux (Heath *et al*. 2009b). In this case, fifty measurements were made for each structure of the ex-type isolate and, where possible, at least ten measurements were made for each structure of the remaining isolates.

2. 5. Mating studies

To confirm the identity of the isolates obtained in this study, they were crossed with strains derived from ex-type isolates of *C. savannae* and *C. oblonga*. In all cases, sporulating isolates were sub-cultured onto standard half-strength potato dextrose agar (½ PDA) (Biolab, South Africa). Zones in cultures where perithecia and protoperithecia were absent occurred on some of the resultant sub-

cultures. Sub-cultures were made from these sterile zones onto fresh ½ PDA. After two weeks, sub-cultures were inspected under a dissection microscope to ascertain that perithecia were absent. To verify the mating type of resultant sub-cultures, DNA was extracted as previously outlined and a PCR done to determine whether the MAT-2 HMG Box gene was present, using the primer pair EUM2-1 and EUM2-2 (Witthuhn *et al*. 2000). PCR reactions, cycling conditions, and visualization of PCR products were the same as those used previously, except that 45 ng template DNA was used in the PCR reactions.

Based on the results of the PCRs, sub-cultures could be distinguished as either MAT1-1 or self-sterile MAT1-2 strains. These strains were then used in crosses both against each other and against strains from the other isolates considered. Crosses were done as outlined by Harrington and McNew (1997) and resultant perithecia examined microscopically for the presence of ascospores.

2. 6. Pathogenicity tests

Twenty trees in the Sudwala Caves area were selected for branch inoculations with the collected *Ceratocystis* isolates in March and September 2007, and in September and October 2008. As each tree was genetically unique, trees were assigned numbers for both inoculation trials when lesion lengths were measured. Branch diameters of inoculated branches were also measured.

Twenty branches (4.15 – 28.6 mm diam.) were randomly selected for inoculation with each isolate. Cork borers, 5 mm and 9 mm diam. were used to make wounds on the branch by removing a disc of bark. Cork borers were sterilized between inoculations by immersion in 70 % ethanol and flaming. Mycelial plugs (5 mm and 9 mm diam.) from 7-day old cultures were placed in the wounds with the mycelium facing the cambium, using a sterile scalpel. A sterile MEA plug was used for inoculations that served as negative controls. Wounds were sealed with Parafilm to prevent desiccation. Lesion lengths were measured six weeks after the branches had been inoculated. Re-isolations were made from all branches on every fifth tree to ascertain whether the inoculated fungi remained present at the termination of the experiment. Inoculation trials were repeated once.

2. 7. Statistical analyses

Data generated for both the morphological characteristics of the various isolates investigated and the pathogenicity trials, were subjected to a two-way analysis of variance (ANOVA) using the General Linear Model Procedure from SAS, Type III Sum of Squares, F-test of SAS, and Fisher's Pairwise

Test (SAS Institute 2004). For the data from the morphological characters, the model considered the interaction between isolate, structure nested within species, and species. For the data from the pathogenicity trials, the model included the tree, the branch diameter, the fungal species, and the isolate nested within the fungal species as predictors of lesion length where trees were considered as blocks. When results were significantly different ($P \leq 0.05$), the Least Squares Means were generated, and in conjunction with Fisher's Pairwise Test, the differences amongst the means were evaluated for statistical significance.

3. RESULTS

3. 1. Isolate collection and morphology

Wood samples taken from artificially induced wounds frequently displayed streaked discoloration and ascomata typical of the ophiostomatoid fungi were occasionally observed on the wound surface. Attempts to induce sporulation and ascomatal production using both carrot baiting and moist chambers were unsuccessful. Direct isolations from perithecia and dried ascospore masses resulted in the collection of four isolates of a *Ceratocystis* sp.

Ascomata of the *Ceratocystis* sp. isolated had elongate bases with conical spines, a disc-like structure at the base of the ascomatal necks, divergent ostiolar hyphae and hat-shaped ascospores. Both primary and secondary conidiophores were present and these produced cylindrical conidia as well as oblong- to barrel-shaped conidia. These characteristics are typical of *Ceratocystis* spp. in the *C. moniliformis sensu lato* species complex (Heath *et al*. 2009b, Kamgan *et al*. 2008a). One of the four isolates (CMW26363) produced only anamorphic structures.

3. 2. DNA sequencing and phylogenetic analyses

PCR amplification of the ITS, BT and EF-1α gene regions yielded fragments of ~500 bp, ~490 bp and 860-870 bp, respectively. Individual and combined gene datasets, identities of isolates, culture and accession numbers are listed in Tables 2 and 3. Phylogenetic trees including majority consensus bootstrap values were generated for each dataset and deposited in TreeBASE (Figs. 1-5, SN4882). Of the individual gene regions, the EF-1α dataset provided the best resolution of isolates obtained in this study amongst those in the *C. moniliformis sensu lato* species complex (Fig. 3). Thus, all species in this complex could be clearly defined in the analyses of sequences for this gene region. This gene region also contained several species-specific single nucleotide polymorphisms that can

be used to distinguish between *C. savannae* and *C. oblonga* (Table 4). Based on phylogenetic inference of the EF-1α gene region, isolates from *P. angolensis* grouped with known isolates of *C. savannae*. Results for the other two gene regions did not provide sufficient resolution to delineate between *C. oblonga* and *C. savannae*, although the isolates from *P. angolensis* grouped with these taxa. Partition homogeneity tests indicated congruency between the ITS and BT gene regions (P = 0.158) but incongruency in the remaining combinations of gene regions. Majority consensus bootstrap trees were generated for the combined dataset of ITS and BT (Fig. 4), and the combined dataset of all three gene regions (Fig. 5). Sequences generated for the phylogenetic analyses in this study were deposited in GenBank (Table 2).

3. 3. Morphological comparisons

To confirm that the isolates from *P. angolensis* were *C. savannae*, measurements were made of morphological structures and statistical analysis done on the measurements. For every structure in the four isolates from *P. angolensis* measured, the range of measurements of the isolates overlapped with those of known isolates for *C. savannae* and *C. oblonga* (Table 1). Measurements of the ex-type isolates of both species also indicated a larger size range in the morphological characters than initially provided in both species descriptions (data not shown). The means of some of the structures produced by an isolate could be distinguished from the same structures of the remaining isolates, such as the longer ascomatal neck in CMW17298 (*C. savannae*) or the longer primary conidiophore base width in CMW23802 (*C. oblonga*). These results, however, indicate the contributing effect of genetic variation between isolates of a species. Consequently, the isolates from *P. angolensis* could not be grouped with either *C. oblonga* or *C. savannae* based on morphology, even though phylogenetic inference of the EF-1α gene region indicated that they were *C. savannae*. In addition, the overlap in measurements of morphological characters could not distinguish or delineate between *C. oblonga* or *C. savannae*. Consequently, the two species are cryptic relatives and morphologically indistinguishable from each other (Bickford *et al*. 2007).

3. 4. Mating studies

Strains of both the MAT1-1 and MAT1-2 mating types were derived from the three isolates obtained from *P. angolensis* that produced sexual structures. From isolate CMW23423, ten strains were derived, of which five are MAT1-1 and five MAT1-2. From isolate CMW23424, eight strains were derived, of which seven are MAT1-1 and one MAT1-2. From isolate CMW23426, eleven strains were derived, of which five are MAT1-1 and six were MAT1-2. Strains of each isolate were

randomly chosen and crossed with each other to verify that they represented effective tester strains of the same species. Most crosses resulted in the production of ascomata with viable ascospores (Table 5) providing support that the three isolates were of the same biological species.

Mating type strains were also derived from the ex-type isolates of *C. savannae* (CMW17300) and *C. oblonga* (CMW23803). For *C. savannae* (CMW17300), ten strains were derived of which five are MAT1-1 and five MAT1-2. These strains were crossed with strains derived from CMW23423 and most of the crosses resulted in cultures producing ascomata with viable ascospores (Table 6). For *C. oblonga* (CMW23803), six strains were derived of which five are MAT1-1 and one MAT1-2. These strains were crossed with strains derived from two of the *P. angolensis* isolates (CMW23424 and CMW23426) and the crosses did not result in cultures producing ascomata with viable ascospores (Table 7). Consequently, the *P. angolensis* isolates obtained in this study belong to the same biological species, *C. savannae*.

3. 5. Pathogenicity tests

Inoculations resulted in the development of lesions (Fig. 6) within six weeks for both inoculation trials for all isolates, as well as for the controls (Fig. 7). For isolates CMW23424 and CMW23426, only tree genotype was a significant predictor of lesion length ($F = 15.63$, $P < 0.0001$) in the first trial. For isolates CMW23423 and CMW26363, only tree genotype was a significant predictor of lesion length in both inoculation trials ($F = 2.28$, $P = 0.0028$ and $F = 2.36$, $P = 0.0019$ respectively). Lesions associated with inoculations of the test isolates did not differ significantly from the control inoculations in either of the trials. *Ceratocystis savannae* was re-isolated from the branches of every fifth tree inoculated with this fungus but never from the lesions associated with the control inoculations.

4. DISCUSSION

In this study, four isolates of a *Ceratocystis* sp. residing in the *C. moniliformis sensu lato* species complex were obtained from artificially induced wounds on *P. angolensis* trees. These isolates were identified as *C. savannae* based on comparisons of DNA sequence data, particularly data from the EF-1α gene region. The results were confirmed based on mating tests between strains derived from three of these *C. savannae* isolates and those of the ex-type strains of *C. savannae* and *C. oblonga*.

The discovery of *C. savannae* on *P. angolensis* in the Sudwala Caves area confirms both the

plurivorous nature and previously known distribution of this fungus. Isolates of the species were first collected from several trees including *Acacia nigrescens*, *Combretum zeyheri*, *Sclerocarya birrea* and *Terminalia sericea* from Skukuza in the Kruger National Park (Mpumalanga Province), and from *Burkea africana* and *T. sericea* from Leeuwfontein Collaborative Nature Reserve (Gauteng Province) (Kamgan *et al*. 2008a). It is likely that the fungus is native to South Africa and its presence on wounds of *P. angolensis* is not surprising.

Isolates of *C. savannae* in this study and the previous study of Heath *et al*. (2009a) were morphologically very similar to *C. oblonga*, a species recently isolated and described from freshly cut *Acacia mearnsii* stumps in Piet Retief (Mpumalanga Province) (Heath *et al*. 2009b). Results of the present study showed clearly that morphological characteristics of the two fungi overlap and cannot be used to distinguish between them. The two fungi do, however, have different growth optima in culture with *C. oblonga* growing best at 20 – 25 ºC and *C. savannae* displaying optimal growth at 30 ºC (Kamgan *et al*. 2008a, Heath *et al*. 2009b). However, the two species are able to coexist in the same area; both have been reported from Leeuwfontein although *C. oblonga* was isolated from nitidulid beetles in the area (Heath *et al*. 2009a) while *C. savannae* was isolated from wounds on several tree species (Kamgan *et al*. 2008a).

The fact that *C. savannae* and *C. oblonga* are morphologically indistinguishable from each other but different based on phylogeny and biological habit is evidence of cryptic speciation. Cryptic species result when "speciation is not accompanied by a morphological change," also referred to as morphological stasis (Bickford *et al*. 2007). This morphological stasis may be the result of environmental conditions that do not select for a morphological character or it may reflect the fact that organisms are limited in their ability to adapt to environmental conditions (Bickford *et al*. 2007). Although there is some dispute (Bickford *et al*. 2007), Giraud *et al*. (2008) argue, using several examples, that cryptic species arise via allopatric divergence, or when species are reproductively isolated from each other. Even when cryptic species occur in the same geographic location, they can be host-specific (Giraud *et al*. 2008). In the context of this study and other species reports (Kamgan *et al*. 2008a, Heath *et al*. 2009b), *C. oblonga* may be host-specific on exotic *A. mearnsii* and consequently an introduced pathogen. In contrast, *C. savannae* is probably a host generalist on native tree species, including *P. angolensis*.

Mating crosses performed in this study confirmed that isolates obtained from *P. angolensis* represent *C. savannae*. Importantly, they also provided robust data to show that *C. savannae* and *C. oblonga* are distinct biological species. This adds confidence to the phylogenetic data based on the

EF-1α sequences that also distinguish between these species. This study also represents the first example where mating studies have been used to distinguish a species in the *C. moniliformis sensu lato* species complex, although the biological species concept has been used to define species in the *C. fimbriata* (Engelbrecht & Harrington 2005, Johnson *et al*. 2005) and *C. coerulescens* (Harrington & McNew 1998, Harrington *et al*. 2002) *sensu lato* species complexes.

Phylogenetic analyses of the ITS and BT gene regions did not support delineation between isolates of *C. savannae* and *C. oblonga*, whereas DNA sequence data from the EF-1α gene region provided sufficient resolution in the form of several species-specific SNPs to distinguish between the two species. The biological species concept also successfully delineated between the two species. However, the biological species concept has been criticized, both for its inability to properly define relationships (Baum & Donoghue 1995, Donoghue 1995) and because it measures potential rather than actual gene flow between species (Taylor *et al*. 2000). In its place, Taylor *et al*. (2000) advocate the use of genealogical concordance phylogenetic species recognition (GCPSR), a technique based on phylogenetic species concepts that determines concordant topology amongst multiple gene genealogies. Based on GCPSR, *C. savannae* and *C. oblonga* might be treated as different species although gene genealogies based on sequence data for gene regions other than those considered here would need to be analyzed to resolve this question.

Field inoculation trials revealed that *C. savannae* is not pathogenic to *P. angolensis*. Previous inoculation trials evaluating the pathogenicity of the species to *A. nigrescens* and *S. birrea* resulted in lesions that differed significantly from the control inoculations (Kamgan *et al*. 2008a). However, the hosts considered in these studies are very different, probably accounting for the different results. Those of Kamgan *et al*. (2008a) also resulted from trials undertaken in a greenhouse whereas the present investigation was undertaken on trees in the field. Nevertheless, Kamgan *et al*. (2008a) noted that the fungus has a very low degree of pathogenicity, consistent with the results of the present study.

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Table 1. Comparison of measurements (µm) of morphological characters of the *P. angolensis* isolates, isolates of *C. savannae* and isolates of *C. oblonga*. Means of ranges follow in brackets. Means in the same row followed by the same letter are not significantly different at a 5 % level. Ex-type isolates are indicated in bold.

Character	***P. angolensis* isolates**				***C. savannae* isolates**								***C. oblonga* isolates**			
	CMW 23423	CMW 23424	CMW 23426	CMW 26363	**CMW 17300**	CMW 17297	CMW 17298	CMW 17301	CMW 17302	CMW 17306	CMW 17575	CMW 17576	**CMW 23803**	CMW 23802	CMW 23804	CMW 23805
Ascomata																
- neck length	272.1–676.8 (471.9) a	377.3–800.1 (575.3) bc	401.3–789.3 (543.6) b	–	319.3–1070.2 (547.7) b	–	692.2–1198.1 (949.2) g	424.7–818.3 (621.2) d	558.6–732.4 (635.8) d	495.9–881.5 (693.7) e	248.9–680.6 (437.7) a	381.0–993.3 (695.1) e	358.3–765.2 (572.1) c	–	–	591.6–903.1 (802.8)f
- neck tip width	10.0–16.7 (13.5)a	10.6–23.0 (15.3)a	11.0–16.4 (13.4)a	–	11.2–18.6 (13.9)a	–	10.0–20.1 (15.3)a	9.5–14.1 (12.5)a	13.5–18.7 (15.9)a	11.1–16.0 (13.3)a	12.8–15.6 (14.0)a	10.8–17.3 (13.7)a	12.3–22.1 (15.8)a	–	–	11.4–18.1 (15.1)a
- neck base width	25.3–58.3 (42.5)a	32.3–59.5 (45.7)a	27.5–51.6 (41.3)a	–	29.5–62.5 (43.4)a	–	29.7–47.4 (40.9)a	25.0–57.1 (45.6)a	41.0–76.7 (59.2)a	31.5–48.1 (41.8)a	35.1–83.0 (47.2)a	31.6–64.5 (47.9)a	21.8–70.8 (46.5)a	–	–	38.8–58.4 (46.7)a
- base length	153.5–375.4 (250.0) def	145.8–401.7 (254.3) efg	149.1–353.9 (232.7) bcdef	–	123.4–332.6 (245.8) def	–	142.5–320.4 (247.0) cdefg	205.7–445.5 (282.8)fg	351.4–443.7 (393.8) h	201.3–369.1 (287.3) g	139.0–247.5 (176.5) a	145.4–295.9 (206.0) abcd	179.1–402.2 (271.5)fg	–	–	174.2–253.4 (214.7) bcdf
- base width	149.9–362.4 (235.6) def	139.5–356.8 (232.6) cdef	134.7–336.3 (217.1) bcde	–	121.8–304.1 (229.2) cdef	–	95.0–252.3 (196.9) abc	199.4–417.3 (273.0)f	322.0–393.7 (348.8) g	151.2–342.1 (252.8) ef	129.1–225.3 (164.1) abd	106.6–286.6 (184.2) ab	175.1–317.7 (238.6) def	–	–	136.4–230.7 (196.6) abc
- disciform base width	57.6–184.9 (111.1) a	70.4–153.6 (115.7) a	69.5–162.2 (104.4) a	–	51.1–143.9 (96.5)a	–	75.1–144.0 (117.1) a	72.4–173.8 (125.0) a	110.0–129.3 (118.2) a	90.3–169.4 (128.6) a	78.8–154.0 (110.4) a	65.1–127.1 (94.4)a	65.4–153.0 (108.0) a	–	–	86.1–110.0 (96.9)a
- conical spine length	3.6–20.5 (8.5)a	5.7–34.6 (13.2)a	4.4–18.5 (11.3)a	–	4.0–13.3 (8.3)a	–	5.8–16.1 (10.1)a	4.1–8.6 (5.8)a	3.9–17.9 (7.5)a	3.7–8.1 (5.8)a	5.8–17.7 (11.6)a	5.0–13.1 (7.5)a	5.6–39.8 (14.4)a	–	–	6.6–16.8 (11.2)a

Character	*P. angolensis* isolates				*C. savannae* isolates								*C. oblonga* isolates			
	CMW 23423	CMW 23424	CMW 23426	CMW 26363	**CMW 17300**	CMW 17297	CMW 17298	CMW 17301	CMW 17302	CMW 17306	CMW 17575	CMW 17576	**CMW 23803**	CMW 23802	CMW 23804	CMW 23805
Ostiolar hyphae																
- length	9.6–43.6 (20.2)a	9.6–34.0 (22.2)a	19.0–51.6 (29.0)a	–	9.6–35.9 (26.2)a	–	10.7–48.9 (34.8)a	7.3–16.3 (10.3)a	7.4–13.7 (9.7)a	10.6–36.8 (17.6)a	9.6–39.7 (19.6)a	18.2–31.0 (22.4)a	12.6–36.8 (25.8)a	–	–	18.4–42.0 (31.6)a
Hat-shaped ascospore																
- length	3.9–7.1 (5.5)ab	4.3–6.7 (5.6)ab	4.2–9.7 (6.7)c	–	4.4–6.9 (5.3)ab	–	4.9–7.5 (6.2)b	4.5–5.8 (5.3)ab	5.1–6.6 (5.8)ab	3.8–6.6 (5.6)ab	–	4.9–6.3 (5.6)ab	5.4–10.0 (7.1)d	–	–	4.7–6.1 (5.4)ab
- sheath width	2.4–5.2 (4.1)ab	3.3–5.2 (4.2)b	3.0–7.2 (5.0)c	–	2.8–4.6 (3.9)ab	–	3.9–5.6 (4.7)c	3.3–4.3 (3.8)ab	3.3–4.8 (4.0)ab	2.3–5.2 (3.9)ab	–	3.5–4.5 (4.0)ab	3.6–8.1 (5.7)d	–	–	2.9–5.0 (3.8)ab
- width	1.5–2.9 (2.2)abcd	1.9–3.0 (2.3)bcd	1.8–3.8 (2.8)d	–	1.5–3.0 (2.2)abcd	–	1.9–3.2 (2.7)cd	1.7–3.4 (2.3)abcd	1.8–2.8 (2.3)abcd	1.4–3.1 (2.3)abcd	–	1.6–2.7 (2.2)abcd	2.4–6.5 (4.3)e	–	–	1.6–4.0 (2.7)cd
Oblong/barrel-shaped conidia																
- length	3.6–8.9 (6.1)ab	4.6–10.2 (6.5)ab	3.6–10.8 (6.6)ab	3.2–17.6 (7.4)ab	2.8–15.9 (6.5)ab	4.6–10.1 (6.4)ab	5.2–9.6 (6.9)ab	4.0–6.3 (5.2)ab	4.1–7.0 (5.1)ab	3.6–6.1 (4.7)ab	5.4–8.4 (6.6)ab	3.9–4.9 (4.4)ab	3.5–10.0 (6.6)ab	5.4–9.1 (7.2)b	3.6–8.2 (5.7)ab	5.1–9.0 (6.3)ab
- width	2.9–5.6 (4.0)ab	2.8–8.2 (4.8)ab	3.1–7.6 (4.8)ab	2.6–5.7 (3.6)ab	2.1–7.0 (3.9)ab	4.0–6.2 (5.0)ab	2.9–4.4 (3.5)ab	3.1–4.5 (3.6)ab	2.2–4.7 (4.0)ab	2.4–4.5 (3.7)ab	3.7–4.9 (4.4)ab	3.4–4.4 (3.7)ab	3.1–8.5 (5.3)b	3.9–6.8 (5.2)ab	3.1–5.1 (4.0)ab	2.3–3.7 (3.1)ab
Cylindrical/bacilliform conidia																
- length	3.9–8.5 (5.8)a	4.9–8.0 (6.1)a	3.6–9.2 (5.9)a	5.3–8.3 (6.7)a	4.2–10.8 (6.1)a	4.5–6.4 (5.5)a	5.7–8.3 (6.9)a	5.1–7.1 (6.2)a	4.8–6.4 (5.4)a	5.1–7.9 (6.2)a	4.9–9.5 (6.5)a	4.9–12.0 (7.4)a	3.6–11.4 (6.8)a	5.3–8.7 (7.0)a	4.4–8.4 (5.9)a	4.4–6.4 (5.5)a
- width	0.9–2.8 (1.9)a	1.5–3.8 (2.5)a	1.1–2.9 (2.1)a	2.1–2.9 (2.4)a	1.5–2.9 (2.2)a	1.8–2.6 (2.2)a	1.8–2.8 (2.3)a	1.8–2.2 (1.9)a	1.8–2.3 (2.0)a	2.1–2.6 (2.4)a	2.4–3.4 (2.8)a	1.5–2.4 (1.9)a	1.2–3.1 (2.3)a	2.4–4.3 (3.2)a	2.1–3.7 (2.8)a	1.5–2.1 (1.7)a
Primary conidiophore																

Character	*P. angolensis* isolates				*C. savannae* isolates								*C. oblonga* isolates			
	CMW 23423	CMW 23424	CMW 23426	CMW 26363	**CMW 17300**	CMW 17297	CMW 17298	CMW 17301	CMW 17302	CMW 17306	CMW 17575	CMW 17576	**CMW 23803**	CMW 23802	CMW 23804	CMW 23805
- length	11.5–37.8 (20.2)fgh	11.7–51.6 (22.9)h	7.7–41.9 (18.8)efg	5.9–35.9 (14.9)bcde	8.8–32.0 (17.7)def	8.2–24.8 (14.3)bcde	6.8–21.0 (13.7)abcd	9.0–32.2 (16.2)bcdef	8.2–16.7 (10.9)ab	9.2–37.8 (17.1)defg	10.9–50.4 (25.3)i	9.4–26.1 (18.1)defg	6.2–38.2 (19.1)efgh	15.3–23.4 (19.4)efgh	15.5–22.3 (19.3)efgh	14.3–30.7 (21.1)gh
- tip width	1.1–2.9 (1.8)a	1.3–4.1 (2.0)a	1.3–3.7 (1.9)a	1.1–3.2 (1.8)a	1.0–2.6 (1.9)a	1.0–3.9 (2.2)a	1.5–3.1 (1.9)a	1.4–2.6 (1.8)a	1.2–1.9 (1.6)a	1.1–2.3 (1.8)a	1.1–2.6 (1.8)a	1.2–2.1 (1.6)a	0.8–3.0 (1.8)a	1.7–2.9 (2.4)a	1.4–2.5 (1.9)a	1.5–3.1 (2.0)a
- medium width	1.8–5.3 (3.0)a	1.7–8.2 (3.8)a	2.1–7.1 (3.3)a	1.8–4.5 (3.0)a	1.8–4.1 (2.8)a	1.8–4.4 (2.8)a	2.1–3.6 (2.8)a	2.2–5.0 (3.2)a	2.3–3.4 (2.5)a	2.1–5.4 (3.7)a	1.8–2.6 (2.1)a	2.7–4.3 (3.1)a	1.4–6.2 (2.9)a	2.5–4.8 (3.3)a	1.7–3.0 (2.7)a	1.8–3.5 (2.7)a
- base width	2.2–6.9 (3.5)a	1.6–7.6 (3.5)a	1.4–7.7 (2.9)a	1.5–5.1 (2.6)a	1.7–4.7 (3.0)a	1.8–4.5 (3.0)a	2.1–3.5 (2.7)a	2.0–3.2 (2.7)a	1.7–4.9 (2.4)a	1.9–6.4 (3.0)a	1.3–4.8 (2.6)a	2.0–3.3 (2.6)a	1.1–4.8 (2.5)a	2.8–5.8 (4.4)a	1.8–3.8 (2.8)a	2.1–5.2 (3.4)a
Secondary conidiophore																
- length	10.8–71.1 (28.8)f	6.4–82.0 (23.0)e	3.4–40.6 (17.2)bc	5.2–76.0 (17.3)bc	9.4–34.5 (17.3)bc	7.5–23.4 (13.1)a	8.2–20.9 (13.0)a	11.6–37.3 (24.2)e	9.9–40.3 (20.0)bcd	11.9–20.3 (16.7)bcd	11.0–38.0 (23.9)de	7.2–30.6 (16.8)bcd	10.2–38.3 (19.8)cd	17.1–30.8 (23.5)e	9.7–29.7 (20.0)cd	9.9–44.4 (19.3)bcd
- tip width	1.3–3.3 (2.3)a	1.3–3.1 (1.9)a	1.1–2.4 (1.8)a	1.1–2.8 (2.0)a	1.0–4.9 (2.0)a	1.5–2.8 (2.3)a	1.3–2.4 (1.9)a	2.0–2.7 (2.3)a	1.6–2.3 (1.9)a	1.5–3.4 (2.0)a	1.7–2.5 (2.0)a	1.5–2.4 (1.9)a	1.1–3.3 (1.7)a	1.8–3.2 (2.5)a	1.5–3.0 (2.2)a	1.6–3.3 (2.2)a
- medium width	1.6–3.3 (2.4)a	1.3–5.6 (2.3)a	1.3–2.8 (1.8)a	1.1–3.6 (2.1)a	1.1–4.9 (2.1)a	2.0–3.2 (2.4)a	1.3–2.6 (2.0)a	1.9–2.7 (2.3)a	1.6–2.9 (2.1)a	1.5–3.4 (2.2)a	1.7–2.8 (2.3)a	1.6–2.4 (1.9)a	1.1–3.4 (1.8)a	2.0–3.4 (2.8)a	1.5–2.9 (2.2)a	1.7–3.3 (2.5)a
- base width	1.6–3.4 (2.4)a	1.4–5.5 (2.6)a	1.3–4.4 (2.0)a	1.1–4.1 (2.1)a	1.1–5.4 (2.3)a	2.1–4.2 (2.8)a	1.8–2.8 (2.3)a	1.9–3.5 (2.4)a	1.6–3.2 (2.3)a	1.5–3.3 (2.2)a	1.7–2.8 (2.3)a	1.5–2.4 (1.9)a	1.0–5.1 (1.9)a	2.0–3.6 (2.9)a	1.8–3.3 (2.8)a	2.2–4.0 (2.7)a

Table 2. Isolates used in the phylogenetic analyses. Culture numbers in bold indicate ex-type cultures. Accession numbers in italics were obtained from GenBank.

Culture no.	Identity	Host	Location*	Collector	GenBank Accession No.		
					ITS	BT	EF-1α
CMW8217	*Ceratocystis bhutanensis*	*Picea spinulosa*	Jelekha, Bhutan	T Kirisits & DB Chhetri	*AY528957*	*AY528962*	*AY528952*
CMW8242	*C. bhutanensis*	*Pic. spinulosa*	Jelekha, Bhutan	T Kirisits & DB Chhetri	*AY528956*	*AY528961*	*AY528951*
CMW9590	*C. moniliformis*	*Eucalyptus grandis*	Mpumalanga, SA	J Roux	*AY431101*	*AY528985*	*AY529006*
CMW4114	*C. moniliformis*	*Schizolobium parahybum*	Ecuador, South America	MJ Wingfield	*AY528997*	*AY528986*	*AY529007*
CMW9986	*C. moniliformopsis*	*E. obliqua*	Tazmania, Australia	ZQ Yuan	*AY528998*	*AY528987*	*AY529008*
CMW10214	*C. moniliformopsis*	*E. sieberi*	Victoria, Australia	MJ Dudzinski	*AY528999*	*AY528988*	*AY529009*
CMW23803	*C. oblonga*	*Acacia mearnsii*	Piet Retief, Mpumalanga, SA	RN Heath	*EU245019*	*EU244991*	*EU244951*
CMW23804	*C. oblonga*	*A. mearnsii*	Piet Retief, Mpumalanga, SA	RN Heath	*EU245021*	*EU244993*	*EU244953*
CMW11048	*C. omanensis*	*Mangifera indica*	Al Batinah, Oman	AO Al-Adawi	*DQ074742*	*DQ074732*	*DQ074737*
CMW11046	*C. omanensis*	*M. indica*	Al Batinah, Oman	AO Al-Adawi	*DQ074739*	*DQ074729*	*DQ074734*
CMW17300	*C. savannae*	*A. nigrescens*	Skukuza, KNP, SA	NG Kamgan & J Roux	*EF408551*	*EF408565*	*EF408572*
CMW17298	*C. savannae*	*Terminalia sericea*	Skukuza, KNP, SA	NG Kamgan & J Roux	*EF408553*	*EF408567*	*EF408574*
CMW23423	*C. savannae*	*Pterocarpus angolensis*	SCA, Mpumalanga, SA	J Mehl	GU078451	GU078455	GU078459
CMW23424	*C. savannae*	*P. angolensis*	SCA, Mpumalanga, SA	J Mehl	GU078452	GU078456	GU078460
CMW23426	*C. savannae*	*P. angolensis*	SCA, Mpumalanga, SA	J Mehl	GU078453	GU078457	GU078461

Culture no.	Identity	Host	Location*	Collector	GenBank Accession No.		
					ITS	BT	EF-1α
CMW26363	*C. savannae*	*P. angolensis*	SCA, Mpumalanga, SA	J Mehl	GU078454	GU078458	GU078462
CMW13013	*C. tribiliformis*	*Pinus merkusii*	Sumatra, Indonesia	MJ Wingfield	*AY529003*	*AY528993*	*AY529014*
CMW13015	*C. tribiliformis*	*Pin. merkusii*	Sumatra, Indonesia	MJ Wingfield	*AY529004*	*AY528994*	*AY529015*
CMW3276	*C. virescens*	*Quercus* sp.	USA	T Hinds	*DQ061281*	*AY528990*	*AY529011*
CMW11164	*C. virescens*	*Fagus americana*	New Hampshire, USA	D Houston	*DQ520639*	*EF070441*	*EF070413*
CMW15245	*Thielaviopsis ceramica*	*E. grandis*	Zomba Mountain, Malawi	RN Heath	*EU245022*	*EU244994*	*EU244926*
CMW15248	*T. ceramica*	*E. grandis*	Zomba Mountain, Malawi	RN Heath	*EU245024*	*EU244996*	*EU244928*

*Abbreviations: SCA=Sudwala Caves area, KNP=Kruger National Park, SA=South Africa, USA=United States of America

Table 3. Information on sequence datasets generated during this study and information on maximum parsimony trees for each set of analyses.

Gene region	ITS	BT	EF-1α	ITS+BT	ITS+BT+EF-1α
Total characters in dataset	556	524	939	1080	2019
- constant characters	455	445	651	765	838
- parsimony uninformative	29	3	103	27	136
- parsimony informative	72	76	185	288	1045
Excluded characters	485	449	756	898	1623
Characters considered for analysis	71	75	183	182	396
No. of most parsimonious trees	78	7	26	6	4
Tree Length	87	116	280	248	614
Consistency Index (CI)	0.8736	0.7845	0.8	0.8669	0.8436
Retention Index (RI)	0.9127	0.8731	0.8843	0.9147	0.8958
Rescaled Consistency Index (RC)	0.7973	0.6849	0.7074	0.793	0.7557
Figure	1	2	3	4	5

Table 4. Nucleotide polymorphisms observed in the datasets considered in the phylogenetic analysis. Nucleotide positions highlighted in grey delineate between *C. savannae* and *C. oblonga*.

Species	Isolate	Gene Region																									
		ITS	BT							EF1-α																	
		96	46	124	133	144	157	175	184	17	19	22	23	29	44	55	60	83	112	114	115	117	118	121	190	270	331
C. savannae	**CMW23423**	T	T	C	T	T	C	C	C	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
	CMW23424	–	T	C	C	C	T	T	T	C	T	A	T	C	T	G	C	C	C	C	A	C	A	A	G	A	A
	CMW23426	–	T	C	C	C	T	T	T	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
	CMW26363	–	T	T	C	C	T	C	T	C	T	A	T	C	T	A	T	C	G	T	G	G	G	A	A	A	G
	CMW17300	–	T	C	T	T	C	C	T	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
	CMW17297	–	T	C	T	T	C	C	T	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
	CMW17298	–	T	C	T	T	C	C	T	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
	CMW17575	–	C	C	T	T	C	C	T	C	T	A	T	C	T	G	C	C	G	T	G	G	G	G	A	A	G
C. oblonga	**CMW23802**	–	T	C	C	C	T	C	T	T	C	G	C	T	C	G	C	T	G	T	G	G	G	G	A	T	G
	CMW23803	–	T	C	C	C	T	C	T	T	C	G	C	T	C	G	C	T	G	T	G	G	G	G	A	T	G
	CMW23804	–	T	C	C	C	T	C	T	T	C	G	C	T	C	G	C	T	G	T	G	G	G	G	A	T	G

Table 5. Results of the mating crosses performed between strains derived from the *P. angolensis* isolates.

Isolate	23423 (4)	23423 (7)	23423 (10)	23423 (12)	23423 (2)	23423 (5)	23423 (11)	23424 (1)	23424 (3)	23424 (5)	23424 (7)	23424 (4)	23426 (1)	23426 (6)	23426 (7)	23426 (10)	23426 (4)	**Mating type**
23423(4)	–	–	–	–	+	+	+	–	–	–	–	+	–	–	–	–	+	1-1
23423(7)		–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	+	1-1
23423(10)			–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	1-1
23423(12)				–	+	+	+	–	–	–	–	+	–	–	–	–	+	1-1
23423(2)					–	–	–	+	+	+	+	–	+	–	+	+	–	1-2
23423(5)						–	–	+	+	+	+	–	+	–	–	+	–	1-2
23423(11)							–	+	+	+	+	–	+	–	–	+	–	1-2
23424(1)								–	–	–	–	+	–	–	–	–	+	1-1
23424(3)									–	–	–	+	–	–	–	–	–	1-1
23424(5)										–	–	+	–	–	–	–	+	1-1
23424(7)											–	–	–	–	–	–	+	1-1
23424(4)												–	+	+	+	–	–	1-2
23426(1)													–	–	–	–	+	1-1
23426(6)														–	–	–	+	1-1
23426(7)															–	–	–	1-1
23426(10)																–	+	1-1
23426(4)																	–	1-2

Table 6. Results of the mating crosses performed between strains derived from one of the *P. angolensis* isolates, CMW23423, and strains derived from the ex-type isolate of *C. savannae*, CMW17300.

Isolate	17300 (3)	17300 (7)	17300 (11)	17300 (1)	17300 (5)	17300 (12)	**Mating type**
17300(3)	–						1-1
17300(7)	–	–					1-1
17300(11)	–	–	–				1-1
17300(1)	+	–	+	–			1-2
17300(5)	+	–	+	–	–		1-2
17300(12)	+	–	+	–	–	–	1-2
23423(2)	–	–	–	+	+	+	1-1
23423(5)	–	–	–	+	+	+	1-1
23423(8)	–	–	–	–	–	–	1-1
23423(7)	+	+	+	–	–	–	1-2
23423(10)	–	–	–	–	–	–	1-2
23423(11)	+	+	+	–	–	–	1-2
23423(12)	+	+	+	–	–	–	1-2

Table 7. Results of the mating crosses between strains derived from isolates obtained in this study and strains derived from the type isolate of *C. oblonga*, CMW23803.

Isolate	23803 (1)	23803 (2)	23803 (3)	23803 (5)	23803 (6)	23803 (4)	**Mating type**
23803(1)	–						1-1
23803(2)	–	–					1-1
23803(3)	–	–	–				1-1
23803(5)	–	–	–	–			1-1
23803(6)	–	–	–	–	–		1-1
23803(4)	+	+	–	–	+	–	1-2
23424(1)	–	–	–	–	–	–	1-1
23424(2)	–	–	–	–	–	–	1-1
23424(3)	–	–	–	–	–	–	1-1
23424(5)	–	–	–	–	–	–	1-1
23424(7)	–	–	–	–	–	–	1-1
23424(4)	–	–	–	–	–	–	1-2
23426(1)	–	–	–	–	–	–	1-1
23426(6)	–	–	–	–	–	–	1-1
23426(7)	–	–	–	–	–	–	1-1
23426(10)	–	–	–	–	–	–	1-1
23426(11)	–	–	–	–	–	–	1-1
23426(4)	–	–	–	–	–	–	1-2

Figure 1. Majority consensus phylogram of the ITS gene region, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above. The tree was rooted to two isolates of *C. virescens*

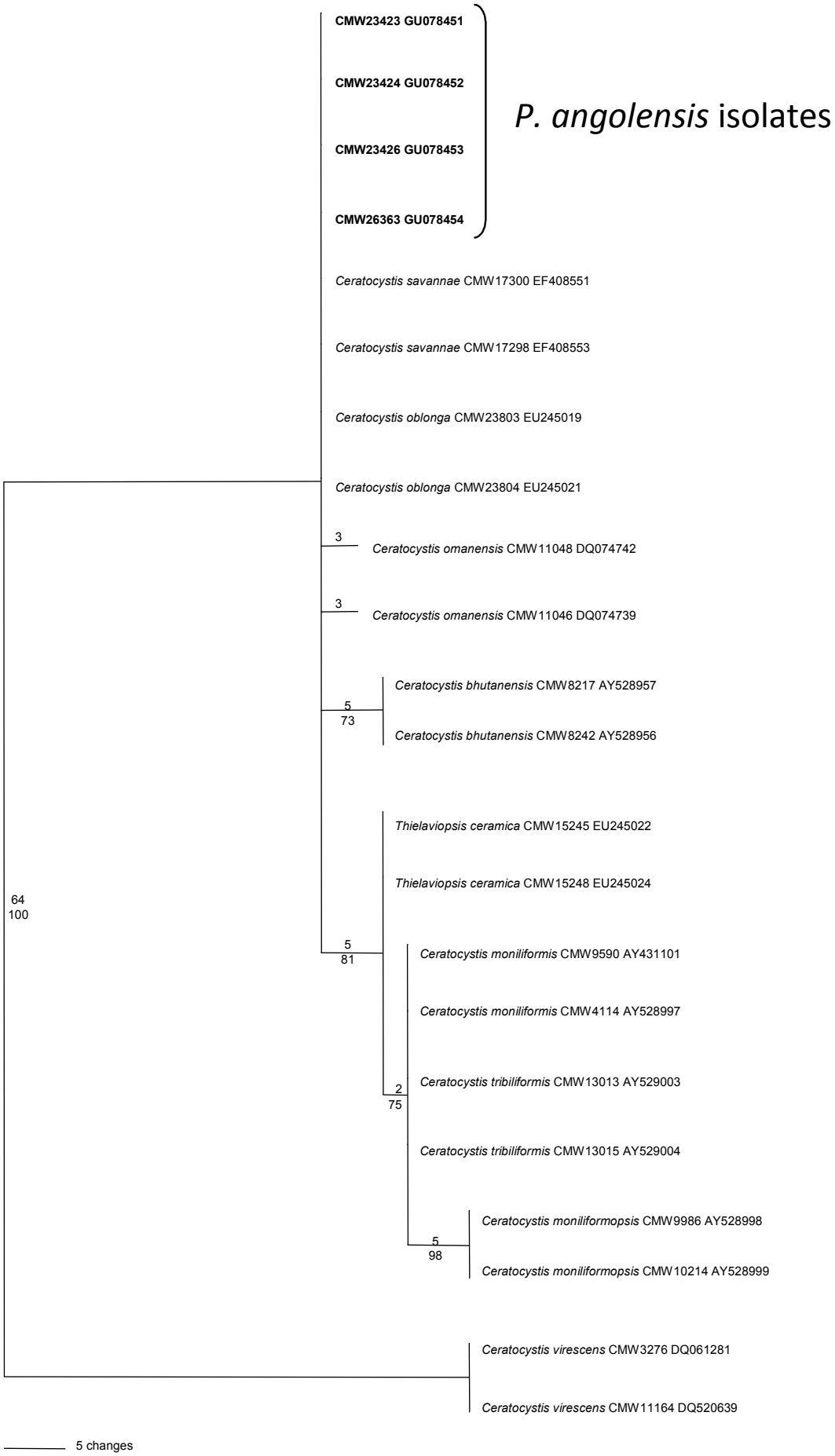

CMW23423 GU078451
CMW23424 GU078452
CMW23426 GU078453
CMW26363 GU078454
P. angolensis isolates
Ceratocystis savannae CMW17300 EF408551
Ceratocystis savannae CMW17298 EF408553
Ceratocystis oblonga CMW23803 EU245019
Ceratocystis oblonga CMW23804 EU245021
3
Ceratocystis omanensis CMW11048 DQ074742
3
Ceratocystis omanensis CMW11046 DQ074739
Ceratocystis bhutanensis CMW8217 AY528957
5
73
Ceratocystis bhutanensis CMW8242 AY528956
Thielaviopsis ceramica CMW15245 EU245022
Thielaviopsis ceramica CMW15248 EU245024
64
100
5
81
Ceratocystis moniliformis CMW9590 AY431101
Ceratocystis moniliformis CMW4114 AY528997
Ceratocystis tribiliformis CMW13013 AY529003
2
75
Ceratocystis tribiliformis CMW13015 AY529004
Ceratocystis moniliformopsis CMW9986 AY528998
5
98
Ceratocystis moniliformopsis CMW10214 AY528999
Ceratocystis virescens CMW3276 DQ061281
Ceratocystis virescens CMW11164 DQ520639
5 changes

Figure 2. Majority consensus phylogram of the BT gene region, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above. The tree was rooted to two isolates of *C. virescens.*

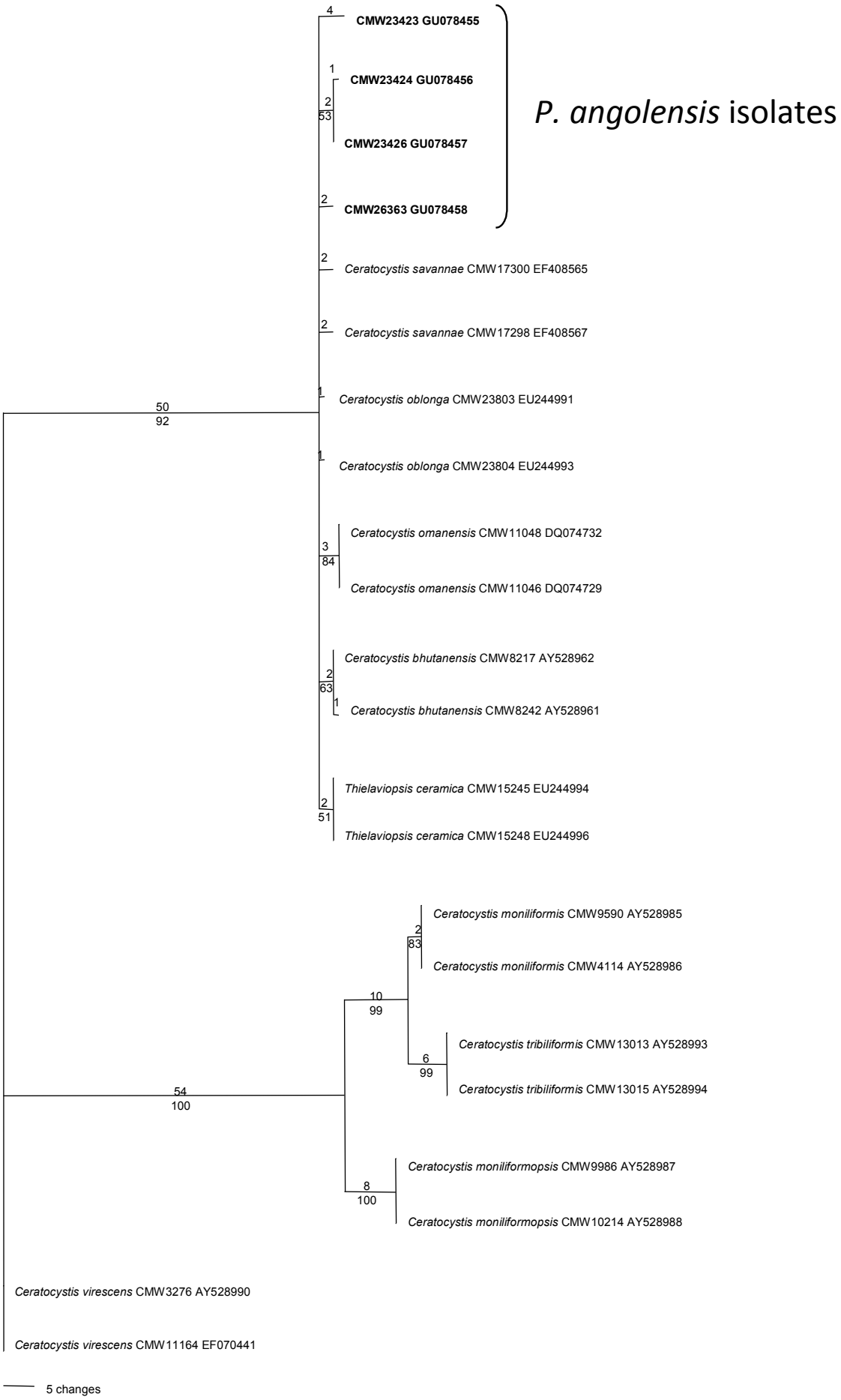

4
CMW23423 GU078455
1
CMW23424 GU078456
2
53
CMW23426 GU078457
2
CMW26363 GU078458
P. angolensis isolates
2
Ceratocystis savannae CMW17300 EF408565
2
Ceratocystis savannae CMW17298 EF408567
1
Ceratocystis oblonga CMW23803 EU244991
50
92
1
Ceratocystis oblonga CMW23804 EU244993
Ceratocystis omanensis CMW11048 DQ074732
3
84
Ceratocystis omanensis CMW11046 DQ074729
Ceratocystis bhutanensis CMW8217 AY528962
2
63
1
Ceratocystis bhutanensis CMW8242 AY528961
Thielaviopsis ceramica CMW15245 EU244994
2
51
Thielaviopsis ceramica CMW15248 EU244996
Ceratocystis moniliformis CMW9590 AY528985
2
83
Ceratocystis moniliformis CMW4114 AY528986
10
99
Ceratocystis tribiliformis CMW13013 AY528993
6
99
Ceratocystis tribiliformis CMW13015 AY528994
54
100
Ceratocystis moniliformopsis CMW9986 AY528987
8
100
Ceratocystis moniliformopsis CMW10214 AY528988
Ceratocystis virescens CMW3276 AY528990
Ceratocystis virescens CMW11164 EF070441
5 changes

Figure 3. Majority consensus phylogram of the EF-1α gene region, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above. The tree was rooted to two isolates of *C. virescens*.

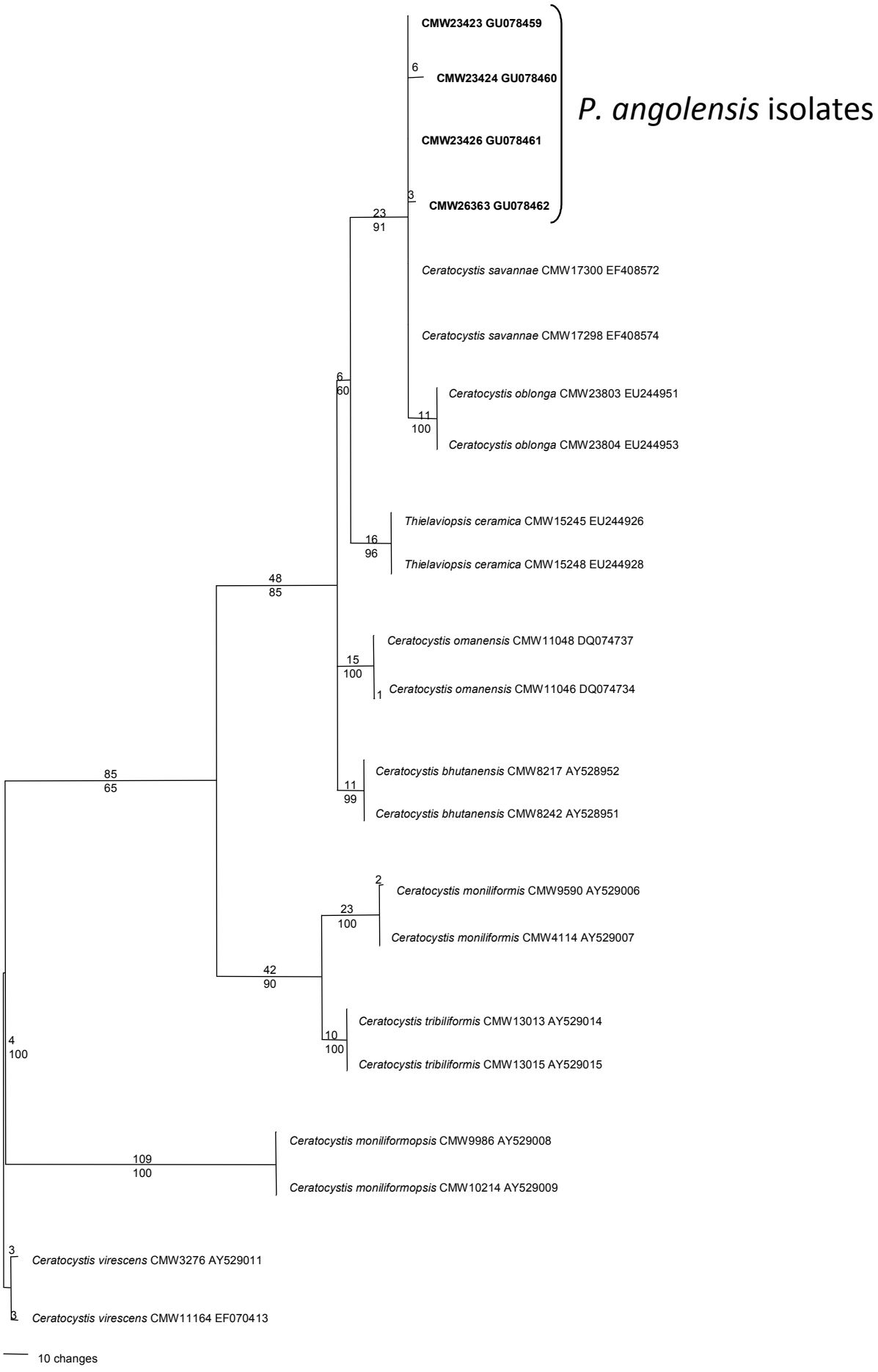

CMW23423 GU078459
6
CMW23424 GU078460
CMW23426 GU078461
3
CMW26363 GU078462
P. angolensis isolates
23
91
Ceratocystis savannae CMW17300 EF408572
Ceratocystis savannae CMW17298 EF408574
6
60
Ceratocystis oblonga CMW23803 EU244951
11
100
Ceratocystis oblonga CMW23804 EU244953
Thielaviopsis ceramica CMW15245 EU244926
16
96
Thielaviopsis ceramica CMW15248 EU244928
48
85
Ceratocystis omanensis CMW11048 DQ074737
15
100
1
Ceratocystis omanensis CMW11046 DQ074734
85
65
Ceratocystis bhutanensis CMW8217 AY528952
11
99
Ceratocystis bhutanensis CMW8242 AY528951
2
Ceratocystis moniliformis CMW9590 AY529006
23
100
Ceratocystis moniliformis CMW4114 AY529007
42
90
Ceratocystis tribiliformis CMW13013 AY529014
10
100
Ceratocystis tribiliformis CMW13015 AY529015
4
100
Ceratocystis moniliformopsis CMW9986 AY529008
109
100
Ceratocystis moniliformopsis CMW10214 AY529009
3
Ceratocystis virescens CMW3276 AY529011
3
Ceratocystis virescens CMW11164 EF070413
10 changes

Figure 4. Majority consensus phylogram of the combined dataset of the ITS and BT gene regions, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above. The trees was rooted to two isolates of *C. virescens*.

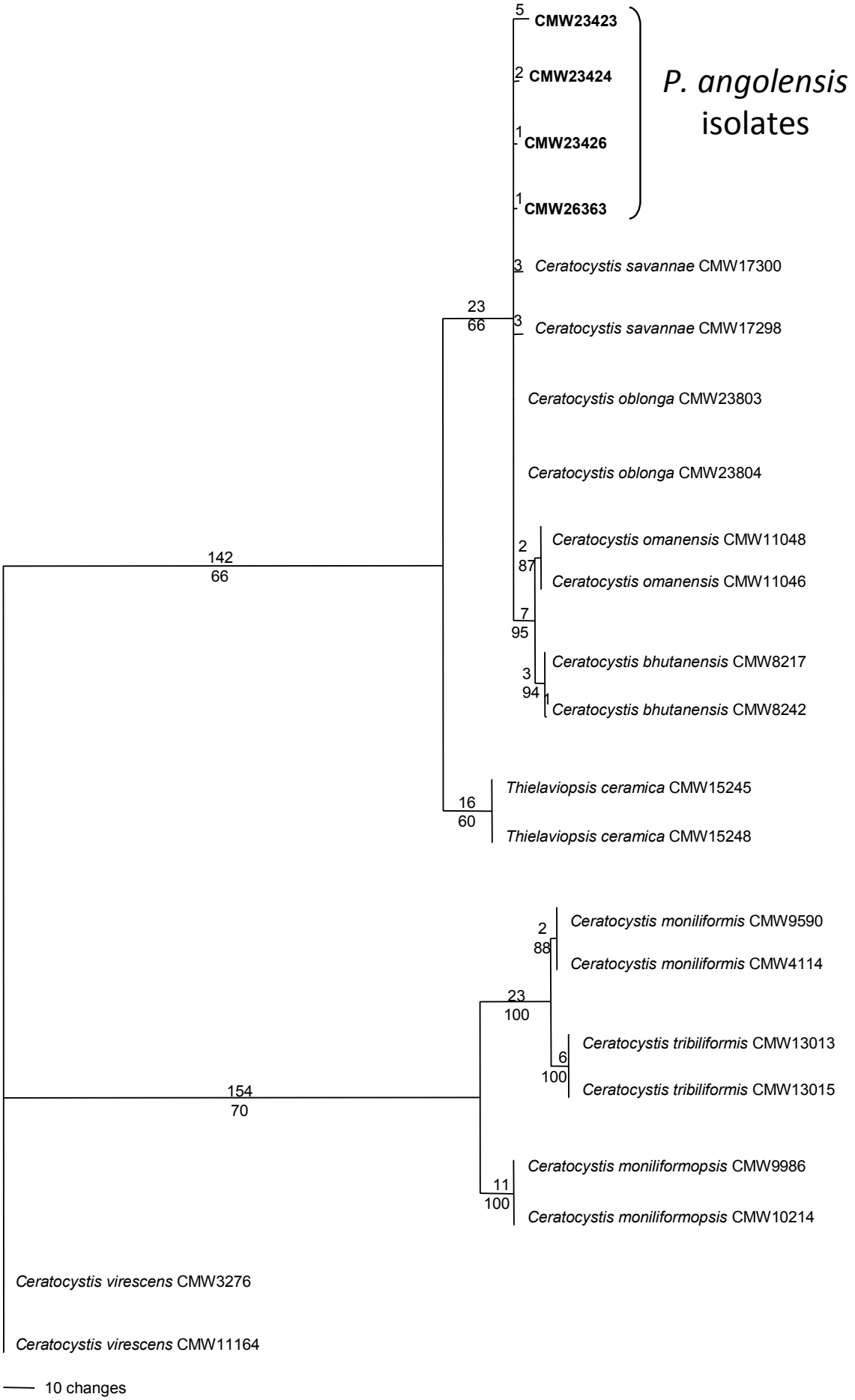

5
CMW23423
2
CMW23424
1
CMW23426
1
CMW26363
P. angolensis isolates
3
Ceratocystis savannae CMW17300
23
66
3
Ceratocystis savannae CMW17298
Ceratocystis oblonga CMW23803
Ceratocystis oblonga CMW23804
2
87
Ceratocystis omanensis CMW11048
Ceratocystis omanensis CMW11046
7
95
3
94
Ceratocystis bhutanensis CMW8217
1
Ceratocystis bhutanensis CMW8242
142
66
16
60
Thielaviopsis ceramica CMW15245
Thielaviopsis ceramica CMW15248
2
88
Ceratocystis moniliformis CMW9590
Ceratocystis moniliformis CMW4114
23
100
6
100
Ceratocystis tribiliformis CMW13013
Ceratocystis tribiliformis CMW13015
154
70
Ceratocystis moniliformopsis CMW9986
11
100
Ceratocystis moniliformopsis CMW10214
Ceratocystis virescens CMW3276
Ceratocystis virescens CMW11164
10 changes

Figure 5. Majority consensus phylogram of the combined dataset of the ITS, BT and EF-1α gene regions, after exclusion of uninformative characters. Bootstrap values (1 000 replicates – values lower than 50% not shown) are indicated below the branches and branch lengths above. The trees was rooted to two isolates of *C. virescens.*

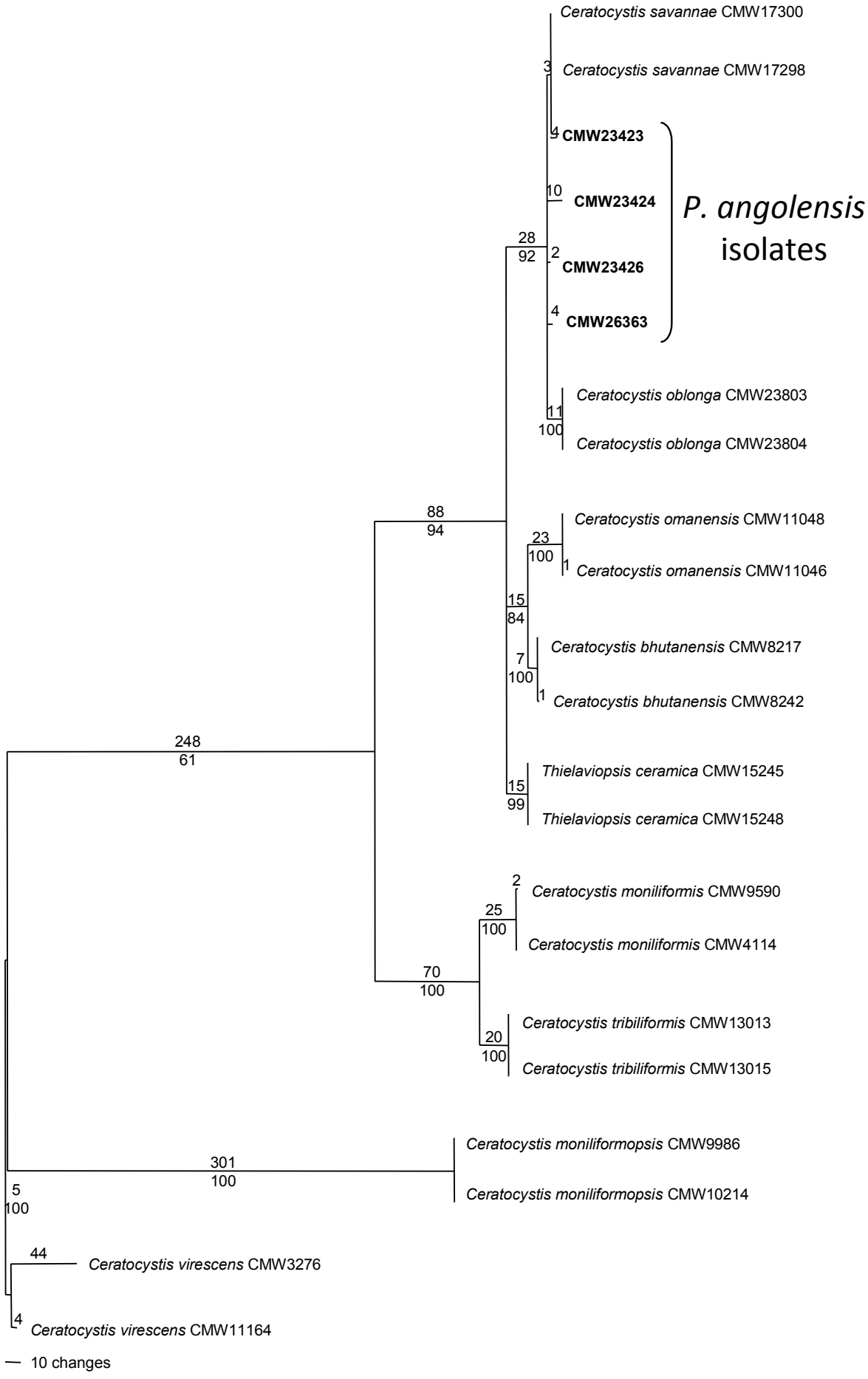
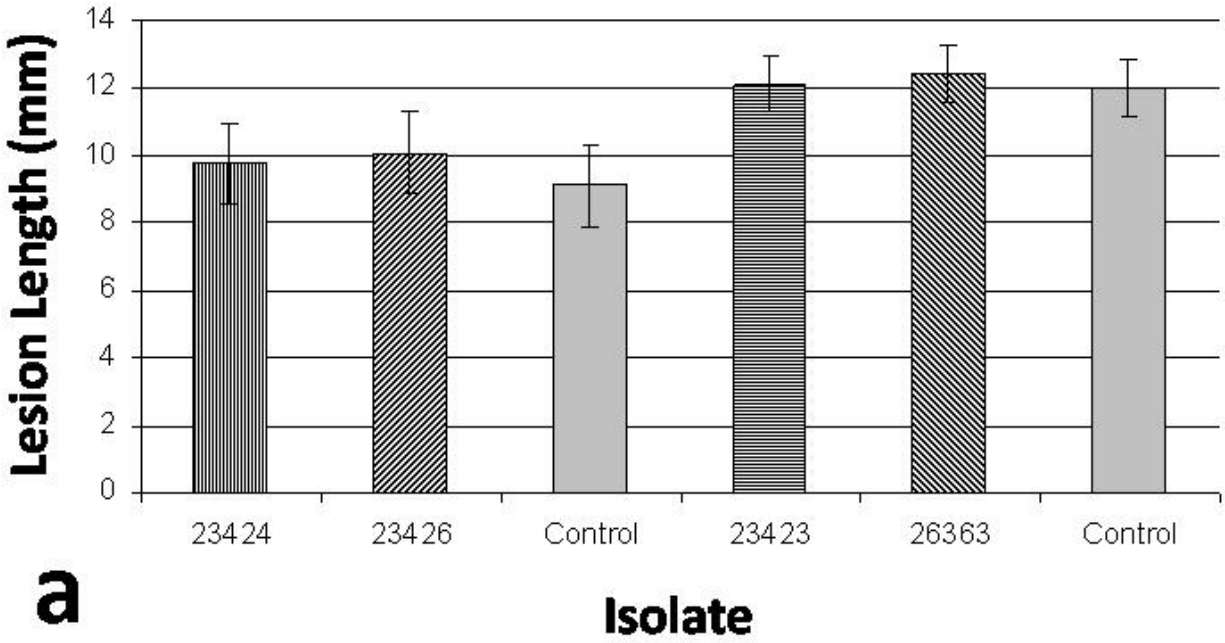
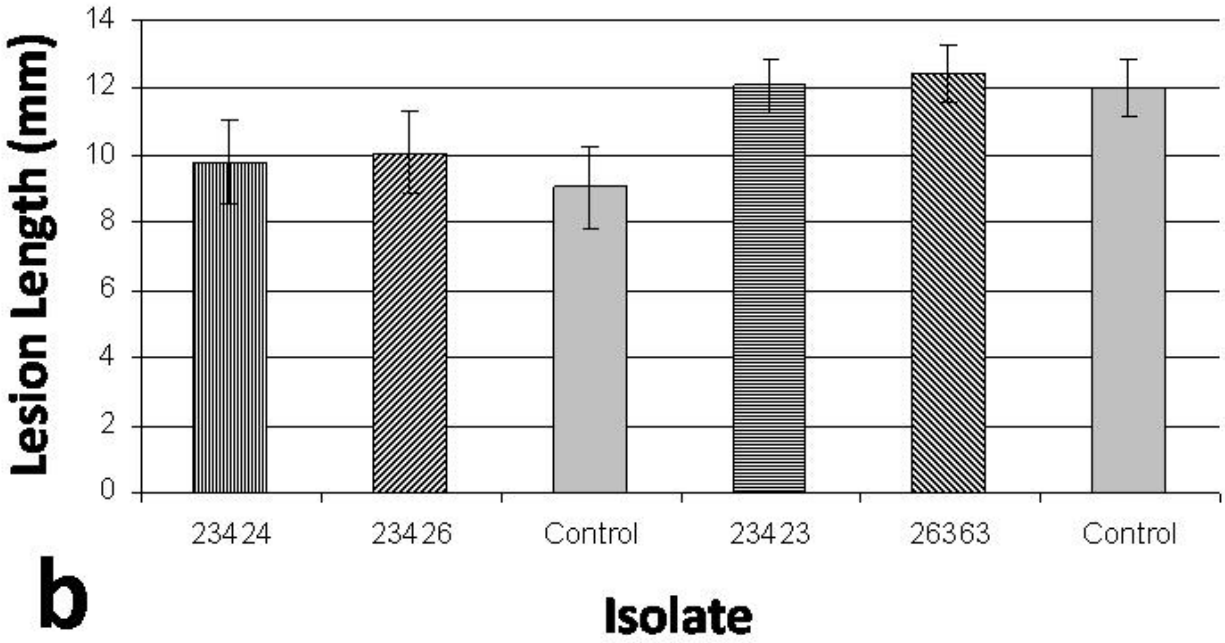

Ceratocystis savannae CMW17300
Ceratocystis savannae CMW17298
CMW23423
CMW23424
CMW23426
CMW26363
P. angolensis isolates
Ceratocystis oblonga CMW23803
Ceratocystis oblonga CMW23804
Ceratocystis omanensis CMW11048
Ceratocystis omanensis CMW11046
Ceratocystis bhutanensis CMW8217
Ceratocystis bhutanensis CMW8242
Thielaviopsis ceramica CMW15245
Thielaviopsis ceramica CMW15248
Ceratocystis moniliformis CMW9590
Ceratocystis moniliformis CMW4114
Ceratocystis tribiliformis CMW13013
Ceratocystis tribiliformis CMW13015
Ceratocystis moniliformopsis CMW9986
Ceratocystis moniliformopsis CMW10214
Ceratocystis virescens CMW3276
Ceratocystis virescens CMW11164
10 changes

Figure 6. Lesions produced on inoculated *P. angolensis* branches in the field inoculations. From left to right: Control inoculation, CMW23423, and CMW26363.Incisions in the branch mark the edge of visible lesions and are indicated by arrows .

Figure 7. Mean lesion lengths (mm) resulting from inoculation of *P. angolensis* branches with isolates of *C. savannae* obtained in this study from the a) first inoculation trial and b) second inoculation trial. Isolates CMW23424 and CMW23426 and their respective control (third column) were inoculated in a) March 2007 and b) September 2007. Isolates CMW23423 and CMW26363 and their respective control (sixth column) were inoculated in a) September 2008 and b) October 2008. Bars represent 95 % confidence limits for each isolate.


Lesion Length (mm)
14
12
10
8
6
4
2
0
23424
23426
Control
23423
26363
Control
Isolate

a


Lesion Length (mm)
14
12
10
8
6
4
2
0
23424
23426
Control
23423
26363
Control
Isolate

b

CHAPTER 4

DIE-BACK OF *PTEROCARPUS ANGOLENSIS* (KIAAT): A CAUSE FOR CONCERN?

ABSTRACT

Pterocarpus angolensis (kiaat) is a well-known southern African tree species of commercial importance that occurs in several vegetation types in the Zambezian regional centre of endemism. The most prominent of these are the Zambezian miombo woodland and undifferentiated woodland. A diverse range of ecosystems within these vegetation types necessitate adaptation by tree species to survive extremes of drought, temperature, altitude and soil nutrition, and to compete with other species in vegetation development. There are several reports of disease and die-back of *P. angolensis* in Zambia, Zimbabwe and South Africa, but very little is known regarding the cause or significance of this problem. In this review, we provide details regarding the history of the disease and consider its possible causal agents. An analysis is made of the importance of the problem and actions that might be taken to alleviate it.

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1. INTRODUCTION

Pterocarpus angolensis (kiaat) is a well-known southern African tree species valued for its timber (Lowore 1993) and use in several traditional medicinal applications (Coates Palgrave 1977). In South Africa, the species is protected by state law (Krynauw 1998) while in Tanzania a minimum stem diameter at breast height (DHB) is prescribed to aid in conservation, thus limiting harvesting of trees to a minimum diameter (Caro *et al*. 2005). Despite these efforts, it is evident that the species is under threat. Size prescriptions are ignored in some areas of Tanzania (Caro *et al*. 2005), past and present exploitation has raised concerns of the extinction of *P. angolensis* in Zimbabwe (Bradley & Dewees 1993, Clarke *et al*. 1996, Mushove 1991) and availability reported by woodcarvers in South Africa indicates that trees are becoming rare (Steenkamp 1999). These concerns are underscored by low seedling recruitment rates during both germination and the suffrutex stage (Boaler 1966, Caro *et al*. 2005, Schwartz *et al*. 2002) and by unsustainable rates of harvesting (Caro *et al*. 2005, Schwartz *et al*. 2002).

Pterocarpus angolensis occurs in several vegetation types in the Zambezian regional centre of endemism. The most prominent of these are the Zambezian miombo woodland, which includes both wetter and drier miombo, and the more species-rich north and south undifferentiated woodland (White 1983). These vegetation types span a range of ecosystems that differ with respect to fire, frost and drought tolerance. *Pterocarpus angolensis* is, therefore, adapted to survive and tolerate extremes stemming from several environmental factors, including fire, drought and moisture availability. The species can tolerate an annual rainfall as low as 500 mm (Van Wyk 1972) and as high as 1250 mm (Von Breitenbach 1973), average temperatures as low as 4 ºC, and altitudes ranging from sea-level to 1650 m above sea-level (Von Breitenbach 1973). The soils of both miombo woodland (Högberg 1992) and undifferentiated woodland (Scholes & Walker 1993) are poor in organic matter, nitrogen and phosphorous but *P. angolensis* possesses both vesicular-arbuscular (VA) mycorrhizae (Munyanziza & Oldeman 1995) and nitrogen fixing root nodules (Boaler 1966), ensuring that the species is able to cope and efficiently utilize available nutrients. The species cannot tolerate competition with dense grass and dense stands of trees and regeneration establishes well with regular fire (Boaler 1966, Geldenhuys 1977).

Despite the adaptations of *P. angolensis* to relatively harsh environments, there are several reports dating back to the late 1950s of these trees becoming diseased and dying (Anon 1973, Krynauw 1998, 2000, Mushove 1996, Piearce 1979, Van Wyk *et al*. 1993). These reports refer to trees in the Livingstone district between Livingstone in Zambia and Bulawayo in Zimbabwe, as well as in

several areas of South Africa. In this review, we discuss disease symptoms observed and the possible reasons for tree death, including ecological conditions that might predispose trees to diseases. Furthermore, advice is provided regarding management options for diseased *P. angolensis* trees and possible strategies that may help to alleviate the disease.

2. THE SITUATION IN THE LIVINGSTONE AREA, ZAMBIA AND ZIMBABWE

2.1. Disease of *P. angolensis* trees

In 1958, a disease resulting in the death of *P. angolensis* trees west of Livingstone, Zambia, was reported. Subsequently in 1964, additional affected trees were found in the Kalahari Sands/Zambezi Teak Forests (Anon 1973). By July 1964, the disease was reported close to the Victoria Falls village in Zimbabwe, thereafter spreading widely (Anon 1973, Geary 1972). In March 1966, the disease was reported in the Bambezi Forest Reserve, about 100 km north-west of Bulawayo (Piearce 1979). Calvert (1972) produced evidence indicating that there had been earlier outbreaks of the disease in Botswana in the early 1930s, in Zambia in the late 1940s and early 1950s, and in Zimbabwe in the early 1930s, late 1940s and early 1950s (Piearce 1979). Since then, the disease has become localized in the border area between Botswana, Zimbabwe and Zambia resulting in stand-level die-back in this area (Van Wyk *et al*. 1993). The disease was named “mukwa disease”, referring to one of the common names (mukwa) given to *P. angolensis*.

Mukwa disease has a range of symptoms, the most prominent being blight and die-back of affected trees (Calvert 1972, Piearce 1979, 1986). Other symptoms include a generally unhealthy appearance of the crown foliage, wilt and chlorosis (Calvert 1972, Piearce 1979, 1986). Defoliation, desiccation, the occurrence of epicormic buds on the healthy parts of the tree trunk and branches and the premature development of new leaves are common symptoms. The bark discolors from brown to grey, cracks or flakes, and can be peeled off in large flaps, and xylem and sapwood become stained. Xylem/sapwood streaks are a dull blue-grey or orange-red in colour that appear as complete flecked rings or discontinuous arcs. In microtome sections, xylem vessels contain amorphous deposits, occlusions, and fungal hyphae (Piearce 1979). Vascular streaking appears to originate from several points in the root system or from a single rotten root (Piearce 1979). Van Wyk *et al*. (1993) noted that phloem streaking was often emphasized in affected trees and Calvert (Calvert 1972, Piearce 1979, 1986) suggested that this was a distinguishing factor on affected trees. The most intensive symptoms are observed at the height of the rainy season. Diseased trees typically occur in patches and usually die within two to three years (Calvert 1972, Piearce 1979, 1986).

2.2. Possible causes of mukwa disease

Mukwa disease has been attributed to *Fusarium oxysporum* Schltdl. based on the consistent isolation of this fungus from discolored wood (Piearce 1979, 1983, 1986, Van Wyk *et al*. 1993). *Fusarium oxysporum* is a well-known pathogen, causing wilt diseases, damping off and crown and root rots of a variety of plants (Leslie & Summerell 2006). It has also been recorded as a wilt and die-back pathogen of several leguminous tree species, including *Acacia koa* and *Albizia julibrissin* (Anderson *et al*. 2002), and was recently associated with cankers on *Cedrelinga cateniformis* in the Amazon basin (Lombard *et al*. 2008). The fungus is typically associated with plant roots and Gordon and Martyn (1997) suggest that either the fungus grows beyond the cortex into the xylem to initiate plant disease or that it already exists as an endophyte within affected plants. Strains of the fungus are often non-pathogenic in nature and it is more likely that some change, either physiological or biological, in the host or the fungus allows disease to develop (Gordon & Martyn 1997).

Drought has been mentioned in every report of mukwa disease and stress might be an important factor in its development (Anon 1973, Calvert 1972, Geary 1972, Piearce 1979, 1986). For example, affected trees in Matabeleland in Zimbabwe experienced 12 consecutive seasons of poor rainfall up to 1973 (Anon 1973). Rainfall data from the South African Weather Bureau for the area (Fig. 1), from the two closest weather stations (Bulawayo in Zimbabwe and Livingstone in Zambia), based on World Weather Records (vol. 5: Africa) for 1951 to 1980 (Anon 1967, 1979, 1987) show a correlation between annual rainfall in the area and mukwa disease. From 1958 to 1959, there was a drop in rainfall at Livingstone from 1410 mm to 620 mm, which is considerably lower than the average ~ 800 mm in preceding years. In general, rainfall remained at this level at both weather stations until 1965, other than in 1962 when 1078 mm of rainfall was recorded in Livingstone). From 1972, rainfall improved at Livingstone. New outbreaks of Mukwa disease were reported in Zimbabwe in 1958/9, 1964, 1966, 1967 and 1968 (Calvert 1972, Piearce 1979) and this is supported by low rainfall reported (Fig. 1) in Bulawayo. From 1973 to 1977, the disease was studied at Livingstone by Piearce (1979) and it is likely that his observations centred on trees that were dying from the sustained drought period of previous years.

Fire is a critical factor in the ecology of *P. angolensis* that has frequently been overlooked or ignored in studies on mukwa disease. The species is a pioneer in areas where fire has occurred (Von Breitenbach 1973); seed germination being dependent on the removal of the underlying grassy layer

and the wings and bristles of the fruit enabling seed to come into contact with the ground (Van Daalen 1991, Von Breitenbach 1973), and subsequent exposure to rain (Boaler 1966). Seedlings of the species produce an extensive, although shallow (Geldenhuys 1977), underground root system in several successive years of annual die-back (the so-called suffrutex stage) to collect sufficient water and nutrients for stem production (Boaler 1966, Lowore 1993, Vermeulen 1990, Von Breitenbach 1973) and survive subsequent fires (Vermeulen 1990). Fire benefits *P. angolensis* by suppressing competing plant species and eliminating root competition for soil water from neighbours (probably exacerbated by the shallow root system of the species), helping to prune side-branches and secondary stems and providing nutrients in the form of ash (Boaler 1966, Orpen 1982, Vermeulen 1990). In fact, both Boaler (1966) and Von Breitenbach (1973) noted positive effects of fire; Boaler (1966) reporting an increase of 20 % in sapling shoot length after burning of the herb layer and Von Breitenbach (1973) a rapid growth in trunk diameter and crown size following removal of competing dense grass and scrub growth. Saplings are also light-demanding, developing a zig-zag appearance to maximize absorption, and fire eliminates competition for sunlight from neighbouring plants (Boaler 1966, Orpen 1982, Vermeulen 1990). A lack of sunlight results in a shorter shoot length, a greater proportion of annual shoot die-back (Boaler 1966, Vermeulen 1990) and eventual death (Groome *et al*. 1957, Vermeulen 1990).

Several other tree species in the same stands, including *Burkea africana*, *Erythrophleum africanum*, *Lannea schweinfurthii* var. *stuhlmanni*, *Strychnos cocculoides* and *Terminalia sericea* (the most severely affected), have also been reported suffering from die-back and death (Anon 1973, Piearce 1979). Of these; *B. africana* (Burke 2006, Holdo & Timberlake 2008), *E. africanum* (Holdo & Timberlake 2008) and *T. sericea* (Geldenhuys 1977, Holdo & Timberlake 2008, Rutherford 1983, Yeaton 1988) are known to have shallow root systems. In contrast, *S. cocculoides* has a deep rooting system but is physiologically limited in that there are almost no fine or adventitious roots in the first 20 cm of soil and this low root surface area density continues at lower soil levels (Oppelt *et al*. 2005). Several of these species are known to be tolerant to fire, including *B. africana* (Burke 2006), *E. africanum* (Lawton 1978), *L. schweinfurthii* var. *stuhlmanni* (Simute *et al*. 1998) and *T. sericea* (Strang 1974, Yeaton 1988). Like *P. angolensis*, some species are ecological pioneers, including *E. africanum* (also light-demanding) (Lawton 1978), *L. schweinfurthii* var. *stuhlmanni* (Welch 1960), *S. cocculoides* (also light-demanding) (Hines & Eckman 1993) and *T. sericea* (also light-demanding) (Strang 1974, Yeaton 1988). Consequently, all of these species share many physiological requirements similar to *P. angolensis* and reports of the same disease symptoms occurring are not surprising.

It has been proposed that mukwa disease is a form of stand-level die-back that encourages regeneration, based on the observation that trees follow a cyclic pattern in response to the disease (Calvert 1986). Indeed, Piearce (1983) noted that mukwa disease was beginning to wane, based on a decrease in the rate of new infections and the recovery of some affected trees. This was confirmed by Mushove (1996) who concluded that mukwa disease is episodic. Also, Van Wyk *et al*. (1993) noted an increase in saplings as mukwa disease progressed, indicating species regeneration, and that the disease affected older trees (with larger diameters) rather than young trees.

The stand-level die-back of *P. angolensis* in the Kalahari Sands/Zambezi Teak Forests has been compared to stand-level die-back of *Metrosideros polymorpha* (Myrtacae) trees in Hawaii (Ohia disease) (Van Wyk *et al*. 1993). In the case of *M. polymorpha*, it has been suggested that die-back is related to primary succession and that the establishment of seedlings and sapling maturation is strongly related to the degree of opening in the forest canopy (Jacobi *et al*. 1983). This is consistent with the cohort senescence theory where many large cohorts/groups of individuals of the same (woody) plant species are established non-uniformly (a patchy distribution) in an area after a catastrophe or a decrease in the forest canopy. These cohorts eventually begin to senesce/age from the effect of one or more environmental stresses that are either abiotic or biotic. Similarly cohorts occurring in areas exposed to the same environmental stress/stresses can begin to senesce at the same time. At this stage a fluctuating/variable environmental site factor begins to contribute to stand-level die-back as its intensity increases over time. The resulting stress predisposes trees to attack by other biotic agents that begin to cause rapid decline or die-back. If trees manage to temporarily recover following the environmental site factor, these biotic agents can cause a lingering decline or die-back (Mueller-Dombois 1983, 1986). Drought, a lack of fire and *F. oxysporum* are considered possible contributors to stand-level die-back of *P. angolensis* (Van Wyk *et al*. 1993).

3. THE SITUATION IN SOUTH AFRICA

3.1. Reports of die-back of *P. angolensis*

In South Africa, *P. angolensis* trees were first reported dying by Krynauw (1998, 2000) in the Mawewe Nature Reserve/Cattle Game Project, close to Malelane, in the east of the country. Although the reserve has a number of giraffe, nyala, kudu and impala; livestock husbandry with cattle is also practised on the reserve (Krynauw 1998). Fire management is not practised on the reserve due to the risk it poses to cattle and the potential effects it may have on their grazing

(Phumzile Khosa, reserve manager of Mawewe Nature Reserve and Cattle Game Project, Mpumalanga Parks Board, pers. comm.). When fires do occur, typically every five to six years, they are extremely intense.

Krynauw (1998) mentioned that *P. angolensis* trees had been reported dying near Bushbuckridge due to the 1991/2 drought in the area (C. M. Shackleton, University of the Witwatersrand, 1998, pers. comm.). The effects of this drought had also been noticed on trees nearby in the Kruger National Park (Krynauw 1998) and Viljoen (1995) recorded that trees were severely affected by the drought. In particular, Viljoen (1995) found that although only 16 % of trees greater than 8 m in height were dead at the Napi area in the Kruger National Park, 80 % of the total tree population had less than 10 % growth, based on assessments of coppice, a single living branch, or a stem or shoot sprouting from the roots, although even coppicing was poor. In the Pretoriuskop area, Viljoen (1995) noted that survival was considerably better and only 21 % of the trees sampled had drought-induced damage of more than 50 %. In addition to the reports from the Kruger National Park and Mawewe, John Burrows (reserve manager of Buffelskloof Nature Reserve, Lydenburg, pers. comm.) indicated that *P. angolensis* trees on the reserve appeared unhealthy.

3.2. Observations in pathology study (2005)

To determine whether the reports of disease and death of *P. angolensis* in South Africa were similar to mukwa disease, samples from trees in three locations in the Mpumalanga Province of South Africa (Fig. 2) were collected during November and December 2005. Samples of diseased material were collected from trees at Mawewe Nature Reserve (near Malelane) (7 trees) and Buffelskloof Nature Reserve (near Lydenburg) (3 trees) and fungi isolated from symptomatic tissue. Samples were also collected from stem wounds on trees in the Sudwala Caves area (10 trees) and Mawewe Nature Reserve (8 trees). To investigate the possible correlations between the observed tree mortality and drought, rainfall data for each area were obtained from the South African Weather Bureau.

Diseased and dying *P. angolensis* trees (Fig. 3) had a range of disease symptoms. These included crown and branch die-back, wood stain and rot (Fig. 3), production of epicormic shoots (Fig. 3a), lack of fruit and oozing of sap. Basidiomycete fruiting bodies were present on some trees (Fig. 3b). Many trees, especially those in the Mawewe Nature Reserve, had signs of fire damage. In many cases these fire scars were most severe at the bases of the trees and associated with internal wood rot (Fig. 3c,d).

A total of 199 fungal isolates were obtained from samples collected from diseased and dying trees (Table 1). Most isolates were of common saprophytes such as species of *Candida*, *Penicillium* and *Humicola*. A number of isolates did not sporulate in culture and are probably basidiomycetes. Other common genera and species isolated that could contribute to disease include *Lasiodiplodia theobromae*, *Cytospora* spp. and *Fusarium* spp.

The species of *Cytospora*, *Fusarium* and *L. theobromae* obtained in our isolations from diseased *P. angolensis* trees, are commonly associated with stress induced decline of trees globally. *Lasiodiplodia theobromae* is known to cause a variety of tree diseases, including die-back and bluestain (Punithalingam 1980). Inoculation trials in the field on *P. angolensis* trees (Mehl *et al*. 2010) did not demonstrate pathogenicity. However, disease expression following infection by species of the Botryosphaeriaceae, including *L. theobromae*, has been linked to drought stress (Schoeneweiss 1975, Lewis & van Arsdel 1978, Mullen *et al*. 1991, Ma *et al*. 2001, Desprez-Loustau *et al*. 2006). The genus *Cytospora* includes species that cause cankers and die-back on infected trees (Adams *et al*. 2006). Disease expression by these species has also been linked to a variety of stresses, including drought stress (see Adams *et al*. 2005, and cited references). Likewise, species of *Fusarium* cause a variety of plant diseases, including vascular wilts, root rots and damping-off (Bloomberg 1981, Leslie & Summerell 2006).

To ascertain the identity of isolates of *Fusarium* species obtained in this study and to determine whether any isolates were *F. oxysporum*, we sequenced the translation elongation factor 1α. Based on phylogenic inference, isolates grouped into five clades in the *Fusarium* genus (total isolates collected in brackets), namely the *F. oxysporum* (5), *F. equiseti* (13), *F. solani* (5), *F. graminearum* (3), and *Gibberella fujikuroi* (3) clades. Most of the isolates grouping in the *F. oxysporum* clade were obtained from stem wounds while most of the isolates obtained from diseased plant tissue grouped into the *F. equiseti* clade. *Fusarium equiseti* is known to colonize damaged or senescent plant tissues (Leslie & Summerell 2006) and has been associated with wilt and decline of date palm in Iraq (Abbas *et al*. 1991). However, *F. equiseti* is classified as primarily a saprophyte or secondary invader of diseased plant tissues (Leslie & Summerell 2006), arguing against a role in facilitating disease of *P. angolensis*.

3.3. Possible contributing factors

A review of the rainfall data for the sample areas showed that rainfall was probably a principal

factor in decline of *P. angolensis*. Die-back and death of trees was not immediate, but occurred several years after drought, which is typical for stress-related diseases (McDowell *et al*. 2008 and references cited therein). Stress resulting from drought predisposes trees to attack from other biotic agents, including insects and fungi, and weakens them, increasing their vulnerability to subsequent periods of drought (Bréda *et al*. 2006, McDowell *et al*. 2008). Other biotic agents have been noted such as mistletoes (*Loranthus* spp.) in the crowns of *P. angolensis* trees dying from mukwa disease (Boaler 1966, Orpen 1982, Vermeulen 1990) and other symptoms such as black spots on the leaves, fungal fruiting bodies and lichens were frequently seen in Krynauw's (2000) study area.

The shallow root system of *P. angolensis* (Geldenhuys 1977), particularly when in competition with a dense grass or shrub layer, undoubtedly contributes to susceptibility to drought and disease development when annual rainfall is lower than 500 mm. A deep, dense and ramified root system is known to confer drought tolerance in trees by providing access to both larger reserves of water already present between soil particles, as well as to the water table which may be several layers deep (Bréda *et al*. 2006). In addition, a lack of soil water and the resultant unfavourable soil conditions explain the production of epicormic shoots by *P. angolensis* trees suffering from mukwa disease (Piearce 1979). Epicormic shoot production indicates degeneration of feeder roots and mycorrhizae (Manion 1991, Jurskis 2005) and was also observed in our field studies. Drought-like conditions in Mawewe Nature Reserve, Pretoriuskop and Bushbuckridge (Fig. 4) in 1993/4 could thus have influenced the disease noted by Krynauw (1998) in South Africa. Several subsequent periods of drought in the years 2002-5 could similarly have contributed to the disease problem we observed during 2005 and 2006 in Mawewe Nature Reserve, Pretoriuskop, Bushbuckridge and Buffelskloof Nature Reserve (Fig. 4).

In most areas where sampling occurred, fire management was not practised. Burning in these areas suppresses competition that *P. angolensis* trees face from neighbouring vegetation (Boaler 1966, Vermeulen 1990, Von Breitenbach 1973). Fire also suppresses bush encroachment, a phenomenon where the density of woody plants present, in the form of a thicket of fire-sensitive species (Geldenhuys 1977), progressively increases (Scholes & Archer 1997 and references cited therein). Both the crowns and roots of neighbouring trees and grasses compete with *P. angolensis* for resources including sunlight, soil moisture, soil nutrients and space (Boaler 1966, Orpen 1982, Vermeulen 1990). Competition for these resources is undoubtedly exacerbated by the shallow root system of *P. angolensis* (Geldenhuys 1977) and heightened in times of drought, probably contributing to the development of disease.

Fire damage represents a possible contributing factor in *P. angolensis* decline and death. Fire is particularly important for *P. angolensis*. Both fire and the clearing of vegetation for cultivation remove competing plant species that adversely affect the growth and development of *P. angolensis* (Boaler 1966, Von Breitenbach 1973). "Cold fires" enable the germination of seedlings by burning off the wings and bristles of fruit and removing the grassy layer, allowing the enclosed seed to encounter water during an ensuing rainfall (Von Breitenbach 1973, Van Daalen 1991). However, fire damage to the bole of trees can occur, resulting in attack by woodborers and fungi (Schoeman 1982, Vermeulen 1990). It is likely that this develops due to an accumulation and buildup of litter, grass and thicket at the base of trees (Geldenhuys 1977). After a fire, this debris smolders and the sustained heat can contribute to, if not cause, the observed damage of the stem (Braam van Wyk, pers. comm.).

It is recommended that fire be applied biannually just before the annual rainfall season (Geldenhuys 1977) to maintain populations of *P. angolensis* and enhance recruitment (Geldenhuys 2005) as annual burning adversely affects developing seedlings until the suffrutex root system has fully developed (Boaler 1966, Groome 1955, Högberg 1986). Field observations indicate that fires that occur in areas where biannual burning is not practised, are inordinately hot for the sustainable growth of mature trees and can inhibit recruitment of seedlings and saplings. Previous studies support these findings. In areas where biannual burning was not practised, a thicket of woody species developed that inhibited *P. angolensis* recruitment and fires that subsequently occurred were so intense that they damaged trees severely by burning the phloem and xylem (Van Wyk *et al*. 1993). In addition, these "hot fires" can destroy both seedlings and saplings (Banda *et al*. 2006, Van Daalen 1991) and the damage to stems and roots of mature trees, especially shallow-rooted trees, can easily enable rot fungi to invade burnt tissues and commence heart rot. Wounds created by fires are further exacerbated by drought conditions (Desprez-Loustau *et al*. 2006) and provide entry points for fungi responsible for heart rot and disease (Chungu *et al*. 2007, Roux *et al*. 2004, 2005).

Fire aids pruning of side branches and multiple, secondary stems of *P. angolensis* trees (Boaler 1966, Orpen 1982, Vermeulen 1990). A lack of fire can result in poor growth form, a common field observation (Fig. 5a, b). In fact, the umbrella-like form of mature, healthy *P. angolensis* trees (Fig. 5c) was virtually absent in all the areas where diseased trees occurred. As the species is light-demanding (Boaler 1966, Orpen 1982, Vermeulen 1990), lower branches shaded by newer branches higher up, progressively die (Boaler 1966, Vermeulen 1990). As the branches break off, they can provide entry points for rot fungi. If the branch partially breaks off, the remainder can become encased in the bark, serving as a residual entry point for rot fungi. Consequently, if side branches

and multiple stems are not pruned or burnt off when branches are small enough to enable active bark recovery, then their presence can contribute to die-back and death of trees. In *Acacia mangium*, the presence of multiple stems results in "intra-stem competition" and a reduced height and diameter of the main stem (Beadle *et al*. 2007). Thus, multiple stems in *P. angolensis* could result in premature death due to other neighbouring trees overshadowing the light-demanding individuals of the species. Apart from these obvious health benefits to *P. angolensis* trees, pruning also has commercial importance: it can be used to correct stem defects (Boaler 1966, Geldenhuys 2005, Vermeulen 1990) and can result in an increased length of planks and logs (Boaler 1966, Vermeulen 1990) as well as a trunk increase of 20-25% (Groome *et al*. 1957, Vermeulen 1990).

4. GENERAL CONCLUSIONS

A number of factors noted in this review most likely impact on the health of *P. angolensis* trees. Drought has been implicated in the disease of these trees in both Zambia and South Africa. Rainfall datasets gathered from these areas indicate that reports of die-back and death of trees can be correlated with several years of drought, either intermittent or sustained over several years. The effect of these drought periods is observed in subsequent years, when trees begin to die back and decline. Along with *P. angolensis*, other tree species in the same area are affected by disease. The symptoms of mukwa disease noted in this review, along with these facts, argue that the primary causal agent is most likely not biotic.

Along with rainfall, fire is a crucial factor impacting on the health and sustainability of *P. angolensis*. Fires enable germination and regeneration of *P. angolensis*, ensuring that seedlings and saplings are not impeded during growth. Fires also suppress neighbouring plant and tree species that compete with *P. angolensis* for soil nutrients, moisture, space and sunlight. Competition for these is likely elevated during times of drought and predisposes *P. angolensis* individuals to stress. Consequently, the interaction between adverse environmental conditions or improper management in combination with the biology of *P. angolensis*, likely predisposes individuals of the species to disease.

The timing of application of fire is critical. An annual fire inhibits regeneration of *P. angolensis* by negatively affecting recruitment of seedlings. A fire applied too infrequently has a similar effect, but can also damage mature trees, contributing to subsequent die-back and disease of stressed *P. angolensis* trees.

Die-back and death of *P. angolensis* remains a cause for concern, especially in the light of global climate change. It is likely that droughts in areas where the species occurs will continue to increase in frequency and intensity, adding to conservation concerns. At present, reserve managers and nature conservation agencies are advised to practise biannual burning just prior to the annual rainfall season in areas where *P. angolensis* occurs, to practise natural side-branch pruning at an early age on small branches and secondary stems where necessary and to prevent and prosecute wounding of trees where possible.

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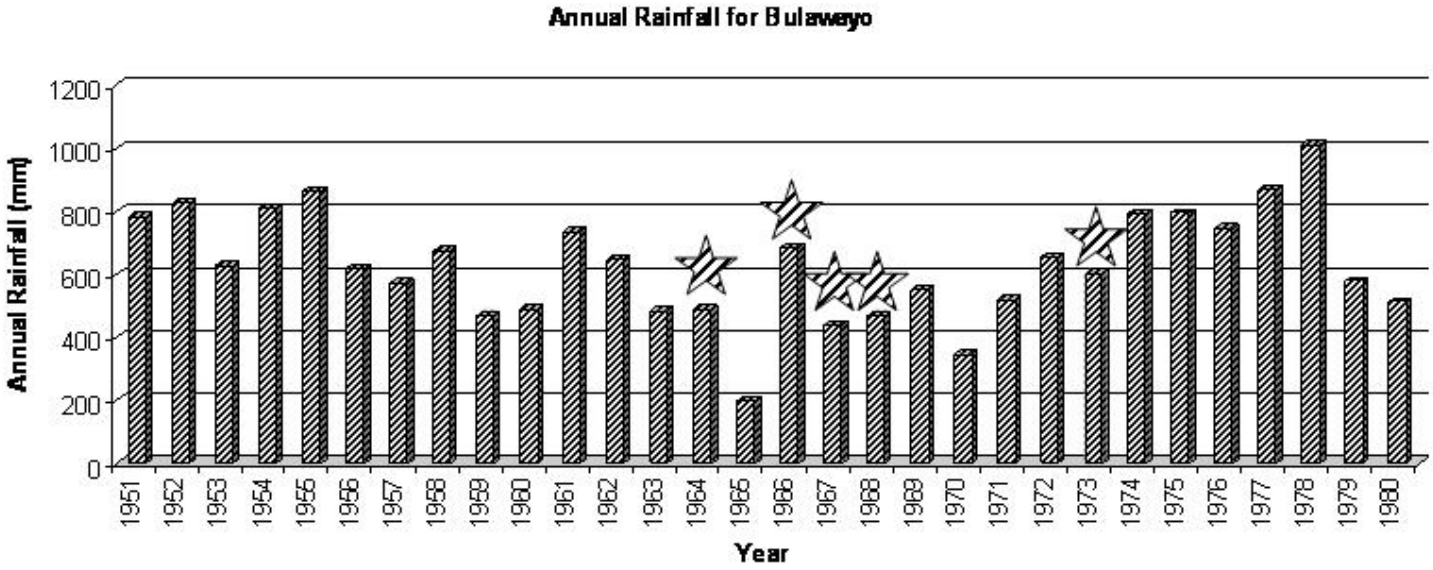
Table 1. Overview of fungi isolated from *P. angolensis* trees in South Africa with locations noted.

Fungus	Location*
Acremonium sp.	BNR, MNR, SCA
Alternaria sp.	MNR
Aspergillus sp.	MNR, SCA
Candida sp.	BNR, MNR, SCA
Chrysosporium sp.	BNR, MNR, SCA
Cylindrocarpon sp.	BNR, MNR, SCA
Cytospora sp.	MNR, SCA
Diplodia alatafructa	BNR, SCA
Fusarium sp.	MNR, SCA
Geotrichum sp.	BNR
Gliocladium sp.	BNR, MNR, SCA
Humicola sp.	BNR, MNR, SCA
Lasiodiplodia crassispora	MNR
L. pseudotheobromae	MNR, SCA
L. theobromae	BNR, MNR
Mycelia sterilia	BNR, MNR, SCA
Myriodontium sp.	MNR
Penicillium sp.	BNR, MNR, SCA
Rhizopus sp.	MNR
Staphylotrichum sp.	BNR, MNR, SCA
Trichoderma sp.	BNR, MNR, SCA
Trichosporon sp.	MNR

*BNR = Buffelskloof Nature Reserve, MNR = Mawewe Nature Reserve, SCA = Sudwala Caves area

Figure 1. Charts of rainfall data from the Livingstone and Bulawayo areas. Mukwa disease reports are denoted by stars above bars.



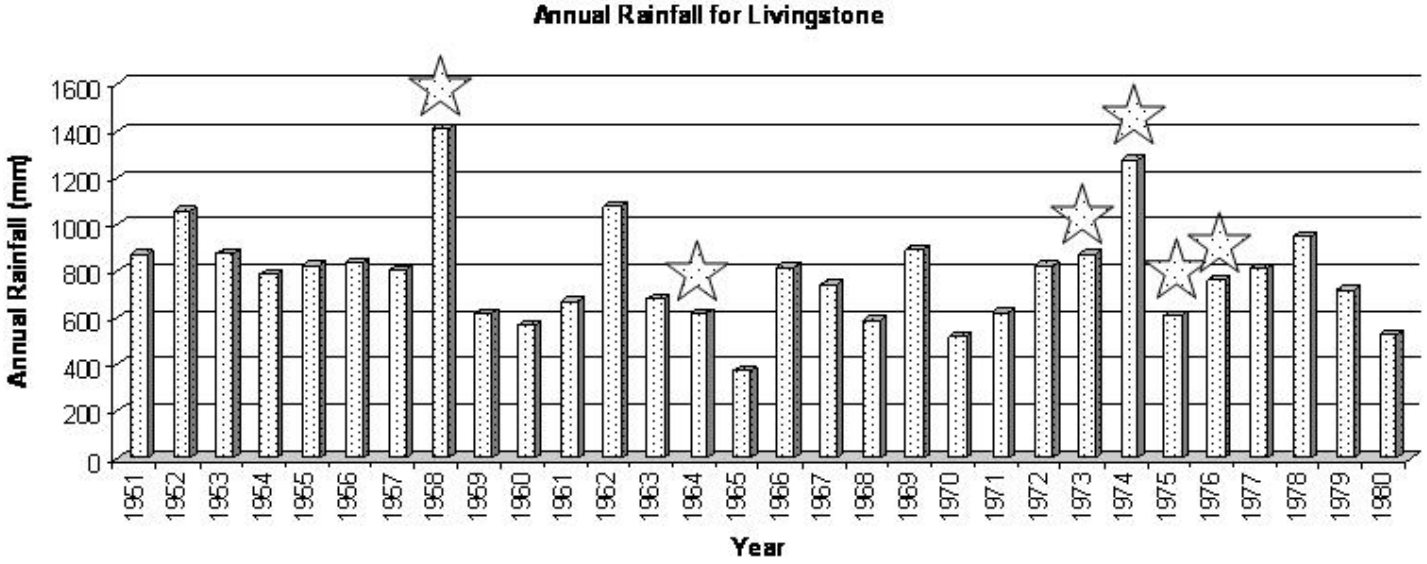


Figure 2. Map of the sites sampled. The shaded area on the South African map indicates the Mpumalanga Province where samples were collected from. Sampling sites on the larger map of Mpumalanga Province are denoted by a grey dot with adjacent white stars (South Africa map source: http://upload.wikimedia.org/wikipedia/commons/thumb/b/bc/Map_of_South_Africa_with_Mpumalanga_highlighted.svg, accessed 27/7/2009, Mpumalanga Province map source: http://www.stayinsa.co.za/southafrica/mpumalanga_hotels.html, accessed 27/7/2009)


ZIMBABWE
BOTSWANA
MOZAM-BIQUE
NAMIBIA
LIMPOPO
NORTH WEST
GAUTENG
MPUMALANGA
KWAZU-LAND
FREE STATE
KWAZULU NATAL
NORTHERN CAPE
LESOTHO
EASTERN CAPE
WESTERN CAPE
Bushbuckridge settlement
Buffelskloof Nature Reserve
Lydenburg
Sabie
Sudwala Caves area
Nelpruit
Hazyview
Skukuza
Pretoriuskop
KRUGER PARK
Malelane
Mawewe Nature Reserve
Standerton

Figure 3. Disease symptoms observed on *P. angolensis* trees in South Africa. a. Diseased trees in the field. b. *Ganoderma* fruiting body at the base of a fire-damaged tree. c. Heart rot with evidence of zone lines present. d. Heart rot initiated due to fire damage of the stem.

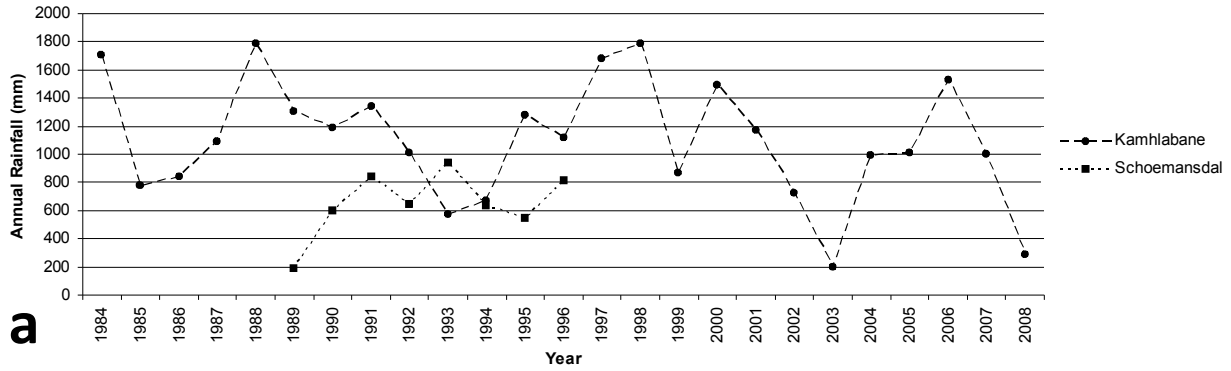

a

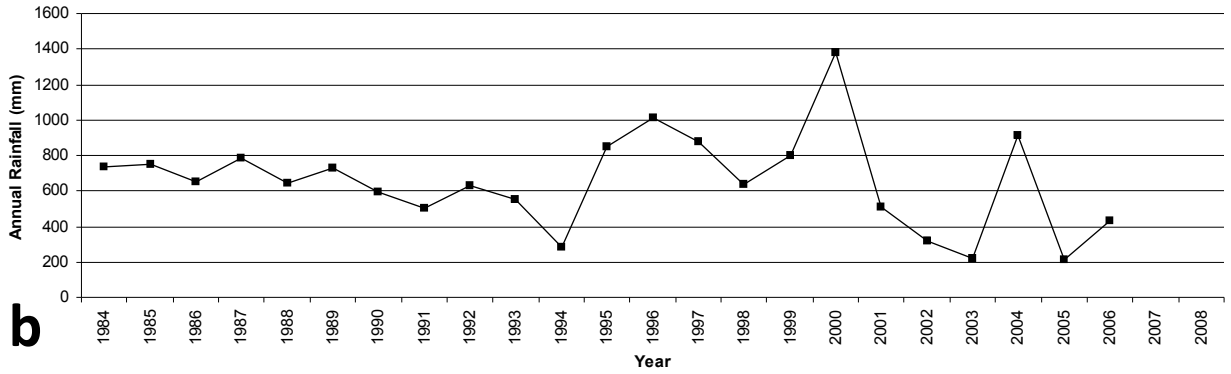

b

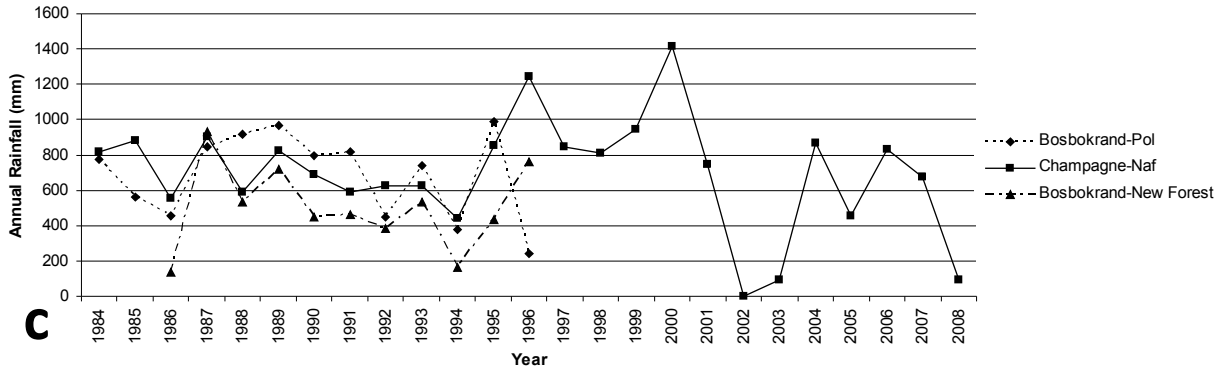

c


d

Figure 4. Rainfall data (mm) for the various areas considered in this study. Weather stations noted are closest to the sites from where we sampled. a) Mawewe Nature Reserve area. b) Pretoriuskop. c) Bushbuckridge area. d) Buffelskloof Nature Reserve area.


Annual Rainfall (mm)
Year
Kamhlabane
Schoemansdal
a


Annual Rainfall (mm)
Year
b


Annual Rainfall (mm)
Year
Bosbokrand-Pol
Champagne-Naf
Bosbokrand-New Forest
c

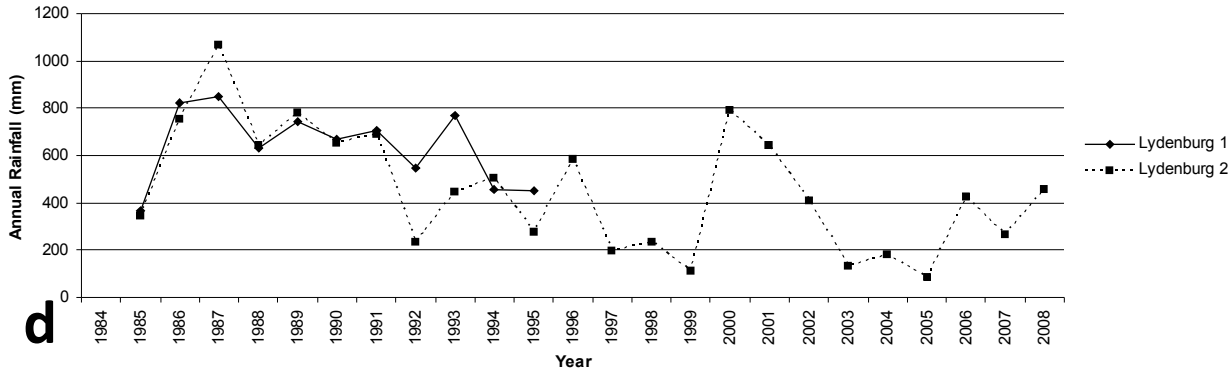

Annual Rainfall (mm)
Year
Lydenburg 1
Lydenburg 2
d

Figure 5. Incorrect growth forms of *P. angolensis* due to side branches and secondary stems during field observations (a, b) and correct growth form (c). Correct growth form can be obtained through fire management and by manually pruning. Note competing plants at the base of the tree in (a). (c) copyright Geldenhuys (2005).


a


b


c

SUMMARY

Studies undertaken in this dissertation have expanded our knowledge regarding the fungal diversity of *P. angolensis* trees in South Africa and identified several important pathogens of this species. The results emphasize the virtually unexplored fungal biodiversity present on native tree species in Southern Africa. In contrast, comparable research undertaken on commercially grown plantation tree species has been extensive. Prior to research undertaken in this dissertation, only saprophytes and wood-rotting fungi were known to occur on *P. angolensis*. The discovery of previously unreported fungal species in these studies, has important implications for the conservation and management of *P. angolensis*, as well as its utility in other Southern African countries.

The literature review presented in chapter one highlighted the limited knowledge regarding fungal diseases of *P. angolensis*. A single disease, mukwa, attributed to *Fusarium oxysporum*, has been reported from the species in the Livingstone district but has not been reported in South Africa. Reports of fungi from the tree have been limited to individual isolations, are based solely on morphology and include one or more Southern African countries.

The second chapter represents the first investigation of Botryosphaeriaceae associated with *P. angolensis* trees in Southern Africa. Seven species were isolated; four of these are new to science and are provided with the names *Pseudofusicoccum olivaceum*, *Ps. violaceum*, *Diplodia alatafructa* and *Fusicoccum atrovirens*. Species distribution varied amongst the sites sampled although the most species represented by the most isolates collected originated from a single site, Mawewe Nature Reserve. Field inoculation trials revealed that two of the species collected, *D. alatafructa* and *Lasiodiplodia pseudotheobromae*, were potential pathogens of *P. angolensis*, although these results highlighted that pathogenic ability is often characteristic of an isolate. Although most of the species of Botryosphaeriaceae isolated are not virulent pathogens of *P. angolensis*, they might contribute to the decline of this species.

Several isolates of *Ceratocystis* obtained from a stem wounding trial on *P. angolensis* trees provided the focus of chapter three. These isolates were all identified as those of *C. savannae*. Phylogenetic analyses highlighted that *C. savannae* and *C. oblonga*, a related species, are cryptic relatives, and only sequence data for a single gene region, EF-1α, successfully delineated between the two species and grouped isolates in this study with those of *C. savannae*. Morphological characterization also indicated overlapping morphological characters between the two species.

Sexual crosses amongst isolates confirmed that they represent the same species. Additional crosses between these isolates and the ex-type isolates of *C. savannae* and *C. oblonga* revealed that these are biologically the same species as *C. savannae* and represent a biological species distinct from *C. oblonga*. This represents the first study to apply the biological species concept to delineate species within the *C. moniliformis sensu lato* species complex. Branch inoculation trials revealed that this fungus is probably not pathogenic to *P. angolensis*.

The final chapter of this dissertation reviews and compares mukwa disease and the disease affecting *P. angolensis* trees in South Africa. Field observations on the symptoms of diseased trees and the fungi isolated from these trees are noted. Possible contributory factors, especially fire and drought, are also considered for both situations. Finally, implications for the management and conservation of *P. angolensis* are considered.

Studies undertaken in this dissertation resulted in a number of fungi newly reported from *P. angolensis* trees in South Africa. Some of these represent potential pathogens of the tree and should be considered in efforts to conserve and manage the species. The discovery of these fungi emphasizes the unexplored fungal diversity of native tree species in Southern Africa. In addition, it lays a foundation and motivates for continued research in the Livingstone district to clarify the cause of mukwa disease.