TABLE OF CONTENTS

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Preface

The pantropical genus *Terminalia* (Combretaceae, Myrtales) accommodates hundreds of species, of which about fifty occur naturally in Africa. These trees are planted throughout the sub-saharan region where they constitute a source of timber and traditional medicine. *Terminalia* trees are also found associated with agricultural crops to establish a "taungya" agri-sylvicultural system where food crops are grown together with tree species. Despite the importance of *Terminalia* spp., very little research has been done regarding the fungal diseases affecting these trees.

The aim of the research presented in this dissertation was to provide a foundation for understanding health issues affecting *Terminalia* spp. in selected regions in Africa. Both native African and an introduced Asian species of *Terminalia* were included in the study, allowing comparison of fungal communities of the same species between different areas. Special emphasis was placed on the identity and diversity of the Botryosphaeriaceae, since these fungi have a wide host and geographic range and represent a fairly well studied group of fungal tree pathogens.

The first chapter of the dissertation is a review of the literature, providing a summary of the knowledge pertaining to *Terminalia* spp. in Africa. A specific focus is given to their origin and distribution, botanic description, ecology**,** propagation, management, functional uses and international trade. Also, the limited knowledge regarding pests and diseases on these trees is reviewed, providing a background for the contents of the dissertation.

Among all the species of *Terminalia* present on the African continent, *T. catappa* is one of the few species planted widely in West, Central, East and Southern Africa. *T. catappa* has a Meridional Asian origin, but its broad distribution over the continent made it an ideal candidate to characterise endophytic species of the Botryosphaeriaceae under variable geographic and climatic conditions. The results of this study are presented in chapter two.

Evergreen forests dominate the vegetation in West and Central Africa. In this area, *Terminalia* spp. represents some of the most imposing and common tree groups. Native *T. ivorensis* and *T. superba* are of great economic importance as they are among the most important export timbers. However, current management of native *Terminalia* spp. in their natural environment does not take into consideration the impact of diseases, and limited information is available regarding the

fungal pathogens of these trees. Fungal species in the Botryosphaeriaceae are important threats resulting in wood stain, cankers, die-back and death of trees, particularly when trees are under some form of stress. The third chapter of this dissertation presents the results of an investigation aimed to identify and characterize the Botryosphaeriaceae occurring on *Terminalia* spp. in Cameroon.

The flora of Southern Africa is remarkably diverse with over a thousand indigenous tree species including at least 13 species of *Terminalia.* These trees are small shrubs to medium sized trees, found in open woodlands and wooded savannahs where they contribute to biodiversity and provide various benefits to rural populations. In contrast to Western and Central Africa, very few indigenous tree species are suitable for timber and pulp production in Southern Africa. Therefore, the domestic need for wood in this area is resorbed through plantations of non-native tree species such as *Eucalyptus* or *Pinus* spp. Many introduced, commercially propagated trees are related to native woody plants and growing evidence are showing that pathogens are able to move between them. Knowledge of potentially pathogenic fungi on native trees contributes to assessments of the vulnerability of both native forests and plantations of non-native trees. Fungi belonging to the Botryosphaeriaceae represent interesting model organisms to study the movement of fungal pathogens between native and introduced hosts. Therefore, the study presented in chapter four considers the diversity of the Botryosphaeriaceae associated with native *Terminalia* spp. in southern Africa.

A “taungya” agri-sylvicultural system is an agroforestry system where food crops are grown together with tree species. Some trees, such as *T. ivorensis* and *T. superba* are successfully mixed with *T. cacao* to establish such an agroforestry system in Western Africa. Under such associations, fungal species in the Botryosphaeriaceae, that can exploit more than one host species, can readily move between these hosts and infect plants without producing clear symptoms. Both *L. theobromae* and *L. pseudotheobromae* have been reported from native *Terminalia* spp. and introduced *T. cacao* in Cameroon. Therefore, in order to better understand the ecology and the evolution of interacting partners in a taungya system, the genetic structure of these species was studied in chapter five.

The last chapter of this dissertation considers a stem canker disease on *Terminalia* spp. in Cameroon. The aim of the study was to identify the causal agent of the disease. Symptoms were

typical of those of a fungal disease and fruiting structures of the pathogen were abundant on the dying bark. Thus, the fungus was isolated, identified using a suite of techniques including DNA sequence comparisons and pathogenicity tests were conducted to assess its ability to cause disease.

Studies in this dissertation expand our knowledge pertaining to diseases on native and non-native *Terminalia* spp. in Africa. In addition to previous diseases reports, it appears that *Terminalia* spp. are far from immune to fungal diseases. Although few serious diseases were found during the surveys, the risk of disease outbreaks in the natural ecosystems is considerable since these trees accommodate various latent pathogens that can cause disease under stress conditions. Therefore, sustainable production of *Terminalia* spp. in Africa must rely on the implementation of effective monitoring systems, supported by appropriate management structures.

Chapter 1

Literature Review

Terminalia spp in Africa with special reference to its health status

ABSTRACT

The genus *Terminalia* is the second largest genus in the Combretaceae. The family is distributed throughout the tropical and sub-tropical regions of the world and approximately fifty species of *Terminalia* are naturally distributed throughout western, eastern and southern Africa. *Terminalia* spp. range from small shrubs or trees to large deciduous forest trees. Some species, such as *T. ivorensis* and *T. superba* develop as elements of the canopy or sub-canopy layer in evergreen, semi-deciduous to deciduous, primary and secondary forests, whereas species such as *T. sericea*, thrive well in open woodlands and mixed deciduous forests. *Terminalia* spp. can be propagated naturally by seeds or through vegetative methods with wildings, seedlings, stump plants or striplings. *Terminalia* spp. provide economical, medical, spiritual and social benefits. Limited information on the pests and diseases affecting *Terminalia* spp. exists. Many insect species are associated with *Terminalia* spp. but no widespread pest problems have been recorded. Nevertheless, some locally common species are potentially dangerous, mostly affecting the early stages of trees. Very few pathogens have been reported from *Terminalia* spp. The majority of reports include limited detail, often representing no more than a brief mention. Often the causal agents were identified based only on morphology and were not classified to species level. Scanty information regarding the pathogens associated with introduced and native *Terminalia* is a limitation that might be detrimental for the survival and the successful exploitation of these trees.

1. INTRODUCTION

Terminalia (Combretaceae, Myrtales) is a pantropical genus accommodating about 200 species (McGaw *et al.* 2001). About fifty of these are native to Africa and distributed throughout the sub-saharan region (Lebrun and Stork 1991). Based on both their functional uses and distribution in Africa, the most important are *Terminalia ivorensis* A. Chev. and *T. superba* Engl. and Diels. in West and Central Africa and *T. prunioides* M.A. Lawson and *T. sericea* Burch : DC in Southern Africa (Irvine 1961; Lamb and Ntima 1971; Coastes-Palgrave 1977; Groulez and Wood 1985; Schmidt *et al.* 2002; Lawes *et al.* 2004).

Terminalia trees are planted in several countries in the tropics as a source of high quality solid timber for fine carpentry, joinery, building, flooring and plywood manufacture (Schmidt *et al*. 2002; Smith *et al.* 2004). *Terminalia ivorensis* and *T. superba*, especially, form an important component of the forestry industries in many countries (Anonymous 1997). *Terminalia* spp. are also commonly planted in mixed crop systems to establish a "taungya" agri-sylvicultural system in which they provide shade and play a major role in increasing soil fertility (Nichols *et al*. 2001; Norgrove and Hauser 2002a). Furthermore, members of the genus *Terminalia* are among some of the plants most widely used for medicinal purposes in Africa (Masoko *et al*. 2005; Kamtchouing *et al.* 2006).

Despite the importance of *Terminalia* spp., very little research has been done regarding the fungal diseases affecting these trees. Evidence of die-back, leaf spot and canker has been reported from *Terminalia* spp. (Lamb and Ntima 1971; Ofosu Siedu and Cannon 1976; Hodges and Fereira 1981). Gryzenhout *et al.* (2005), recently reported a serious disease problem that emerged on non-native *T. ivorensis* in Ecuador, while in South Africa, two *Ceratocystis* spp. have been reported from *T. sericea* (Roux *et al*. 2004; Kamgan *et al*. 2008).

The last or the 20th Century was marked by an increasing requirement for timber, fuel and medicine from trees. This has resulted in unsustainable logging of native trees in Africa. To supplement this requirement, plantations of non-native trees, including *Eucalyptus* spp., *Pinus* spp., *Acacia* spp. and *Cupressus* spp. are been established in many parts of the tropics and the southern hemisphere (Turnbull 1991; Wingfield *et al*. 2002; Anonymous 2007). In Africa, as in most other countries, these non-native trees are established in close proximity to native trees. This close association may in the long run, expose trees to new pests and diseases. One

might thus see the movement of native pests and pathogens onto introduced tree species. This is of great concern since this could provide pathogens with an elevated opportunity to spread to the country of origin of its new host, through reciprocal international trade of wood and wood products, causing large-scale mortality of trees in their native ecosystems (Wingfield 2003; Slippers *et al.* 2005; Wingfield *et al*. 2008). On the other hand, the non-native tree might be the source of non-native pathogens and pests, which may spread to the native trees in its new country, resulting in disease epidemics. An increasing number of examples for both case scenarios exist. For example, it has been shown that *Chr. austroafricana* Gryzenh. & M. J. Wingf., the cause of canker and death of plantation grown *Eucalyptus* spp. in South Africa, also occurs on native Myrtales in Africa (Heath *et al*. 2006; Nakabonge *et al*. 2006). This pathogen is thought to have originated from Africa (Heath *et al.* 2006). On the other hand, in California, native Monterey pine (*Pinus radiata* D. Don) are seriously affected by the pitch canker pathogen, *Fusarium circinatum* Niremberg & O'Donnell, following its introduction with Mexican pines (Gordon *et al.* 2001). In this respect, knowledge of indigenous tree diseases would be useful to establish firm risk assessment programs.

In Africa, some species of *Terminalia* generally occur as elements of the canopy or subcanopy layer in evergreen, semi-deciduous to deciduous primary and secondary forests. Other species thrive in open woodlands and littoral areas. Within this natural habitat of *Terminalia* spp., several non-native tree species are frequently encountered. There is, therefore, a good chance of introduced pathogens spreading onto native *Terminalia* trees, or innocuous fungi on *Terminalia* spp., moving onto the non-native trees. The objective of this review is to present a summary of knowledge pertaining to *Terminalia* spp. in Africa. A specific focus is given to their origin and distribution, botanic description, ecology**,** propagation, management, functional uses and international trade. Also, the limited knowledge regarding pests and diseases on these trees is reviewed, providing a background for the contents of the dissertation that follows this review and that focuses on fungi associated with native and introduced *Terminalia* spp. on the African continent.

2. THE GENUS *TERMINALIA*

2.1. Origin and distribution

The family Combretaceae is comprised of 20 genera and about 475 species (Thiombiano *et al.* 2006). Of these about 200 belong to the genus *Terminalia,* making it the second largest genus of the family after *Combretum* (McGaw *et al.* 2001). The family is distributed throughout the tropical and sub-tropical regions of the world (Lamb and Ntima 1971). Approximately 54 species of *Terminalia* are naturally distributed throughout western, eastern and southern Africa (Lebrun and Stork 1991; Smith *et al.* 2004). *Terminalia ivorensis* and *T. superba* are the most important species found in West and Central Africa (Norgrove and Hauser 2002b), but are also established in plantations within and outside their natural range, e.g. in South and Central America, east Africa, Hawaii, Fiji and the Solomon Islands (Jones 1969). Within the Malaysian region, further trials exist in Sabah, Kalimatan and the Philippines (Lamprecht 1989). On the other hand, *T. prunioides*, *T. brachystemma* Welw. ex Hiern, *T. sericea*, *T. gazensis* Bak., *T. mollis* Laws. and *T. sambesiaca* Engl. & Diels. are the most common species in eastern and southern Africa (Coates-Palgrave 1988; Masoko *et al.* 2005).

2.2. Botanical description and ecology

The genus *Terminalia* derives its Latin name (terminalis = end) from the position of the leaves, which are crowded at the ends of the shoots (Lamb and Ntima 1971; Rogers and Verotta 1996). The taxonomy of the genus has not been without problems, with the assignment of sub-genera, especially presenting differing views (Stace 1965). However, the different species have now been grouped into three sections according to characteristics of the fruits. These sections include the Section *Abbreviatae* Excell with shorter fruits, Section *Psidioides* Excell with longer fruits and the Section *Platycarpae* Engels & Diels emend. Excell, with fruits that are broader in the centre (Carr 1988).

Terminalia spp. range from small and medium sized shrubs or trees to large deciduous forest trees, ranging in height from 1.5 to 75 m tall (Lebrun and Stork 1991; Schmidt *et al.* 2002). Some species, such as *T. ivorensis, T. superba* and *T. trichipoda* Diels have cylindrical boles that are very straight and long (Figure 1a) with small to large, flat buttresses (six meters above the soil surface) and are sometimes branchless for up to 30 m and 2-5 m in girth (Lemmens *et*

al. 1995). Mature trees are extensively flat topped, with a wide horizontal canopy of evenly distributed foliage arising from the apex of the straight bole (Dupuy and Mille 1993). It is these characteristics, and their relatively fast growth, that make *T. ivorensis* and *T. superba* popular timber species. Other species, such as *T. sericea* and *T. prunioides* are common as small shrubs (Figure 1b) to bushy trees that may be single or multi-stemmed with a girth of up to 1.5 m (Coates-Palgrave 1977).

The bark of *Terminalia* trees is smooth and light grey to dark brown when young and on branchlets. The inner bark and contact zone with the cambium is frequently yellow. In mature trees, the bark surface cracks and flakes off in long thin strips or small patches, often becoming blackish and developing deep longitudinal fissures as the trees grow (Keay 1989; Lemmens *et al.* 1995).

The root systems of *Terminalia* trees are frequently fairly shallow. As the trees age, the tap roots disappear. In the tall species such as *T. ivorensis*, buttresses from which descending roots arise at some distance from the trunk, develop to support the trees (Keay 1989; Lamprecht 1989).

The leaves are frequently simple and obovate, clustered spirally at the ends of the dwarfed lateral branchlets, or crowded near the ends of the branches. Some species, like *T. brassii* Exell., have prominent glands at the leaf bases (Lamb and Ntima 1971). In mature trees the crown is usually flat or very slightly domed, giving *Terminalia* trees a distinctive shape.

Terminalia trees are bi-sexual or hermaphroditic with male and female flowers carried on the same plants. These flowers are apetalous, small, and cream to pale, bright yellow or greenish-white, in spicate inflorescences. The stalked male flowers tend to be grouped towards the apex and the bisexual flowers towards the base of the inflorescences (Coates-Palgrave 1977). *Terminalia* spp. have an effective system of self-incompatibility. Although male and female flowers are in the same plant, self-pollination cannot produce viable zygotes (Newbegin *et al.* 1994). The flowers are pollinated by various insects (*Coleoptera, Diptera, Hemiptera, Hymenoptera* and *Lepidoptera*) (Uzoechina 1978). The flowering-to-fruiting period may last about 4 months, depending on the species and the locality where it is grown (Coates- Palgrave 1977; Keay 1989).

Terminalia fruits, in combination with leaf characteristics, are of great diagnostic value and absolutely essential to distinguish between species (Coode 1969; Excell and Stace 1972). The fruits are hard, flattened, two-winged and to some extent inconsistent in size, especially the length and width of the wings. The general seed shape is consistent however, specific characters such as fruit colour when ripe (yellow, red, purple green, brown or pink depending on the species) and fruit morphology (elongate, broad, narrow, ovoid, oblong or elliptic) (Figure 2) vary and are helpful in species differentiation (Dale and Greenway 1961; Coates Palgrave 1977; Dale and Keay 1989). The fruits of some *Terminalia* trees, together with the bark, are important sources of tannin (Lemmens and Wulijarni-Soetjipto 1991; Ellery and Ellery 1997; Mabberley 1997). *Terminalia* fruits differ from those of closely related *Combretum* spp. in having a sclerenchymatous endocarp (Lamb and Ntima 1971). Most of the African *Terminalia* spp. produce fruits from January to September, with the exception of species such as *T. ivorensis* and *T. glaucescens* Planch.: Benth. that produce fruits from July onwards (Coates Palgrave 1977; Keay 1989).

The African species of *Terminalia* generally occur in various environments. Some species, such as *T. ivorensis* and *T. superba* develop as elements of the canopy or sub-canopy layer in evergreen, semi-deciduous to deciduous, primary and secondary forests (Keay 1989). Species such as *T. sericea* on the other hand, thrive well in open woodlands to wooded savannahs and mixed deciduous forests (Dale and Greenway 1961; Lebrun and Stork 1991; Carr 1994). *Terminalia* trees can tolerate light to moderate shade, when young (Jones 1969). Thereafter, they should receive full overhead light for optimal growth (Veenendaal *et al.* 1996). Few individuals of this genus are able to grow at high altitude; most species perform well at altitudes less than 2000 m a.s.l. The climates in which *Terminalia* trees grow varies from areas with year round rain for species occurring in the forest areas (> 2000 mm per annum) to seasonal with moderate rainfall (< 1200 mm per annum) for those occurring in savannah zones (White 1983).

2.3. Propagation and management

Terminalia spp. can be propagated naturally by seeds or through vegetative methods with wildings, seedlings, stump plants or striplings (Lemmens *et al.* 1995). However, obtaining plants is difficult and with most *Terminalia* spp., propagation is not easy (Carr 1994).

2.3.1. Seed propagation

Freshly fallen seed should be collected from the ground, as seed still on the tree may not be fully mature. Sometimes the seeds are collected from the trees by cutting off the branches, because fallen seed are often parasitized by insects, leading to low viability. As far as possible, seeds should be collected from healthy mother trees with a vigorous stem and crown (Browse 1979; Hartman and Kester 1983). The number of fruits and seeds (per kilogram) vary greatly between *Terminalia* spp. For example, *T. ivorensis* can produce 5500 - 7300 seeds per kg (Lamb and Ntima 1971), *T. superba* can produce 8000 - 10000 seeds/kg (Groulez and Wood 1985) and *T. prunioides* 8200 seeds/kg (Palmer and Pitman 1972). Seeds are extracted from the fruits by placing them in a heap, spraying them with water and then covering them with grass or leaves. After a day or so the fruit wing is stripped off and the seeds are extracted manually (Carr 1994).

In general, seeds vary in the length of time that they remain viable. The viability of seed varies between species. The viability of most species diminishes rapidly, with the exception of *T. superba*, of which the seeds can be stored in sealed containers at 2-4 °C for one year, adding to its suitability for commercial exploitation (Groulez and Wood 1985).

Seeds of *Terminalia* spp. must undergo a period of dormancy before germination occurs. There are two types of dormancy in plants, namely physical and physiological dormancy (Weber and Stoney 1986). Several methods of pre-treatment can be used to overcome the two types of dormancy in seeds of *Terminalia* spp. As seeds of *Terminalia* spp. are covered by a hard protective coat, the physical dormancy ends when the seed coat is opened through different processes such as mechanical abrasion, nicking or soaking in water (Browse 1979; Weber and Stoney 1986). Most often, for some species of *Terminalia*, seeds are pre-treated by soaking in water for 12-48 hours, by manual scarification, or, in the case of *T. ivorensis*, by alternate soaking and drying for one week (Lamb and Ntima 1971). For *T. ivorensis* the germination rate is 10-50 %, but up to 93 % under experimental temperature fluctuations, while for *T. superba* it is 60-80 % (Lemmens *et al.* 1995), 15-35 % for *T. prunioides* and 1-2 % for *T. sericea* (Carr 1994). However, as the viability in seeds is not assured, covering seed or fruit in the seedbed is important for increasing the germination percentage. Light shade is generally applied during germination, but it should be removed after one to two months. The seedbed should be watered frequently to provide adequate moisture during germination

(Browse 1979). The sowing medium should be sand with low levels of "fines" or a light soil, half mixed with sifted compost to promote good drainage and avoid water logging during heavy rain periods (Carr 1994).

The physiological dormancy of *Terminalia* seed ends within two weeks after sowing and is followed by epigeous germination which lasts two to five weeks. Pricking out should be done early enough to avoid disturbing the rapidly developing taproot (Lemmens *et al.* 1995). For *T. superba,* pricking out is recommended six weeks after sowing when two leaves have developed (Groulez and Wood 1985), whereas for *T. ivorensis*, it should be as soon as the two cotyledons unfold and the seedlings are 20-30 cm tall (Lamb and Ntima 1971).

2.3.2. Vegetative propagation

As an alternative to overcome seed viability problems, long growth periods and inconsistency of seed germination (Carr 1994), vegetative propagation through cuttings and stump plants can be used (Lemmens *et al*. 1995). However, vegetative propagation has in Africa been used early for species such as *T. ivorensis* and *T. superba* in Africa (Fisher 1976).

The planting stock for vegetative propagation should be taken from young, vigorous shoots or suckers from healthy, mature trees, during the dormant season, to encourage fast-growing stems (Browse 1979). Short cuttings are taken from different parts of the stem (e.g. from the rejuvenated stump plants and from branches). These stems (25-35 cm long) are cut just above the proposed top bud and horizontally at the base, if possible dipping in a rooting hormone before planting. Depending on the species, the cuttings can be placed either in pots filled with water, or directly into a trench which is kept moist (Hartman and Kester 1983). After a period of time shoots will produce roots, and they can then be transplanted to a permanent site. Stumps should have a diameter of at least 1.3 cm. Cuttings of *T. superba* and *T. ivorensis* produce roots within two weeks, with a rooting percentage of 11-100 % for *T. superba* according to the degree of rejuvenation (Lemmens *et al.* 1995).

2.3.3. Tending of trees

Planting of *Terminalia* trees should be done early in the rainy season to ensure an adequate water supply, and thus to avoid loss through drought (Lamb and Ntima 1971). Where necessary, hand watering should be used to supplement insufficient rainfall. Weeding is

necessary during the first 3-4 years to give the seedlings adequate light and air circulation, and to prevent competition for nutrients from weeds (Carr 1994).

Most *Terminalia* spp. have a good to extremely good self-pruning capacity (Lamb and Ntima 1971; Swaine and Hall 1983), adding to their popularity as plantation trees. The tall *T. superba* tree is occasionally branchless up to 90 % of the total tree height (Lemmens *et al.* 1995). Pruning by hand is, therefore, not required in commercial plantations. However, because of the wide spreading branches, the tree needs considerable space between stems (Groulez and Wood 1985).

Terminalia spp. are planted in Africa on a wide variety of soil types ranging from alluvial, sandy, salty and coral soils to heavy cracking clays (Coates-Palgrave 1988). The coppicing ability is good for a number of *Terminalia* spp. planted in Africa and India. *Terminalia chebula* Retz. and *T. albida* Scott-Elliot are known to withstand fire well, but *T. superba* and *T. ivorensis* are very vulnerable in this respect. The average rotation age for *T. superba* and *T. ivorensis* in Africa is 40 years, with trees reaching heights of 50-60 m and diameters of five meters in this time (Keay 1989; Lemmens *et al.* 1995).

2.4. Functional uses of *Terminalia* trees

Terminalia spp. provide economical, medicinal, spiritual and social benefits. The wood of *Terminalia* spp. is highly appreciated as constructional timber. It is currently used for light construction, door and window frames, coffin boards, mouldings, beams, rafters, joists, flooring, furniture, carts, tool handles, spindles, shuttles, picker sticks, walking sticks, bowls, boat building, masts, mine props, foundation piles, veneer and plywood (Irvine 1961; Lemmens *et al.* 1995; Schmidt *et al.* 2002; Smith *et al.* 2004). The fruits and bark of *T. sericea* and *T. catappa* L. are important sources of tannin, as well as gum and resins for glazing pottery (Irvine 1961; Lemmens and Wulijarni-Soetjipto 1991; Ellery and Ellery 1997). Dyes of various colours (black, red, orange, yellow, brown) are extracted from the leaves, fruits, bark and roots of species such as *T. mollis* Lawson, *T. ivorensis, T. laxiflora* Engl. & Diels., *T. catappa* L. and *T. superba* and used for decorating the walls of houses and buildings with murals, for dyeing clothes, mattings, rattan, spoons and walking sticks (Dalziel 1937; Errington and Chisumpa 1987). The seed of some species is edible and considered one of the best flavoured tropical nuts. Furthermore, consumable oil can be extracted from the

seed of *T. catappa* and used as a substitute for groundnut (*Arachis hypogea* L.), cotton seed (*Gossypium* spp.) and silk cotton seed (*Ceiba* spp.) oils (Irvine 1961).

In Africa, forests are sometimes use for ritual and spiritual purposes. Certain trees can serve to link the living with their ancestors, as this is often symbolized in the relationship between the sky and the earth. In Southern Africa, Tswana people believe that good crops are ensured at harvest and planting times by thrusting a stick of *T. sericea* into the floor of a shrine in homage to ancestral spirits, and that cutting down an entire tree will result in hail-storms (Coates-Palgrave 1977).

The importance of traditional medicines, derived from plants, is of great importance in most parts of both Africa and Asia (Lawes *et al.* 2004; Steenkamp *et al.* 2004; Moshi and Mbwambo 2005). Many *Terminalia* spp. have been identified as sources of medicines, for use in pharmaceuticals and cosmetic production (Dalziel 1937; Irvine 1961). Extracts of the flowers, fruits, bark, leaves, stems and roots from species such as *T. glaucescens*, *T. macroptera* Gill.& Perr., *T. laxiflora*, *T. superba*, *T. prunioides*, *T. brachystemma*, *T. sericea, T. gazensis, T. catappa, T. mollis* and *T. sambesiaca* are used in traditional medicine to treat diseases such as malaria, eczema, candidosis, asthenia, gonorrhoea, diabetics, dermatitis, scurfy affection, leprosy and tuberculosis (Batawila *et al.* 2005; Masoko *et al.* 2005; Fyhrquist *et al.* 2006; Kamtchouing *et al.* 2006). The bark of *T. macroptera* is burned and used by Sudanese women as a perfume (Irvine 1961).

Terminalia spp. have a number of agricultural uses. They are important sources of fodder for animals. *T. sericea* is one of the palatable woody plants found in South African savannahs (Owen-Smith and Cooper 1987; Lawes *et al.* 2004). Its leaves are browsed by domestic cattle, goats and game during the hot, dry season (Ellery and Ellery 1997; Mabberley 1997; Katjiua and Ward 2006).

Terminalia spp. are commonly established in the "taungya system" (Lamb and Ntima 1971), where they are combined in the early stages with the growing of agricultural crops like banana and cocoa (Nichols *et al.* 2001; Norgrove and Hauser 2002b). *T. ivorensis* and *T. superba* have been successfully used in this respect throughout West Africa, particularly in Cameroon (Diaw *et al.* 1999; Norgrove and Hauser 2002a, 2002b). In this part of the continent, cacao farms are managed in harmony with several tree species by adjustment of forest cover to

provide shade for cacao trees. One of the advantages of the system is that it reduces the initial costs of establishment by alleviating the cost of forest clearing and weeding in the first two or three years of the agricultural crop. In addition, many of these shade trees provide additional services and incomes through the provision of medicinal products and timber (Sonwa *et al.* 2000). The cultivation of the crops greatly stimulates the early growth of the trees, providing fertilisation through the leaf litter (Singh *et al.* 2002; Goma-Tchimbakala and Bernhard-Reversat 2006). This form of tree management through enhancement of carbon sequestration confers environmental sustainability to the cocoa plantations and thus justifies its use as a model of promotion within the framework of developing agroforestry systems with perennial crops in Africa.

In West and Central Africa, native *Terminalia* spp. are of great economic importance as they are among the most important export timbers. In 1995, Cameroon exported 62000 m^3 of *Terminalia* logs, together with 15000 m^3 of sawn wood, 10000 m^3 of veneer and an unrecorded amount of plywood. This amount of exported *Terminalia* ranked it third in national export for the country (Anonymous 1999). During the same period, the Democratic Republic of Congo exported 3000 m^3 of *Terminalia* logs, 1000 m^3 of sawn wood and small quantities of veneer, while the other Congo Republic exported 10000 m^3 of logs (Anonymous 1997). Côte d'Ivoire exported 7000 m^3 of logs and a small amount of veneer, while Ghana exported 18000 m^3 of *Terminalia* logs, 3000 m^3 of sawn wood and 1000 m^3 of veneer during 1995 (Anonymous 1997). The wood of African *Terminalia* spp. is used particularly in Egypt, France, Belgium, Germany, Nederland, Switzerland, USA, Philippines, Brazil, Thailand, and Japan (Anonymous 1997).

2.5. Pests and diseases

Relatively few studies of the pests and diseases affecting *Terminalia* spp. have been made. There are many insects species associated with *Terminalia* spp. but, as yet, no widespread pest problems have emerged. Nevertheless, some locally common species are potentially dangerous, mostly affecting the early stages of the trees.

2.5.1. Insects

2.5.1.1. Fruit Borers

A 3-mm-long weevil (*Nanophyes* sp.), is considered the most serious pest of *T. ivorensis* and *T. superba* in Nigeria and Ghana. The weevil deposits its eggs in the ripening seed on the tree and can reduce germination by up to 40 % (Lamb and Ntima 1971). Attacked seed can be recognised by the presence of a dark brown spot, consisting of excrement, on the seed surface.

2.5.1.2. Stem Borers

Stem damage, caused by a thyridid moth, *Tridesmodes ramiculata* Warren, and ambrosia beetles (*Dolipygus* spp.) have been reported from higher altitude zones in Nigeria and Ghana (Lamb and Ntima 1971). The larvae of the moth occur as shoot borers on *Terminalia* seedlings in nurseries as well as in plantations (Lamb and Ntima 1971). The death of infested shoots results in the production of multiple stems. This moth is considered to be of potential importance (Groulez and Wood 1985). The ambrosia beetle prefers to attack newly felled trees and those which are injured or sickly, leading to very serious economical damage as they reduce the tree quality and consequently reduce the financial returns (Robert 1987).

Zeuzera coffeae Nietner is a branch boring caterpillar, primarily infesting branches of *Terminalia* spp. in Africa and Asia (Lamb and Ntima 1971). However, it can also attack saplings where it may be found in the main stems (Bigger 1998). It has a wide host range in the South Pacific Region, including several important plantation species such as *Paraserianthes falcate* Becker, *Casuarina equisetifolia* L., *Eucalyptus deglupta* Blume, *Swietenia* macrophylla King, *Tectona* grandis L., *Terminalia* brassii Excell and *T. ivorensis* (Bigger 1998). Elsewhere, in Panama, multiple xylem borer attacks by a *Cossula* sp. was observed on *T. ivorensis*, affecting wood quality where the incidence is high (Kapp *et al.* 1997).

2.5.1.3. Defoliators

Many insects feed on the leaves of *Terminalia* spp. *Zononocerus variegatus* L., the variegated grasshopper, is widely distributed throughout tropical Africa and feeds on the

leaves of young trees in nurseries and plantations (Akabi and Ashiru 1991, Messi *et al.* 2006). A number of coleopterans (*Trochalus* sp., *Maladera* sp., *Pseudotrochalus* sp.) (Browne 1968) and lepidopterans (*Maurilia phaea* Hamps., *Negeta luminosa* Wkr., *Westermania cuprea* Hamps., *Tortrix dinota* Meyrick, *Trabala lambaurni* Beth-Baker) also feed on the leaves of *Terminalia* spp. in nurseries and plantations (Browne 1968). In Côte d'Ivoire and Nigeria, several species of caterpillars infest plantations of *T. ivorensis* and *T. superba*, but the most serious defoliator is *Epicerura pergrisea* Hampson, which can cause severe damage to its hosts (Akanbi and Ashiru 1991, Kanga and Fediere 1991). Sucking pests, such as *Cryptoflata* spp., as well as *Otionotus* sp. and *Tricoceps albescens* Funkh. have been reported to occur commonly in Ghana where their adults and colonies of nymphs affect the growth of *T. ivorensis* shoots, mainly in nurseries (Browne 1968). In Papua New Guinea, *Roeselia lignifera* Walker causes substantial harm to *T. brassii* plantations. The larva of this moth was associated with defoliation of young entire plantations in that country (Lemmens *et al.* 1995), while leaf cutting ants (*Atta* sp.) have damaged young plantations of *T. ivorensis* in Panama (Kapp *et al.* 1997).

2.5.1.4. Termites

When *Terminalia* trees are split, many insects, notably termites, usually infest them (Lamb and Ntima 1971). However, these insects occur only sporadically and are not a major threat.

2.5.2. Wildlife

In Eastern Nigeria, the foliage of *T. ivorensis* are prone to browsing by small antelopes (C*ephalopus maxwelli* Hamilton-Smith, Maxwell's duiker), which may give rise to injuries. Other serious damage is caused by elephants (Lamb and Ntima 1971). These wounds, from wildlife, could open trees for infection by pathogens, such as those residing in the genus *Ceratocystis*, as recently shown in South Africa for *T. sericea* (Kamgan *et al*. 2008).

2.5.3. Diseases

Terminalia spp. are potential hosts for many fungal pathogens in Africa and elsewhere. It is, however, evident that the incidence of pathogens on *Terminalia* spp. has not been studied thoroughly. Very few pathogens have been reported from *Terminalia* spp. The majority of

the reports that have been made include limited detail, often representing no more than a brief mention. Often the causal agents were identified based only on morphology, and not classified to the species level. This is problematic for the establishment of quarantine guidelines.

2.5.3.1. Root diseases

Howes (cit. Piening, 1962), reported *Armillaria mellea* (Vahl ex Fr.) Kummer, the honey fungus, associated with a *Terminalia* sp. in Ghana. However, no particulars were given. Certainly, modern taxonomic treatments of the genus *Armillaria* in Africa has shown that it represents numerous species (Coetzee *et al.* 2000; Mwenje *et al.* 2006) suggest that the fungus on *Terminalia* was identified only in the broad sense. More recently, it was observed that root rot caused by species of *Rosellinia* and *Phytophthora*, leads to die-back of *T. ivorensis* in Panama and Costa Rica (Kapp *et al.* 1997).

2.5.3.1. Stem diseases

In nurseries and forest plantations in Nigeria, Parker (1964) reported die-back, leaf spot and canker due to a *Sphaeronaema* sp. on *T. ivorensis*. The main symptoms were a cessation of growth, accompanied by die-back of the main shoots, but wilting and yellowing of the foliage occurred occasionally. Otherwise, observations of black stem cankers, frequently associated with reddening of leaves, causing mortality and stagnation in nursery plants were reported in this country (Lamb and Ntima 1971). An *Endothiella* sp. has also been found on cankers on *T. ivorensis* in Ghana (Ofosu Siedu and Cannon 1976), while pink disease, caused by *Erythricium salmonicolor* Berk, causes stem canker on tropical almond (*T. catappa*) in India (Thomson and Evans 2006). Recently, Gryzenhout *et al.* (2005) described of a new pathogen, *Rostraureum tropicale* Gryzenh. & M. J. Wingf. in association with dying *T. ivorensis* in Ecuador.

2.5.3.3. Leaf diseases

Some foliage diseases caused by unidentified species of *Cercospora*, *Ramularia*, *Irenina* and *Spaceloma* have been reported from *T. superba* in Africa (Groulez and Wood 1985). In Brazil, *Korinomyces terminaliae* Hodge & Ferreira causes leaf spots on seedlings and young

T. ivorensis plants (Hodges and Fereira 1981), and *Auerswaldiella parvispora* causes black blotches on leaves (Farr 1989).

2.5.3.4. Stain diseases

When freshly felled, logs are liable to attack by fungi whose effect is mainly aesthetic, but which is nevertheless serious in a wood renowned for its agreeable colour. In this respect, blue stain of logs caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl was described on *T. superba* by Fougerousse (1958). The fungus is able to completely spoil the lustrous creamy-white colour of the log even if only the sheen of the wood is altered.

3. CONCLUSIONS

Terminalia spp. are dominant trees in many African ecosystems and are planted extensively in timber plantations and in intercropping systems. Furthermore, selected species such as *T. sericea* that regenerate easily, are relatively fast growing and are favoured by local communities and grown in protected stands as part of a process to rehabilitate degraded woodlands. *Terminalia* spp. provides medicinal, spiritual and social benefits. However, it appears from the present review that despite the fact that species of *Terminalia* are not immune from disease infections, scanty information regarding the diseases associated with introduced and native *Terminalia* trees are available. This limitation might be detrimental for the survival and the successful exploitation of these trees, but also presents potential threats to surrounding vegetation and crops.

Fungal pathogens and pests present a serious threat to the future of plants and trees on the African continent. This is especially important because of the rate and ease of spread of fungal pathogens between continents that is increasingly more common. Our native African biodiversity is, therefore, under threat from numerous fungal pathogens, non-native to the continent. These pathogens may enter the continent on a number of different hosts, especially those related to our native trees.

The purpose of the studies presented in this dissertation will be to address the above mentioned issues by studying the fungal flora and possible diseases of *Terminalia* spp. in Africa. Surveys will be conducted in Southern and Western Africa in order to identify fungal

pathogens on these trees, as well as their means of infection and spread. Hopefully, results of this study will serve as valuable tools in forestry management in Africa.

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Figure 1: Growth forms of *T. ivorensis* (a) and *T. sericea* (b) in Africa.


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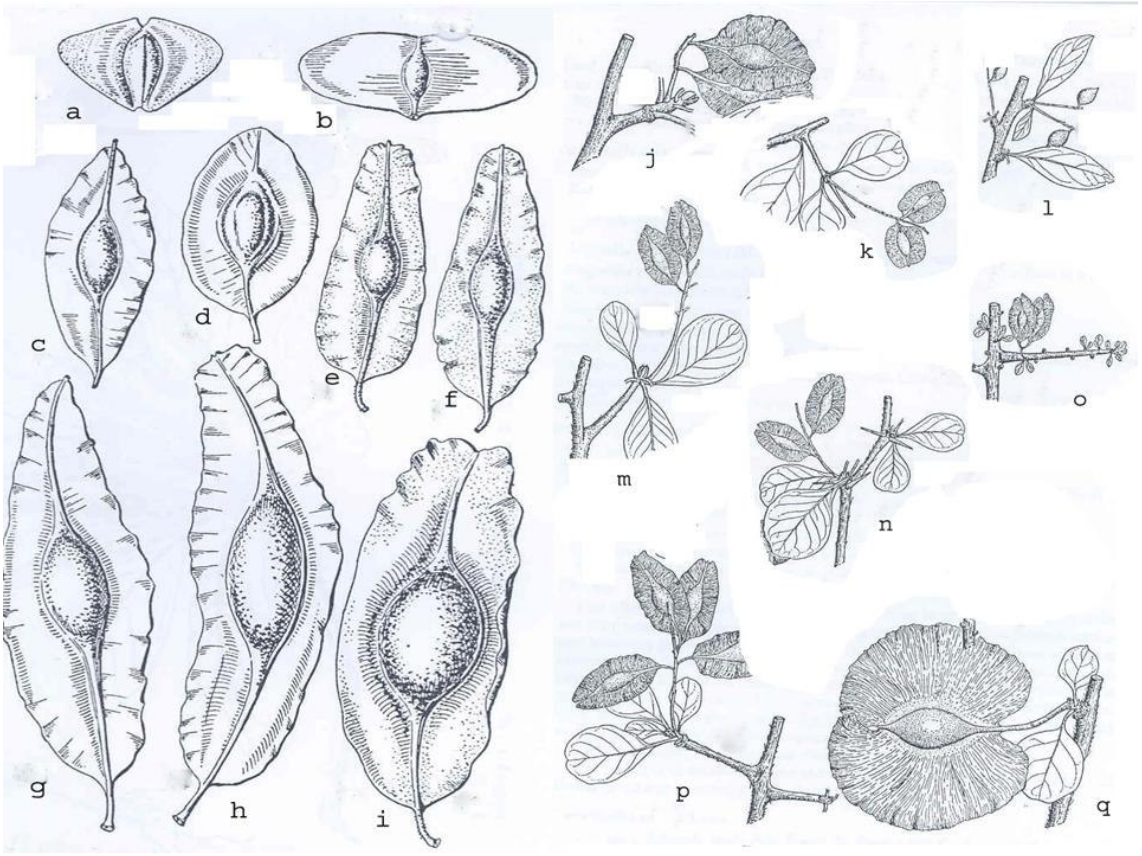
b

Figure 2: Fruits of some African *Terminalia* spp. (a) *T. scutifera* Planch. ex Lawson, (b) *T. superba,* (c) *T. laxiflora*, (d) *T. brownii,* (e) *T. glaucescens,* (f) *T. avicennioides,* (g,h) *T. macroptera,* (i) *T. mollis,* (j) *T. kilimandscharica* Engl., (k,m) *T. brevipes* Pampan, (l) *T. fatraea* (Poir.) DC., (n) *T. spinosa* Engl., (o) *T. parvula* Engl. & Diels, (p) *T. prunioides,* (q) *T. orbicularis* Engl. & Diels (Dale and Greenway 1961; Keay 1969).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Chapter 2

Botryosphaeriaceae associated with *Terminalia catappa* in Cameroon, South Africa and Madagascar

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ABSTRACT

Species in the Botryosphaeriaceae represent some of the most important fungal pathogens of woody plants. Although these fungi have been relatively well studied on economically important crops, hardly anything is known regarding their taxonomy or ecology on native or non-commercial tree species. The aim of this study was to compare the diversity and distribution of the Botryosphaeriaceae on *Terminalia catappa*, a tropical tree of Asian origin planted as an ornamental in Cameroon, Madagascar and South Africa. A total of 83 trees were sampled, yielding 79 Botryosphaeriaceae isolates. Isolates were initially grouped based on morphology of cultures and conidia. Representatives of the different morphological groups were then further characterized using sequence data for the ITS, *tef 1-α*, *rpb2*, BOTF15 and *β-tub* gene regions. Five species of the Botryosphaeriaceae were identified, including *Neofusicoccum parvum*, *N. batangarum sp. nov.*, *Lasiodiplodia pseudotheobromae*, *L. theobromae* and *L. mahajangana sp. nov*. *Lasiodiplodia pseudotheobromae* and *L. theobromae,* were the most commonly isolated species (62%), and were found at all the sites. *Neofusicoccum parvum* and *N. batangarum* were found in South Africa and Cameroon respectively, whereas *L. mahajangana* was found only in Madagascar. Greenhouse inoculation trials performed on young *T. catappa* trees showed variation among isolates tested, with *L. pseudotheobromae* being the most pathogenic. The Botryosphaeriaceae infecting *T. catappa* appear to be dominated by generalist species that also occur on various other hosts in tropical and sub-tropical climates.

1. INTRODUCTION

The Botryosphaeriaceae is a diverse group of fungi that accommodates numerous species spread over many anamorph genera, the best known of which are *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum, Dothiorella* and *Sphaeropsis* (Crous *et al.* 2006). Members of the Botryosphaeriaceae have a worldwide distribution and occur on a large variety of plant hosts including monocotyledons, dicotyledons, gymnosperms and angiosperms, on which they are found as saprophytes, parasites and endophytes (Slippers and Wingfield 2007; von Arx 1987).

It has long been recognized that species of the Botryosphaeriaceae are important pathogens of several plants (Von Arx 1987). Infected plants can exhibit a multiplicity of symptoms such as die-back, canker, blight and rot on all above ground plant organs (Punithalingham 1980; Slippers *et al.* 2007). A particularly dangerous feature of these fungi is that they can live as endophytes in plant organs, in a latent phase, without producing clear symptoms, and diseases only emerge following the onset of unfavourable conditions to the tree (Smith *et al.* 1996). This implies that they can easily, and unobtrusively, be moved around the world with seeds, cuttings and even fruit.

Extensive studies have been conducted on diseases of economically important species of fruit (e.g. Lazzizera *et al.* 2008; Phillips 1998; Slippers *et al.* 2007; van Niekerk *et al.* 2004) and timber trees (e.g. Mohali *et al.* 2007; Sanchez *et al.* 2003) caused by fungi in the Botryosphaeriaceae. Much less is known about the Botryosphaeriaceae on plants with no large-scale international commercial value (Denman *et al.* 2003; Pavlic *et al.* 2008), such as *Terminalia catappa*, but which have social and environmental significance (Gure *et al.* 2005). Without knowledge of the Botryosphaeriaceae on hosts with limited or no commercial value, and hosts in their native environments, the impact and biology of the important pathogens in this group will never be fully understood.

Terminalia catappa, frequently referred to as “tropical almond”, belongs to the Combretaceae and originates from Southern India to coastal South-East Asia (Smith 1971). These trees are widely cultivated in tropical and subtropical coastal areas and utilised by local communities for a number of household uses. The multitude of non-wood products and services pertaining

to this tree species make it an important component, especially for coastal communities. The tree is planted for shade and ornamental purposes in urban environments, the timber is converted into decorative tools, furniture and many other applications, leaves and bark are commonly used in traditional medicine and its fruits contain edible kernels from which high energy oil is extracted and which can also be admixed into diesel fuel (Chen *et al.* 2000; Hayward 1990; Kinoshita *et al.* 2007).

The diversity and spatial distribution of the Botryosphaeriaceae, associated with a specific host, is important. Whether it accommodates similar or different fungal assemblages depending on the environment, is useful in understanding the ecology and host-pathogen relationships of these fungi. This knowledge in turn can be applied where recommendations for disease management strategies are required. Several studies have compared assemblages of fungal endophytes in different geographic regions (Fisher *et al.* 1994; Gallery *et al.* 2007; Gilbert *et al.* 2007; Taylor *et al.* 1999). However, such studies dealing with a specific endophytic group of fungi are limited. Similarly, very few studies have compared the assemblages of Botryosphaeriaceae from a specific host at a regional level (Taylor *et al.* 2005; Urbez-Torrez *et al.* 2006).

Among all the species of *Terminalia* present on the African continent, *T. catappa* is one of the few species planted widely in West, Central, East and Southern Africa. As part of a larger project in which we explore diseases of *Terminalia* spp. in Africa, the broad distribution of this species over the continent made it an ideal candidate to characterise endophytic species of the Botryosphaeriaceae under variable geographic and climatic conditions. The aims of this study were, therefore, to investigate the diversity of the Botryosphaeriaceae occurring on introduced *T. catappa* and to analyse the patterns of their distribution in three African countries. Pathogenicity trials were also undertaken to assess the ecological significance of the Botryosphaeriaceae collected from *T. catappa*.

2. MATERIALS AND METHODS

2.1. Isolates

Collections were made from *T. catappa* trees in Cameroon, Madagascar and South Africa. In Cameroon, samples were collected along the beach front of Kribi, a seaside town within the tropical forest and bordering the Atlantic Ocean (N2 58.064, E9 54.904, 7 m asl). The climate in this area is characterized by high humidity, precipitation up to 4000 mm per annum and relatively high temperatures, averaging 26 ˚C. In South Africa, sampling was done in Richardsbay (S28 46.886 S, E32 03.816, 0 m asl), a harbour city on the Indian Ocean where *T. catappa* trees are planted to provide shade in open spaces and in parking areas. Climatic conditions in this area are typically subtropical to tropical. The average temperature in summer is 28 °C and 22 °C in the winter. The humidity levels tend to be very high in summer and the annual rainfall is ~1200 mm. In Madagascar, samples were collected from the towns of Morondava (S20 17.923, E44 17.926, 3 m asl) and Mahajanga (S15 43.084, E46 19.073, 0 m asl), both located on the west coast of the country. In these areas, the climate is between semi-arid and tropical humid with mean annual temperatures of 23.5 °C and average rainfall between 400 and 1200 mm per annum.

Samples were collected from 83 *T. catappa* trees in all three countries in 2007. Forty trees were randomly sampled in Kribi, 15 in Richardsbay, 20 and eight in Morondava and Mahajanga, respectively. Except for the trees in Richardsbay, that were showing symptoms of die-back at the time of collection, those at all the other sites were healthy. One branch (~0.5 - 1 cm diameter) per tree was cut and all the samples placed in paper bags and taken to the laboratory where they were processed after one day.

From each branch, two segments (1 cm in length each) were cut and split vertically into four halves. Samples were surface sterilized by dipping the wood pieces in 96 % ethanol for 1 min, followed by 1 min in undiluted 3.5 % sodium hypochlorite and 1 min in 70 % ethanol, before rinsing in sterile distilled water and allowing them to dry under sterile conditions. The four disinfected branch pieces from each tree were plated on 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri dishes

were sealed with Parafilm and incubated at 20 °C under continuous near-Ultra Violet (UV) light. One week later, filamentous fungi growing out from the plant tissues and resembling the Botryosphaeriaceae were transferred to new Petri dishes containing fresh MEA.

All cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representatives of all species have also been deposited at the Centaalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands). Herbarium materials for previously underscribed species have been deposited at the National Fungal Collection (PREM), Pretoria, South Africa.

2.2. Morphology and cultural characteristics

Fungal isolates were grown on plates containing 1.5 % water agar (Biolab, S.A.) overlaid with three double-sterilized pine needles and incubated at 25 °C under near UV-light for two to six weeks to induce the formation of fruiting bodies (pycnydia and/or pseudothecia). Morphological features of the resultant fruiting bodies were observed using a HRc Axiocam and accompanying Axiovision 3.1 camera (Carl Zeiss Ltd., München, Germany). For previously undescribed species, sections of fruiting bodies were made with a Leica CM1100 cryostat (Setpoint Technologies, Johannesburg, South Africa) and mounted on microscope slides in 85 % lactic acid. For the undescribed species 50 measurements of all relevant morphological characters were made for the isolate selected as the holotype and 30 measurements were made for the remaining isolates. These measurements are presented as the extremes in brackets and the range calculated as the mean of the overall measurements plus or minus the standard deviation.

The morphology of fungal colonies growing on 2 % MEA at 25 °C under near UV-light for two weeks was described and colony colours (upper and reverse surfaces) of the isolates were recorded using the colour notations of Rayner (1970). Growth rates of cultures on 2 % MEA in the dark was determined at 5 °C intervals from 10 to 35 °C. For growth rates, evaluations of five plates were used for each isolate at each temperature. Two measurements, perpendicular to each other, were made after three days for each plate resulting in 10 measurements for each isolate at each temperature. The experiment was repeated once.

2.3. DNA extraction

Mycelium was scraped from 10-day-old cultures representing different morphological groups, using a sterile scalpel and transferred to 1.5 µl Eppendorf tubes for freeze-drying. The freeze-dried mycelium was mechanically ground to a fine powder by shaking for 2 min at 30.0 $1s^{-1}$ frequency in a Retsch cell disrupter (Retsch Gmbh, Germany) using 2 mm-diameter metal beads. Total genomic DNA was extracted using the method described by Möller *et al.* (1992). The concentration of the resulting DNA was determined using a ND-1000 uv/Vis Spectrometer (NanoDrop Technologies, Wilmington, DE USA) version 3.1.0.

2.4. PCR amplification

The oligonucleotide primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'TCCTCCGCTTATTGA TATGC 3') (White *et al.* 1990), EF1 (5' TGCGGTGGTATCGACAAGCG T 3') and EF2 (5' AGCATGTTGTC GCCGTTGAAG 3') (Jacobs *et al.* 2004), BT2A (5'GGTAACCAAATCGGTGCTGCTTTC3') and BT2B (5'ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass and Donaldson 1995), RPB2bot6F (5'GGTAGCGACGT CACTCCC3') and RPB2bot7R (5'GGATGGATCTCGCAATGCG3') (Sakaladis 2004), BOT15 (5' CTGACTT GTGACGCCGGCTC3') and BOT16 (5' CAACCTGCTCAGCAAGCGAC3') (Slippers *et al.* 2004c) were respectively used to amplify and sequence the internal transcribed spacer regions (ITS), including the complete 5.8S gene, the translation elongation factor 1-α gene (*tef 1-α*), partial sequence of the β-tubulin gene (*β-tub*), part of the second largest subunit of RNA polymerase II gene (*rbp2*) and an unknown locus (BotF15) containing microsatellite repeats. A "hot start" polymerase chain reaction (PCR) protocol was used on an Icycler thermal cycler (BIO-RAD, Hercules, CA, USA). The 25 µl PCR reaction mixtures for the ITS, BT and RPB2 regions contained 0.5 µl of each primer (10 mM) (Integrated DNA Technology, Leuven, Belgium), 2.5 µl DNTPs (10 mM), 4 µl of a 10 mM $MgCl_2$ (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µl of 10 mM reaction buffer (25 mM) (Roche Diagnostics GmbH, Mannheim, Germany), 1 U of *Taq* polymerase (Roche Diagnostics GmbH, Mannheim, Germany), between 60-100 ng/µl of DNA and 13.5 µl of sterile distilled water (SABAX water, Adcock Ingram Ltd, Bryanston, S.A.). The amplification conditions were as follows: an initial denaturation step at 96 °C for 1 min, followed by 35 cycles of 30 seconds at 94 °C, annealing for 1min at 54 °C, extension for

90 seconds at 72 °C and a final elongation step of 10 min at 72 °C. To amplify the *tef 1-α* gene region, the 25 µl PCR reaction mixture contained 0.5 µl of each primer (10 mM), 2.5 µl DNTPs (10 mM), 2.5 µl of 10 mM reaction buffer with $MgCl_2$ (25 mM) (Roche Diagnostics GmbH), 1 U of *Taq* polymerase, between 2-10 ng/µl of DNA and 17 µl of sterile SABAX water. The amplification conditions used were similar to those of Al-Subhi *et al.* (2006) and the conditions used to amplify the BotF15 locus were the same as those of Pavlic *et al.* (2009a). The PCR amplification products were separated by electrophoresis on 2 % agarose gels stained with ethidium bromide in a 1x TAE buffer and visualized under UV light.

2.5. DNA Sequencing

Amplified PCR fragments were cleaned using 6 % Sephadex G-50 columns with 50-150 µm bead size (Sigma, Steinheim, Germany) following the manufacturer's instructions. Thereafter, 25 amplification cycles were carried out for each sample on an Icycler thermal cycler to generate sequences in both the forward and reverse directions using 10 µl mixtures. Each mixture contained 1µl reaction buffer, 2 µl ready reaction buffer (Big dye), 1 µl primer (10 mM), 3 µl of the PCR product and 3 µl Sabax water. The following PCR conditions were followed: One step at 96 °C for denaturation of the double stranded DNA (10 s), followed by an annealing step at 50 °C (5 s) and primer extension at 60 °C (4 min). The BigDye Terminator v 3.1 Cycle sequencing Kit (PE Applied Biosystems) was used for sequencing reactions, following the manufacturer's protocols, on an ABI PRISM 3130xl genetic analyzer using Pop 7 polymer (Applied Biosystems, Foster City, California, USA).

2.6. DNA Sequence Analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using MEGA version 4 (Tamura *et al.* 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in Genbank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh *et al.* 2005). The aligned sequences were transferred to PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2001) where a final manual alignment was made. All the ambiguously aligned regions within each data set were excluded from the analyses. Single gene phylogenetic analyses were run

for the datasets representing the different gene regions and three combinations of analyses were also done: ITS and *tef 1-α* for all the isolates; ITS, *tef 1-α*, *β-tub*, *rbp2* and BOTF15 for *Neofusicoccum* and ITS, *tef 1-α* and *β-tub* for *Lasiodiplodia* isolates. In the analyses, gaps were treated as fifth characters and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition sequences for the construction of maximum parsimonious trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. *Guignardia philoprina* (Berk. & M.A. Curtis) Van der Aa was used as outgroup in analyses of ITS and *tef 1-α* data sets whereas no outgroup was inserted in additional analyses for the *Neofusicoccum* and *Lasiodiplodia* groups of isolates as the trees generated were unrooted. The support for branches of the most parsimonious trees was assessed with a 1000 bootstrap replications (Felsenstein 1985). Other measures used to assess the trees were tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992). A partition homogeneity test (Farris *et al.* 1995) was conducted in PAUP to assess the possibility of combining the ITS and *tef 1-α* data sets in analyses of all the isolates whereas Incongruence Length Difference (Farris *et al.* 1995) was used in combined analyses for the groups of *Neofusicoccum* and *Lasiodiplodia* isolates.

Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with PAUP. Before launching the Bayesian analyses, the best nucleotide substitution models for each dataset were separately determined with MrModelTest version 2.2 (Nylander 2004) and included in each partition in MrBayes v3.1.2. (Huilsenberg and Ronquist 2001). GTR+I+G and HKY+G were chosen as best-fitting models for the ITS and *tef 1-α* data sets respectively for the general analyses. In the following independent analyses, K80, HKY, GTR, and HKY + I, models were chosen for the ITS, *tef 1-α*, *rbp2*, *β-tub* and BotF15 data sets respectively to analyse sequences of species in the *Neofusicoccum* group. In the second analyses for species in the *Lasiodiplodia* group, the following models were chosen: K80, HKY + I and HKY for the ITS, *tef 1-α* and *β-tub* data sets respectively. The MCMC analyses, with four chains, started from random tree topology and lasted one million generations. Trees were saved every 100th generation. The burn-in

number was graphically estimated from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after 50 % majority rule.

2.7. Pathogenicity

Two-year-old nursery grown *T. catappa* plants with stems ranging from 50-100 cm in height and 1- 1.5 cm in diameter, growing in peat moss soil in 20 L plastic bags were maintained in the greenhouse at 22 °C and watered once a day for pathogenicity experiments. For inoculations, 15 isolates of Botryosphaeriaceae representing all the species identified in the study (Table 1) were grown on 2 % MEA for 10 days prior to inoculation. To inoculate trees, wounds were made on the stems by removing the outer bark with a 7 mm diameter cork-borer. A 7 mm-diameter plug of the test isolates was placed into each wound, with the mycelium facing the cambium, and covered with a strip of Parafilm to prevent desiccation of the wound and inoculum. Five trees, arranged in a completely randomized design, were used for each isolate and the trial was repeated once. For the controls, sterile MEA plugs were used instead of a fungal culture. After six weeks, the lengths of the bark and cambium lesions were measured to obtain an indication of the pathogenicity of the isolates tested. Small pieces of necrotic tissue from the edges of lesions were incubated on MEA to show that the inoculated fungi were associated with the lesions. The trial was repeated once. As no significant differences were noticed between the two repeats of the pathogenicity test, the data for all isolates of a particular species were pooled in a single dataset for analyses. Variations in the lengths of the lesions were assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Isolates

In total, 79 isolates of Botryosphaeriaceae were obtained from 40 of the 83 *T. catappa* trees sampled in Cameroon, Madagascar and South Africa. Of these, 19 originated from branches on *T. catappa* in Kribi (Cameroon), 29 from Morondava, 8 from Mahajanga (Madagascar), and 25 from Richardsbay (South Africa). Only one isolate per tree was used for further

morphological and molecular studies. The isolates obtained were grouped according to their colony and conidial morphology, and representative isolates of each group were selected for DNA sequence comparisons.

3.2. Morphologic characterization

All the isolates from *T. catappa* could group into two categories based on conidial morphology (Table 2). The isolates in the first category (Group A) produced hyaline, elongate and thin-walled, fusoid conidia. In the second category (Group B), isolates were characterized by hyaline or dark, thick-walled, aseptate or one-septate, ovoid conidia sometimes exhibiting longitudinal striations. Only anamorph structures were produced by the isolates collected from *T. catappa* when incubated on pine needles.

Based on colony morphology, only one group could be distinguished for all isolates collected in this study. All isolates on MEA grew fast, filling the Petri dishes within five days. The aerial mycelium was originally white, turning dark greenish-grey or greyish after four to five days at 25 °C under near UV-light (Table 2). Based on a combination of colony morphology and morphology of conidia, it was possible to distinguish two groups of Botryosphaeriaceae from *T. catappa* with confidence and these were used in DNA sequence comparisons.

3.3. DNA extraction and PCR amplification

A total of 40 isolates, each originating from a separate *T. catappa* tree, were selected for ITS sequence comparisons to obtain a broad indication of their identities and to select isolates for the data sets used in the final analyses. These comprised 12 from Group A and 28 from Group B. Of these, 19 isolates were selected for *tef 1-α* sequence comparisons and were considered in the final analyses. Sequences from the *β-tub, rbp2* and BotF15 gene regions were used to clarify the relationships between isolates that could not be clearly resolved with ITS and *tef 1-α* sequences. DNA extraction and PCR was conducted successfully for all gene regions selected**.** PCR fragments for the ITS were ~ 580 bp in size, while those for *tef 1-α, β-tub, rbp2* and BOTF15 were 710 bp, 440 bp, 615 bp and ~350 bp, respectively.

3.4. DNA sequence analyses

ITS analyses. The ITS dataset comprised 82 sequences of which 40 originated from *T. catappa* and 42 sequences were retrieved from GenBank. Of the 543 characters present in the ITS sequence data set, 24 % were parsimony informative. The MP analyses generated 11 trees with identical topology (TL = 401, CI = 0.840, RI = 0.977, RC = 0.821). Isolates from *T. catappa* grouped into five well separated clades, representing *Neofusicoccum* [Bootstrap support (BS) = 74 % and Bayesian posterior probabilities (BPP) = 1] and *Lasiodiplodia* (BS = 51 %, BPP = 0.61), which also corresponded with the two groups defined based on isolate morphology (Figure 1).

Within the *Neofusicoccum* clade, isolates from *T. catappa* were divided into two groups. The first comprised only isolates from Cameroon, grouping in a single clade (with no Bootstrap value) close to the recently described *N. umdonicola* Pavlic, Slippers, & M.J. Wingf. The second clade accommodated isolates from *T. catappa* in South Africa, together with isolates of *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips., with no sequence variation among them. Bayesian analyses supported the separation of the isolates in the *Neofusicoccum* group as observed with MP analyses.

Isolates from *T. catappa* formed three clades within *Lasiodiplodia* based on ITS sequence data. Isolates in the first two clades grouped with *L. theobromae* (Pat.) Griff. & Maubl. and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous., and included isolates from all three countries. Three isolates (CMW27801, CMW27818, CMW27820) from *T. catappa* in Madagascar constituted a clade (BS = 63 % and BPP = 0.91), which did not group with any known *Lasiodiplodia* sp., suggesting a possibly undescribed species, most closely related to *L. parva* A.J.L. Phillips, A. Alves & Crous. The topology of the consensus tree generated with Bayesian analyses was similar in overall topology to the one obtained with MP analyses.

ITS and tef 1-α analyses. The partition homogeneity test for the ITS and *tef 1-α* data sets showed that they could be combined (P = 0.303) as no conflict was found between the gene genealogies. The combined dataset consisted of 59 isolates and contained 804 characters of which 38 % were parsimony informative. Gaps were treated as a fifth character. After heuristic searches, eight most parsimonious trees were obtained (TL = 884; CI = 0.782, RI =

0.960, RC = 0.750, TreeBase No: SN4517). The topology of the tree generated from the combined analyses with MP, as well as with the 50 % majority rule consensus tree (Figure 2), was congruent with the trees obtained with the individual analyses of ITS and *tef 1-α*, presenting the same clades. However, the Cameroonian isolates with hyaline, thin-walled conidia formed a single sub-group (BS = 56 % and BPP = 0.94) close to *N. umdonicola* within the larger clade containing species close to *N. ribis*, similar to that observed on the tree obtained for the ITS analyses. The clade (BS = 100 % and BPP = 1) accommodating the apparently undescribed *Lasiodiplodia* sp. from Madagascar (CMW27801, CMW27818, CMW27820) was basal to *L. plurivora* as observed on the tree obtained using *tef 1-α* analyses.

Analyses of both ITS and *tef 1-α* separately, as well as combined, identified the same groups amongst the isolates collected from *T. catappa*. These included *N. parvum*, *L. theobromae, L. pseudotheobromae* and two previously unidentified groups. Some uncertainty was, however, present regarding the Cameroonian isolates with hyaline, thin-walled conidia. Although these isolates consistently grouped in a unique clade within the *N. ribis/N. parvum* complex, only low statistical support was observed in all analyses. These results raised uncertainties regarding their relationship with other closely related species and prompted analyses using additional gene regions in an attempt to clarify their identity.

Additional analyses using five loci: To resolve uncertainties in the relationships among the Cameroonian isolates (CMW28315, CMW28363, CMW28320, CMW28637) grouping close to *N. ribis*, additional independent multilocus analyses were used for taxa included in the genus. Twenty-one isolates taken from the *N. ribis* and *N. parvum* complex were included in the ITS, *tef 1-α*, BOTF15, *rbp2* and *β-tub* data sets (Table 5). For each data set, trees obtained from both MP and Bayesian analyses showed identical topologies. Isolates from *T. catappa* in Cameroon grouping in the *N. ribis/ N. parvum* complex formed a distinct clade in four of the five individual partitions (Figure 3a, 3b, 3d, 3e). These isolates were more closely related to *N. ribis* and *N. umdonicola* than to any other species of the complex. The clade accommodating the Cameroonian *Neofusicoccum* isolates was characterized by Bootstrap and Bayesian posterior probabilities values between 60 and 97 %. The ITS and BOTF15 gene regions contained one unique fixed polymorphism each, while nucleotide sequences of *tef 1-α* and *β-tub* showed a number of base variations (one deletion and two substitutions for *tef 1-α* and one substitution for *β-tub*) from those representing *N. ribis* but no fixed polymorphism

was observed (Table 3). No differences were observed in *rbp2* sequences between Cameroonian isolates and those representing *N. ribis* (Table 3) (Figure 3c). However, analysis of the data from the *rbp2* locus remained informative and could not cancel out the lineage sorting of the Cameroonian isolates. To provide a better resolution in the relationship of these isolates, we combined the data from each partition into one phylogenetic analysis.

The Incongruence Length Difference calculated for all the data sets related to all the isolates included in the *N. ribis* and *N. parvum* complex (I = 0) (Table 5) indicated that the gene phylogenies were congruent. This was illustrated by strong statistical (BS = 86 %; BPP = 1) support observed on the consensus tree obtained from both MP and Bayesian analyses of the combined data set (Figure 3f). Analyses of the combined data sets confirmed the grouping of the isolates from Cameroon into an undescribed and unique lineage (Figure 3f).

Additional analyses for the *Lasiodiplodia* clade, including nine isolates representing *L. theobromae, L. pseudotheobromae, L. parva* and isolates of the apparently undescribed species from Madagascar were conducted using part of the ITS, *tef 1-α* and *β-tub* gene regions (Table 6). From the MP tree topologies, which were identical to those obtained in Bayesian analyses, for each partition, as well as the combined analysis, it was clear that isolates from Madagascar consistently formed a clade distinct from *L. theobromae, L. pseudotheobromae* and *L. parva* (Figure 4a, 4b, 4c, 4d). There was considerable sequence variation across the three gene regions among isolates representing the undescribed species and those of *L. theobromae, L. pseudotheobromae* and *L. parva* (Table 4). The resolution in the relationship of these isolates was improved by combining individual data sets in one phylogenetic analysis. The Incongruence Length Difference calculated for all the data sets for this group of isolates (I = 2) (Table 6) indicated a congruence in the phylogenies of all three genes. Strong statistical (BS = 100 %; BPP = 1) support was observed for the consensus tree obtained for both MP and Bayesian analyses (Figure 3d) of the combined data set. Analyses of the combined data sets confirmed the monophyly of isolates (CMW27801, CMW27818, CMW27820) from Madagascar in the *Lasiodiplodia* clade (Figure 4d).

3.5. Taxonomy

DNA sequence data for the ITS, *tef 1-α*, BOTF15, *rbp2* and *β-tub* gene regions revealed the presence of two previously undescribed species of Botryosphaeriaceae amongst the isolates collected from *T. catappa* in this study. A study of the morphology of these isolates confirmed that they are distinct from previously described species and they are consequently described as new here:

Lasiodiplodia mahajangana Begoude, Jol. Roux, Slippers, sp. nov. MB514012 FIGURE 5.

Etymology: the name refers to the locality where this fungus was collected for the first time.

Conidia pycnidialia usque ad 300.0 µm lata, in foliis Pini in MEA in 14 diebus facta, solitaria mycelio tecta, superficialia conica. Conidiophorae ad cellulas conidiogenas reductae. Cellulae conidiogenae holoblasticae discretae hyalinae cylindricae. Conidia primo non septata, hyalinae ellipsoideae vel ovoideae parietibus crassis < 2.5 µm, contentis granularibus, demum semel septataet liberata colorata, matura verticaliter striata 17.5 x 11.5 µm.

Conidiomata: pycnidial (up to 300 µm wide), produced on pine needles on MEA within 14 days, solitary and covered by mycelium, superficial, conical, unilocular, with long necks (up to 200 µm) and single ostioles at the tips, locule walls thick, consisting of two layers: an outer dark brown *textura angularis*, lined with inner thin-walled, hyaline cells. *Paraphyses*: rare, cylindrical, hyaline, aseptate 1-celled (27.5) 33.5-52.5 (66.0) x (2) 2.5-3.5 (5.0) µm, (average 50 paraphyses 43.0 x 3.0 µm), rounded at the tips, unbranched. *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, discrete, hyaline, cylindrical, proliferating percurrently to form a periclinal thickening (10.0) 10.5-18.0 (26.0) x (3.0) 3.5-5.5 (6.0) µm (average 50 conidiogenous cells 14.5 x 4.5 µm, l/w 3.2). *Conidia*: initially aseptate, hyaline, ellipsoid to ovoid, thick-walled (< 2.5 µm), granular content, becoming one-septate and pigmented after release, vertical striations observed at maturity, (13.5) 15.5-19.0 (21.5) x (10.0) 11.5-13.0 (14.0) µm (average 50 conidia 17.5 x 11.5 µm, l/w 1.4). *Cultural characteristics*: white fluffy and abundant aerial mycelium, becoming pale olivaceous grey (23’’’’’f) after 4 days, with the reverse sides of the colonies olivaceous grey (23’’’’’b).

Optimum temperature for growth 25-30 °C, covering a 90 mm diameter Petri dish after 3 days on MEA in the dark, no growth observed at 10 °C.

Teleomorph: not observed

Host: *Terminalia catappa*.

Distribution: Madagascar, Mahajanga.

Specimen examined: Madagascar, Mahajanga, 15° 43'.084 N, 46° 19'.073 E, 0 m asl: isolated from healthy branches of *Terminalia catappa*, Oct 2007, J. Roux, holotype (PREM 60288), a dry culture on pine needles CMW27801 = CBS124925; ex-type culture CMW27820 = CBS124927.

Additional specimens: Madagascar, Mahajanga, 15° 43'.084 N, 46° 19'.073 E, 0 m asl: isolated from healthy branches of *Terminalia catappa*, Oct 2007, J. Roux, ex-paratype (PREM 60289) CMW27818 = CBS124926.

Neofusicoccum batangarum Begoude, Jol. Roux, Slippers, sp. nov. MB514013 FIGURE 6.

Etymology: Name refers to the Batanga people who live in the area where the type specimen was collected.

Conidia pycnidialia in foliis Pini in 14 diebus facta, solitaria mycelio tecta, primo immersa, matura ¾ per foliis emergentia, obpyriformia vel ampulliformia. Conidiophorae ad cellulas conidiogenas reductae. Cellulae conidiogenae holoblasticae hyalinae cylindricae. Conidia non septata, hyalinae fusoideae vel ovoideae parietibus tenuis, 15.5 x 5.5 µm.

Conidiomata: pycnidial produced on pine needles within 14 days, solitary and covered by mycelium, initially embedded, ¾ erumpant through the pine needles at maturity, obpyriform to ampulliform with a central and circular ostiole at the neck, unilocular, locule wall thick consisting of two layers: an outer layer of dark brown *textura angularis*, lined with an inner layer of thin-walled, hyaline cells. *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, hyaline, cylindrical, proliferating percurrently, sometimes forming a periclinal thickening, smooth producing a single conidium, (11.0) 12.5-19.0 (27.0) x (2.0) 2.5-3.0 (3.5) µm (average of 50 conidiogenous cells 15.5 x 2.5 µm, l/w 6). *Conidia*: aseptate, hyaline, smooth, fusoid to ovoid, thin-walled, (12.0) 14.0 -17.5 (20.0) x (4.0) 4.5-6.0

(6.5) µm (average 50 conidia 15.5 x 5.5 µm, l/w 2.9). *Cultural characteristics*: colonies forming concentric rings on MEA, mycelium white and immersed at the leading edge, becoming smokey grey (21''''d) to grey olivaceous (21''''b) from the old ring after 5 days on MEA. *Optimum temperature for growth* 25 °C, covering the 90 mm diameter Petri plate after 4 days on MEA in the dark, little growth observed at 10 and 35 °C.

Teleomorph: not observed

Host: *Terminalia catappa*.

Distribution: Cameroon, Kribi.

Specimen examined: Cameroon, Kribi, Beach , 2° 58'.064 N, 9° 54'.904 E, 7 m asl, isolated from healthy branches of *Terminalia catappa*, Dec 2007, D. Begoude and J. Roux, ex-paratype (PREM 60285), a dry culture on pine needles CMW28315 = CBS124922; ex-type culture (PREM 60286) CMW28363 = CBS124924.

Additional specimens: Cameroon, Kribi, Beach, 2° 58'.064 N, 9° 54'.904 E, 7 m asl, isolated from healthy branches of *Terminalia catappa*, Dec 2007, D. Begoude and J. Roux ex-paratype (PREM 60284) CMW28320 = CBS124923; (PREM 60287) CMW28637.

3.6. Distribution of the Botryosphaeriaceae

In total, five species of Botryosphaeriaceae were isolated from *T. catappa* in South Africa, Madagascar and Cameroon. Two cosmopolitan species, *L. pseudotheobromae* the most commonly isolated species, which represented 42 % of the isolates collected and *L. theobromae*, were collected from trees in all three countries. The other three species, *N. parvum*, *N. batangarum* and *L. mahajangana,* were each isolated only in South Africa, Cameroon and Madagascar respectively (Figure 8).

3.7. Pathogenicity

All inoculations with isolates of Botryosphaeriaceae collected in this study resulted in visible lesions on the bark and cambium of *T. catappa* trees after six weeks. Analysis of variance showed that there were significant differences in the pathogenicity among species ($P < 0.0001$). Overall *L. pseudotheobromae*, *L. theobromae, N. parvum* and *N. batangarum* produced the longest lesions on both bark and cambium, whereas *L. mahajangana* produced the smallest lesions (Figure 9,10). Considerable variation in levels of pathogencity was also

observed among isolates of the same species. There was a positive correlation (R^2 = 75 %) between lesions produced on the bark and those on the cambium. Re-isolations from lesions on the inoculated trees resulted in the recovery of the inoculated fungi.

4. DISCUSSION

This study presents the first consideration of the possible fungal pathogens of *T. catappa*. It is also the first study of the Botryosphaeriaceae on these popular ornamental trees. In total**,** five species of the Botryosphaeriaceae were identified and two of these were new taxa that were described and provided with the names *N. batangarum* and *L. mahajangana.*

Slippers *et al.* (2004a), in comparing the assemblage of Botryosphaeriaceae on native and introduced *Eucalyptus* trees in Australia and South Africa, emphasized the importance of individually identifying species affecting a specific host in every country or environment where it occurs. This was because they found more pathogenic fungal species on *Eucalyptus* spp. outside their native environment (South Africa), than in the area (Australia) where these trees were native. Although the assemblage of the Botryosphaeriaceae found in the current study varied from one country to another, colonization patterns on *T. catappa* in the three areas showed similar trends. In each country, three species of Botryosphaeriaceae were found, one of which was specific to that country and two species occurring in all three countries. These patterns might be explained by climatic differences as has been shown for the distribution of Botryosphaeriaceae in California (Urbez-Torrez *et al.* 2006).

Phylogenetic relationships for the Botryosphaeriaceae from *T. catappa* and other known members of this fungal family were determined using combined sequence data sets of the ITS and *tef 1-α* gene regions. However, the resulting phylogenies did not clearly separate all the species. This was especially true for isolates from Cameroon grouping in the *N. ribis / N. parvum* complex. Within the Botryosphaeriaceae, species in the *N. ribis / N. parvum* complex have been difficult to distinguish based on phylogenies of single gene regions (Pavlic *et al.* 2007; Slippers *et al.* 2004b). A recent study by Pavlic *et al.* (2009a) thus made use of the Genealogical Concordance Phylogenetic Species recognition (GCPSR) approach (Taylor *et al.* 2000) to resolve species boundaries in the complex. These authors

were able to identify three cryptic species in the *N. ribis / N. parvum* complex. The same approach was used in the present study, to confirm the unique nature of *N. batangarum*. Isolates of *N. batangarum* were distinct from *N. ribis* based only on four fixed unique single nucleotide polymorphisms (SNPs) out of 86 informative characters across four gene regions. The gene genealogies across the five different loci were not different, as illustrated by the similarity in the sums of the length of the gene trees for the observed and resampled data. Under these conditions, a recent clonal mutation, most likely due to geographical and host isolation (Geiser *et al.* 1998), provides the best explanation for these results.

Even though DNA sequence data provided the most important basis used to discriminate *N. batangarum* from other species in the *N. ribis / N. parvum* complex, some morphologically informative characters were also found. The most obvious of these were the fact that colonies of *N. batangarum* formed concentric rings on MEA (Figure 7), a characteristic that was not observed in any other species of the complex.

Neofusicoccum batangarum was found as an endophyte on healthy twigs of *T. catappa* in Cameroon. Although no more information regarding its ecology is available, *N. batangarum* was able to produce lesions on young *T. catappa* in pathogenicity trials. This suggests that *N. batangarum* lives in a latent phase in plant organs and is able to convert to being a virulent pathogen when environmental conditions become unfavorable for the tree host.

The second previously undescribed species, *L. mahajangana*, was found in samples from Madagascar, a country where very few studies of microfungi have been conducted. *L. mahajangana* is phylogenetically most closely related to *L. theobromae* and *L. parva*. However, six and 14 SNPs amongst 60 informative characters across ITS, *tef 1-α* and *β-tub* gene regions distinguish *L. mahajangana* from *L. theobromae* and *L. parva* respectively. Moreover, *L. mahajangana* can also be distinguished from these fungi based on conidial size, its paraphyses and growth characteristics. Conidia of *L. mahajangana* are smaller than those of its closest relatives, *L. theobromae* and *L. pseudotheobromae*, but larger than those of *L. parva*. The paraphyses in this species are aseptate while those of *L. theobromae* and *L. parva* are septate. Moreover, *L. mahajangana* exhibited growth at temperatures as high as 35 °C.

Isolates of *L. mahajangana* were obtained from healthy plant material where they occurred as endophytes. Besides this particular feature, there are no data relating to its ecology, distribution and host range. Our consideration of its pathogenicity on *T. catappa* trees showed that *L. mahajangana* was less pathogenic than the other Botryosphaeriaceae found on this host. Lesions produced by *L. mahajangana,* although smaller than those produced by the other species collected from *T. catappa* in this study, were also significantly different from the control inoculations. The relatively small lesions produced by *L. mahajagana,* together with the fact that is was isolated only from healthy material, provides an indication that it is not a primary pathogen of these trees.

Lasiodiplodia theobromae is considered to be a pantropical pathogen that occurs on numerous hosts worldwide (Punithalingam 1980). Thus, it was not surprising to isolate it from the tropical *T. catappa*. The relatively common occurrence of *L. theobromae* in Cameroon, compared to the other regions sampled in this study could also reflect a climatic influence. *Lasiodiplodia theobromae* appears to occur most commonly in consistently warm areas (Taylor *et al.* 2005; Urbez-Torrez *et al.* 2008) and the climatic conditions in the localities where samples were collected in this study apparently support the findings.

Neofusicoccum parvum was the most common species collected form *T. catappa* in South Africa and produced lesions on young trees of *T. catappa* in pathogenicity trials. *N. parvum* is a well known pathogen of forest and fruit trees (Davidson and Tay 1983; Mohali *et al.* 2007; Slippers *et al.* 2004a; van Niekerk *et al.* 2004). In the current study, isolates of this species were obtained from branches of *T. catappa* showing symptoms of die-back. This might indicate that it is the pathogen responsible for branch die-back and death of *T. catappa* in South Africa. In previous studies conducted in South Africa, *N. parvum* was common on non-native *Eucalyptus* trees and on native *Syzygium cordatum*, where it has been shown to be pathogenic to these hosts (Pavlic *et al.* 2007; Slippers *et al.* 2004a). The common occurrence and wide host range of *N. parvum* in South Africa suggests that this fungus might be native to this area.

Lasiodiplodia pseudotheobromae emerged from a recent separation of cryptic species originally identified as *L. theobromae* (Alves *et al.* 2008). It is known from Africa, Europe and Latin America, where it occurs on forest and fruit trees. However, no

information concerning its pathogenicity to these trees is available. *L. pseudotheobromae* was the most abundant species isolated from *T. catappa* and it occurred in all the sampled areas. The known host range of *L. pseudotheobromae* is very limited, with single isolates obtained from *Rosa* sp. in the Netherlands, *Gmelina arborea* and *Acacia mangium*. in Costa Rica, *Coffea* sp. in Democratic Republic of Congo and *Citrus aurantium* in Suriname (Alves *et al.* 2008). Results of this study have substantially increased the geographic areas from which the fungus is known and they suggest that *L. pseudotheobromae,* like *L. theobromae,* has a worldwide distribution and a very wide host range.

The inoculation trials conducted in this study have shown that, *L. pseudotheobromae* was the most pathogenic of all the species tested. *Lasiodiplodia theobromae* and *N. parvum* have previously been shown to be pathogens of several hosts (Davidson and Tay 1983; Mohali *et al.* 2007; Pavlic *et al.* 2007; Slippers *et al.* 2004a). It was, therefore, not surprising that they caused lesions on *T. catappa* in this study. However, this study has provided the first data for the pathogenicity of *L. pseudotheobromae,* which suggests that its importance has been overlooked in the past, most likely because it was considered collectively with *L. theobromae*. It will now be important to determine its host range and distribution in order to understand the threat that it might pose as a pathogen, as well as to guide possible quarantine and other control measures.

The origins of the species of Botryosphaeriaceae collected from *T. catappa* in this study are unknown. However, its common occurrence on both introduced and native plants has led to suggestions that *N. parvum* might be part of the indigenous fungal flora of South Africa (Pavlic *et al.* 2007; 2008; 2009a). In contrast, *L. theobromae,* which has a wide host range and has been reported on native and introduced hosts on many continents, may have been introduced to Africa. Population genetic studies on this fungus will likely provide answers to the questions related to its origin and movements. As limited information is available regarding the recently described *L. pseudotheobromae*, the origin of this species cannot be considered here. In this study, the close relationship between *N. batangarum* and *N. ribis* suggests that *N. batangarum,* which was commonly isolated form *T. catappa* in Cameroon, could be derived from a clonal mutation possibly arising from geographical and host isolation of *N. ribis,* a fungus that has been reported with certainty only from the United States of America on *Ribes* sp. (Slippers *et al.* 2004b).

More sampling, both in other areas and hosts is clearly needed to address the question of its origin.

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Table 1.Botryosphaeriaceae used for phylogenetic analyses in this study.

Species	Culture number[a]	Origin[b]	Host	Collectors	Genbank Accession No.				
					ITS	*tef 1-α*	*rbp2*	*β-tub*	BotF15
Botryosphaeria dothidea	CMW7999	Switzerland	*Ostrya* sp.	B. Slippers	AY236948	AY236897			
	CMW8000	Switzerland	*Prunus* sp.	B. Slippers	AY236949	AY236898			
Dichomera eucalyptii	CMW15952	Australia	*Eucalyptus diversicolor*	T. Burgess/K.LGoei	DQ093194	DQ093215			
	CMW15953	Australia	*E. diversicolor*	T. Burgess/K.LGoei	DQ093194	DQ093216			
Diplodia mutila	CBS112553	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259093	AY573219			
	CBS230.30	USA	*P. dactylifera*	L.L. Huilllier	DQ458886	DQ458869			
D. seriata	CMW7774	USA	*Ribes* sp.	B.Slippers/G.Hudler	EF445343	EF445382			
	CMW7775	USA	*Ribes* sp	B.Slippers/G.Hudler	EF445344	EF445383			
Guignardia philoprina	CMW7063	Netherlands	*T. baccata*	H.A. van der Aa	AY236956	AY236905			
Lasiodiplodia crassispora	WAC12533	Venezuela	*E. urophylla*	S. Mohali	DQ103552	DQ103556			
	WAC12534	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103550	DQ103557			
	WAC12535	Australia	*S. album*	T.I. Burgess/B. Dell	DQ103551	DQ103558			
L. gonubiensis	CBS115812	South Africa	*Syzigium cordatum*	D. Pavlic	DQ458892	DQ458877			

	CBS116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567	
L. mahajangana	**CMW27801**	Madagascar	*Terminalia catappa*	J. Roux	FJ900595	FJ900641	FJ900630
	CMW27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642	FJ900631
	CMW27820	Madagascar	*T. catappa*	J. Roux	FJ900597	FJ900643	FJ900632
L. margaritacea	CMW26162	Australia	*Adansonia gibbosa*	D. Pavlic	EU144050	EU144065	
	CMW26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066	
L. parva	CBS356.59	Sri Lanka	*Theobroma cacao*	A. Riggenbach	EF622082	EF622062	EU673113
	CBS494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064	EU673114
L. plurivora	STEU-5803	South Africa	*Prunus salicina*	U.Damm	EF445362	EF445395	
	STEU-4583	South Africa	*V. vinifera*	F.Halleen	AY343482	EF445396	
L. pseudotheobromae	**CMW26721**	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900598	FJ900644	
	CMW26716	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900599	FJ900645	
	CMW27802	Madagascar	*T. catappa*	J. Roux	FJ900600	FJ900646	
	CMW27817	Madagascar	*T. catappa*	J. Roux	FJ900601	FJ900647	
	CBS116459	Costa Rica	*Gmelinea arborea*	J.Carranza/Velásquez	EF622077	EF622057	EU673111
	CBS447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060	EU673112

L. rubropupurea	WAC12535	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103553	DQ103571			
	WAC12536	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103554	DQ103572			
L. theobromae	**CMW28317**	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900602	FJ900648			
	CMW28319	Cameroon	*T. catappa*	D. Begoude/J. Roux	FJ900603	FJ900649			
	CMW26715	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900604	FJ900650			
	CMW27810	Madagascar	*T. catappa*	D. Begoude/J. Roux	FJ900605	FJ900651			
	CMW9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054		AY236930	
	CBS164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258		EU673110	
L. venezuelensis	WAC12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568			
	WAC12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569			
Neofusicoccum batangarum	**CMW28315**	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900606	FJ900652	FJ900614	FJ900633	FJ900622
	CMW28363	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900607	FJ900653	FJ900615	FJ900634	FJ900623
	CMW28320	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900608	FJ900654	FJ900616	FJ900635	FJ900624
	CMW28637	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900609	FJ900655	FJ900617	FJ900636	FJ900625
N. cordaticola	CMW13992	South Africa	*S. cordatum*	D. Pavlic	EU821898	EU821868	EU821928	EU821838	EU821802
	CMW14056	South Africa	*S. cordatum*	D. Pavlic	EU821903	EU821873	EU821933	EU821843	EU821807
	CMW14054	South Africa	*S. cordatum*	D. Pavlic	EU821906	EU821876	EU821936	EU821846	EU821810
N. kwambonambiense	CMW14023	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870	EU821930	EU821840	EU821804
	CMW14025	South Africa	*S. cordatum*	D. Pavlic	EU821901	EU821871	EU821931	EU821841	EU821805
	CMW14123	South Africa	*S. cordatum*	D. Pavlic	EU821924	EU821894	EU821954	EU821864	EU821828

N. parvum	CMW9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888	EU821963	AY236917	EU821837
	CMW9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885	EU821961	AY236915	EU821835
	CMW26714	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900610	FJ900656	FJ900618	FJ900637	FJ900626
	CMW26717	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900611	FJ900657	FJ900619	FJ900638	FJ900627
	CMW26718	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900612	FJ900658	FJ900620	FJ900639	FJ900628
	CMW26720	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900713	FJ900659	FJ900621	FJ900640	FJ900629
N. ribis	CMW7772	USA	*Ribes* sp.	B. Slippers/G.Hudler	AY236935	AY236877	EU821958	AY236906	EU821832
	CMW7773	USA	*Ribes* sp.	B. Slippers/G Hudler	AY236936	AY236878	EU821959	AY236907	EU821833
N. umdonicola	CMW14106	South Africa	*S. cordatum*	D. Pavlic	EU821899	EU821869	EU821929	EU821839	EU821803
	CMW14058	South Africa	*S. cordatum*	D. Pavlic	EU821904	EU821874	EU821934	EU821844	EU821808
	CMW14060	South Africa	*S. cordatum*	D. Pavlic	EU821905	EU821875	EU821935	EU821845	EU821809

[a] **CMW**, Research collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa;

[b] Country of collection.

Table 2. Conidial dimensions of *Neofusicoccum* spp. and *Lasiodiplodia* spp. from *Terminalia catappa* and comparison with those reported in previous studies.

Species	Conidial size (µm)		Source of data
	This study	Previous studies	
N. parvum	(10.5-)14.0-19.0(-20.5) x (4-)5.5-6.5(-7.5)	(12-)15-19(-24) x 4-6	Slippers *et al.* 2004b
N. batangarum	(12.0-)14.0-17.5(-20.0) x (4.0-) 4.5-6.0(-6.5)		This study
L. pseudotheobromae	(21.5-)24.5-29.5(-31.0) x (13.5-)14.0-16.5(-18.0)	(22.5-)23.5-32(-33) x (13.3-)14-18(-20)	Alves *et al.* 2008.
L. theobromae	(20.5-)22.5-26.0(-30.5) x (11.5-)12.5-15.0(-17.0)	(19-)21-31(-32.5) x (12-)13-15.5(-18.5)	Alves *et al.* 2008.
L. mahajangana	(13.5-)15.5-19.0(-21.5) x (10.0-)11.5-13.0(-14.0)		This study

Table 3. Polymorphic nucleotides from sequence data of the ITS, *tef 1-α*, *rbp2*, *β-tub* and BOTF15gene regions for isolates in the *Neofusicoccum ribis, N. kwabonambiense*, *N. umdonicola, N. cordicola* and *N. parvum* clade introduced from outgroup comparisons.

Identity	Culture number	ITS									*tef1-α*										*rbp2*																			*β-tub*											BOTF15										
		51	115	141	163	168	173	372	389	416	43	44	66	67	77	81	153	211	227	257	10	22	49	97	100	112	205	265	280	343	382	397	409	421	475	526	42	50	93	106	125	167	185	245	261	326	389	407	97	98	102	105	166	215	252	301	311
N. ribis	CMW 7772	A	G	T	C	T	A	A	A	T	G	I	T	G	T	C	C	A	-	G	T	T	C	T	G	C	T	G	C	C	G	T	T	G	C	T	C	C	C	G	T	C	G	G	C	T	T	G	A	0	C	0	C	G	C	G
	CMW 7773	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N . batangarum	**CMW 28315**	.	.	.	.	.	.	.	G	.	.	0	.	.	.	.	.	G	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
	CMW 28320	.	.	.	.	.	.	.	G	.	.	0	.	.	.	.	.	G	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
	CMW 28363	.	.	.	.	.	.	.	G	.	.	0	.	.	.	.	.	G	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
	CMW 28637	.	.	.	.	.	.	.	G	.	.	0	.	.	.	.	.	G	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
N. kwabonambiense	CMW 14023	.	-	.	T	.	G	.	.	.	-	0	.	.	.	.	.	G	-	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	C	.	.	.	.	A	C	T	A	A	.	C	C	.	T	I	T	I	.	.	T	.
	CMW 14025	.	-	.	T	.	G	.	.	.	-	0	.	.	.	.	.	G	-	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	C	.	.	.	.	A	C	T	A	A	.	C	C	.	T	I	T	I	.	.	T	.
	CMW 14123	.	-	.	T	.	G	.	.	.	-	0	.	.	.	.	.	G	-	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	C	.	.	.	.	A	C	T	A	A	.	C	C	.	T	I	T	I	.	.	T	.
N. umdonicola	CMW 14106	.	.	.	.	C	.	.	.	.	-	0	.	.	.	T	.	G	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	CMW 14079	.	.	.	.	C	.	.	.	.	.	0	.	.	.	T	.	G	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	CMW 14096	.	.	.	.	C	.	.	.	.	.	0	.	.	.	T	.	G	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N. cordaticola	CMW 13992	.	-	.	.	.	.	G	.	C	.	0	.	.	C	.	.	G	-	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	T	.	.	T	.	C	.	.	.	G	C	C	T	C	.	T	.	.	.	T	.
	CMW 14056	.	-	.	.	.	.	G	.	C	.	0	.	.	C	.	.	G	-	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	T	.	.	T	.	C	.	.	.	G	C	C	T	C	.	T	.	.	.	T	.
	CMW 14054	.	-	.	.	.	.	G	.	C	.	0	.	.	C	.	.	G	-	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	T	.	.	T	.	C	.	.	.	G	C	C	T	C	.	T	.	.	.	T	.
N. parvum	CMW 9081	T	-	.	.	.	.	.	.	.	.	0	C	A	.	.	T	G	-	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.	.	T	.	A	C	.	.	.	.	.	.	.	.	.	T	.	.	T	.	T	A
	CMW 9079	T	-	.	.	.	.	.	.	.	.	0	C	A	.	.	T	G	-	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.	.	T	.	A	C	.	.	.	.	.	.	.	.	.	T	.	.	T	.	T	A

Table 4. Polymorphic nucleotides from sequence data of the ITS, *tef 1-α* and *β-tub* gene regions for isolates in the *Lasiodiplodia theobromae, L. parva, L. pseudotheobromae* and *L. mahajangana* clade introduced from outgroup comparisons.

Identity	Culture number	ITS	*tef 1-α*	*β-tub*
		42 46 98 116 439	20 22 23 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 50 53 55 58 59 61 62 68 82 99 232 267 282	85 124 143 157 177 189 230 244
L. theobromae	CBS 164.96	A C C T C	G C C C A C G C A T G T C G T T T T T T A T C A T C - - 1 C C A A A	C C G T T A C C
	CMW 9074	. . . . .	. . . . . - - 1	. . . . .
L. parva	CBS 356.59	. T . . .	- G A A . G A T T . T C - - C C - - - C . G T G C T C A 0 . T . . G	T . C C C G A .
	CBS 494.78	. T . . .	- G A A . G A T T . T C - - C C - - - C . G T G C T C A 0 . T . . G	T . C C C G A .
L. pseudotheobromae	CBS 447.62	G T . C T	- T . A T T T . C C C C - - C C . C C C . G T G C T T A 0 . . G G G	T . . C C . . .
	CBS 116459	G T . C T	- T . A T T T . C C C C - - C C . C C C . G T G C T T A 0 . . G G G	T . . C C . . .
L. mahajangana	CMW 27801	. T T . .	. . . . . G . . . . . T . . . . - - 0 T T . . .	T T C C C G . A
	CMW 27818	. T T . .	. . . . . G . . . . . T . . . . - - 0 T T . . .	T T C C C G . A
	CMW 27820	. T T . .	. . . . . G . . . . . T . . . . - - 0 T T . . .	T T C C C G . A

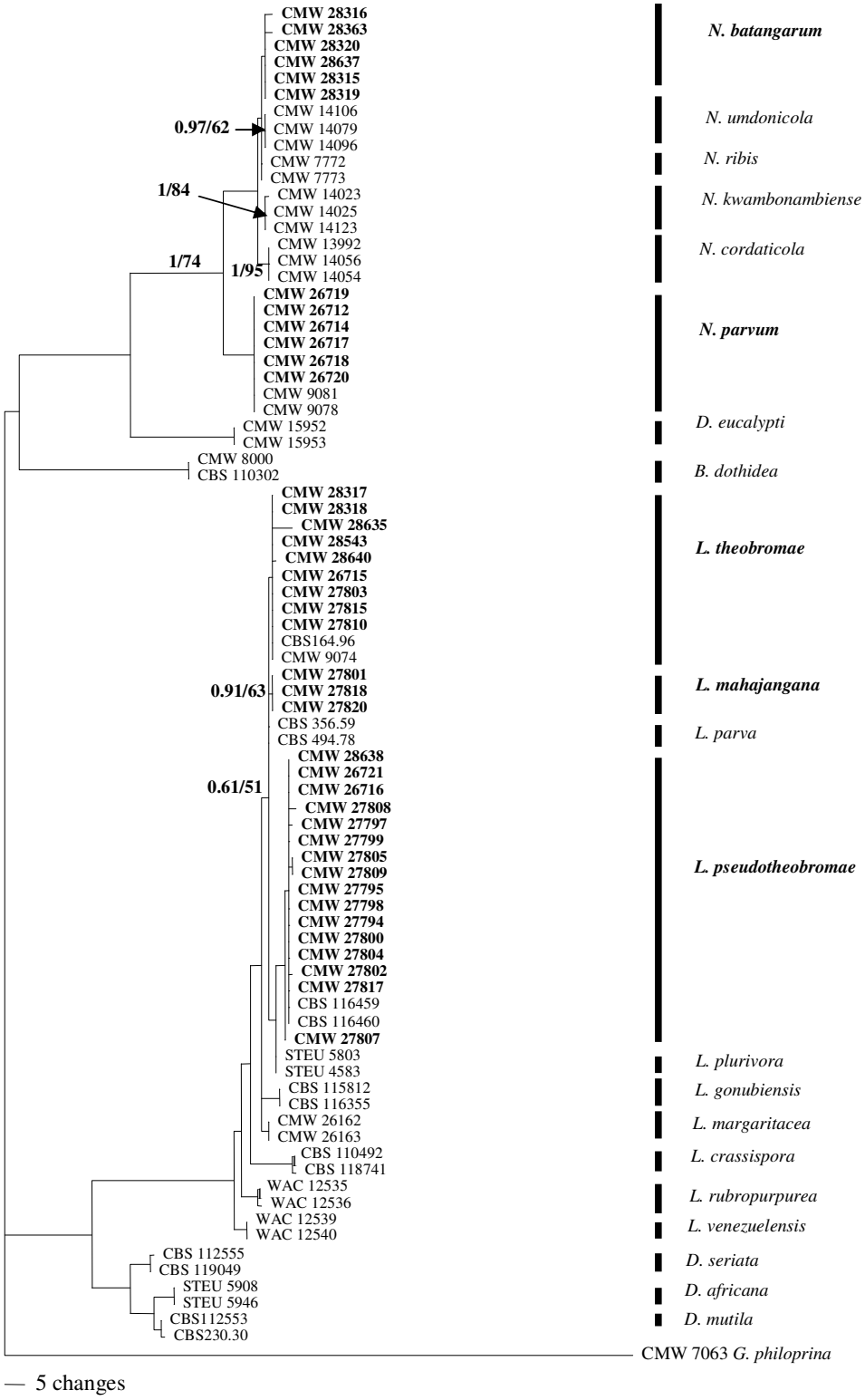
Table 5. Sequence dataset characteristics and phylogenetic information for ITS, *tef 1-α*, *rbp2, β-tub* and BotF15 and combined data sets of *Neofusicoccum* spp.

Data set	Sequence range (bp)	No. variable sites	No. informative sites	No. most parsimonious trees	Tree length	Consistency index	Retention index	Monophyletic taxa
ITS	502	15	10	1	15	1	1	*N. kwambonambiense, N. cordaticola, N. batangarum, N. umdonicola, N. ribis, N. parvum*
tef 1-α	263	24	23	6	25	0.960	0.977	*N. kwambonambiense, N. cordaticola, N. batangarum, N. umdonicola, N. ribis, N. parvum*
rpb2	566	19	16	1	19	1	1	*N. kwambonambiense, N. cordaticola, N. umdonicola, N. parvum*
β-tub	420	12	12	2	13	0.923	0.974	*N. kwambonambiense, N. cordaticola, N. batangarum, N. umdonicola, N. ribis, N. parvum*
BotF15	364	26	25	1	26	1	1	*N. kwambonambiense, N. cordaticola, N. batangarum, N. parvum*
Combined data	2115	96	86	1	98	0.980	0.992	*N. kwambonambiense, N. cordaticola, N. batangarum, N. umdonicola, N. ribis, N. parvum*

Table 6. Sequence dataset characteristics and phylogenetic information for ITS, *tef 1-α* and *β-tub* and combined data sets for *Lasiodiplodia* spp.

Data set	Sequence range	No. of variable sites	No. of informative sites	No. of most parsimonious trees	Tree length	Consistency index	Retention index	Monophyletic taxa
ITS	461	5	5	1	5	1	1	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
tef 1-α	276	51	47	1	55	0.982	0.990	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
β-tub	422	9	8	1	9	1	1	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
Combined data	1159	65	60	1	71	0.958	0.976	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*

Figure 1. One of the most parsimonious trees obtained from Maximum Parsimony analyses of the ITS sequence data of the representative taxa of the Botryosphaeriaceae. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branch (PP/BS). Isolates marked in bold represent those obtained from *T. catappa*.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CMW 28320
CMW 28637
CMW 28315
CMW 28319
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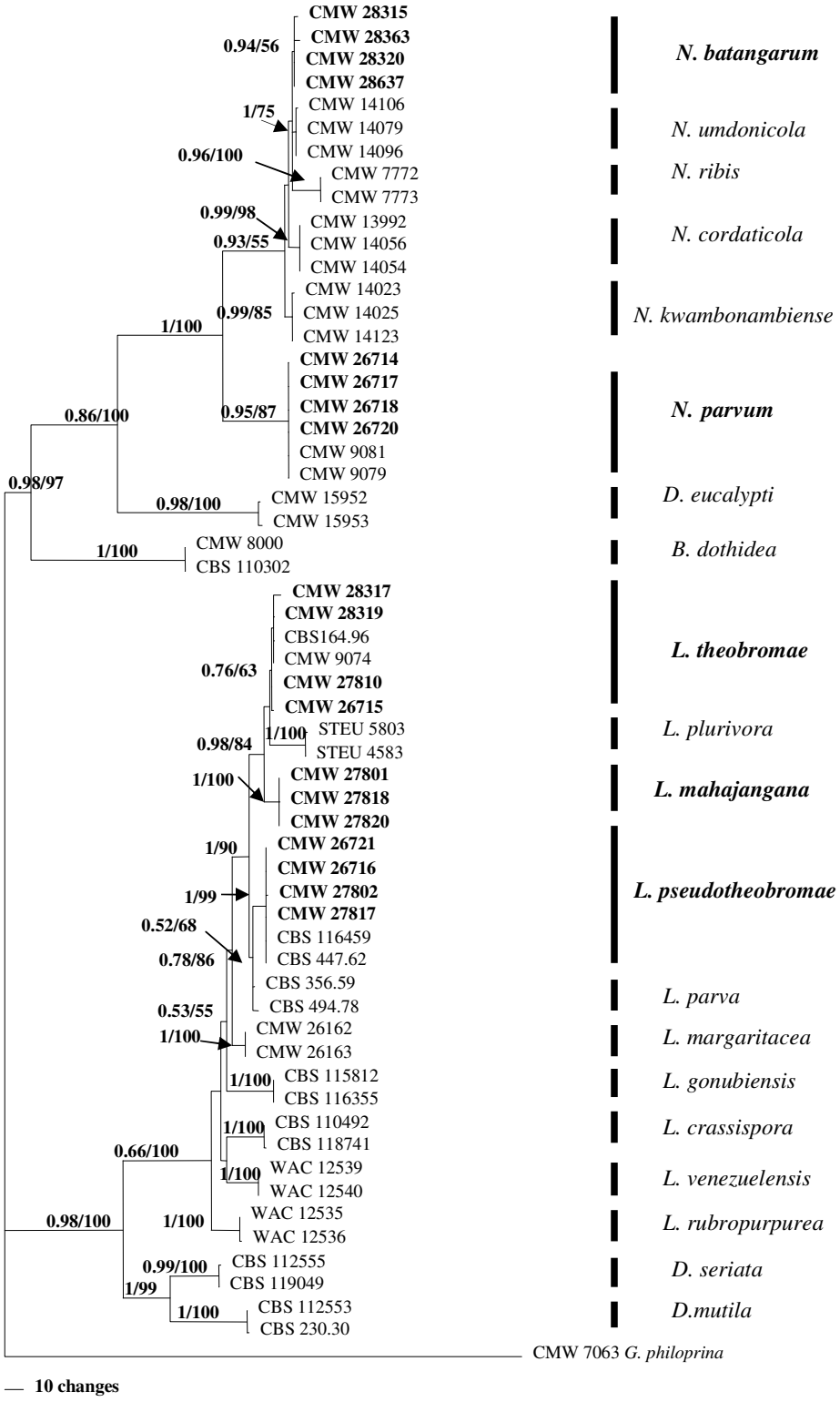
CMW 14079
CMW 14096
CMW 7772
CMW 7773
1/84
CMW 14023
CMW 14025
CMW 14123
CMW 13992
1/74
CMW 14056
1/95
CMW 14054
CMW 26719
CMW 26712
CMW 26714
CMW 26717
CMW 26718
CMW 26720
CMW 9081
CMW 9078
CMW 15952
CMW 15953
CMW 8000
CBS 110302
CMW 28317
CMW 28318
CMW 28635
CMW 28543
CMW 28640
CMW 26715
CMW 27803
CMW 27815
CMW 27810
CBS164.96
CMW 9074
CMW 27801
0.91/63
CMW 27818
CMW 27820
CBS 356.59
CBS 494.78
CMW 28638
CMW 26721
0.61/51
CMW 26716
CMW 27808
CMW 27797
CMW 27799
CMW 27805
CMW 27809
CMW 27795
CMW 27798
CMW 27794
CMW 27800
CMW 27804
CMW 27802
CMW 27817
CBS 116459
CBS 116460
CMW 27807
STEU 5803
STEU 4583
CBS 115812
CBS 116355
CMW 26162
CMW 26163
CBS 110492
CBS 118741
WAC 12535
WAC 12536
WAC 12539
WAC 12540
CBS 112555
CBS 119049
STEU 5908
STEU 5946
CBS112553
CBS230.30
CMW 7063 G. philoprina
N. batangarum
N. umdonicola
N. ribis
N. kwambonambiense
N. cordaticola
N. parvum
D. eucalypti
B. dothidea
L. theobromae
L. mahajangana
L. parva
L. pseudotheobromae
L. plurivora
L. gonubiensis
L. margaritacea
L. crassispora
L. rubropurpurea
L. venezuelensis
D. seriata
D. africana
D. mutila
5 changes

Figure 2. One of the most parsimonious trees obtained from Maximum Parsimony analyses of the combined ITS and *tef 1-α* sequence data of the representative taxa of the Botryosphaeriaceae. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branch (PP/BS). Isolates marked in bold represent those obtained from *T. catappa*.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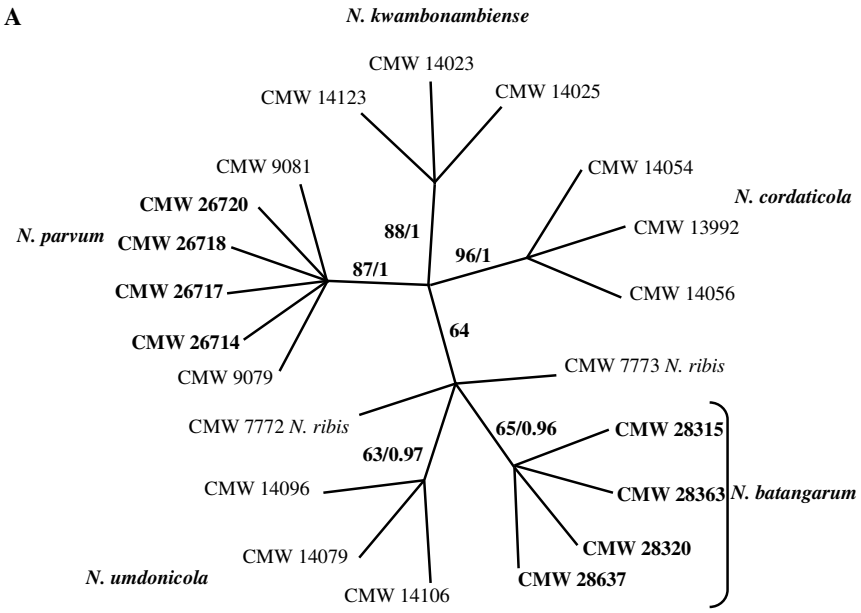
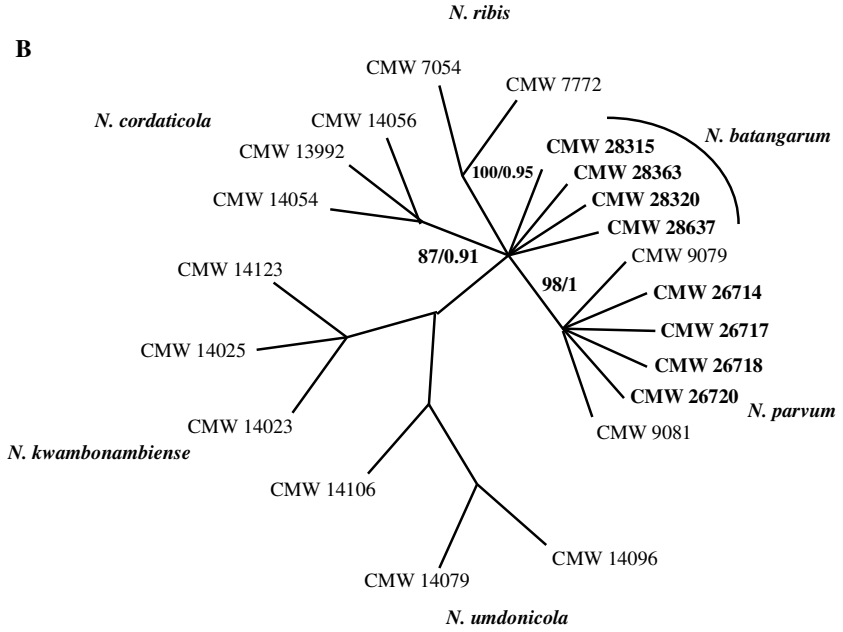
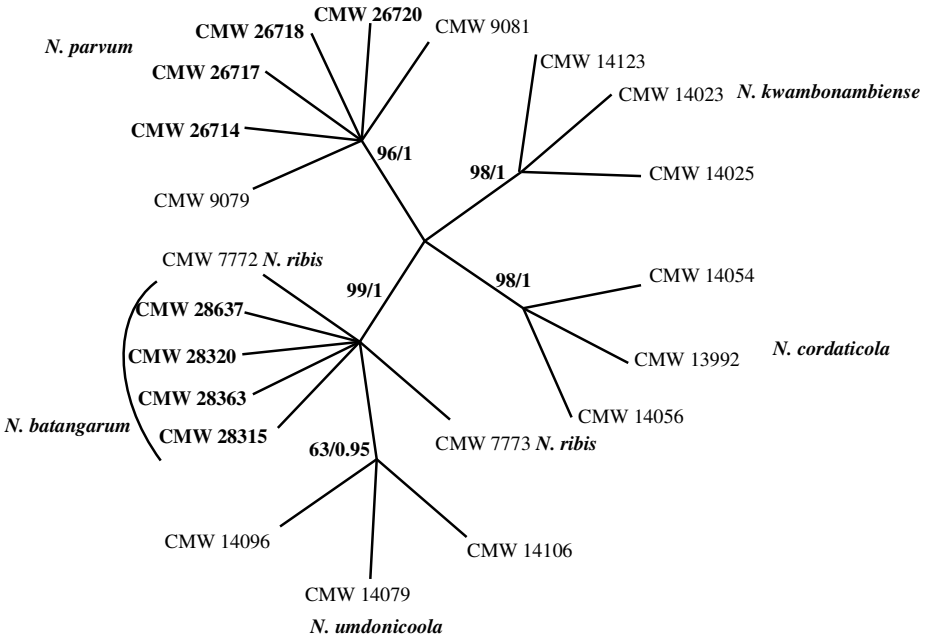
CMW 28320
CMW 28637
CMW 14106
CMW 14079
CMW 14096
CMW 7772
CMW 7773
CMW 13992
CMW 14056
CMW 14054
CMW 14023
CMW 14025
CMW 14123
CMW 26714
CMW 26717
CMW 26718
CMW 26720
CMW 9081
CMW 9079
CMW 15952
CMW 15953
CMW 8000
CBS 110302
CMW 28317
CMW 28319
CBS164.96
CMW 9074
CMW 27810
CMW 26715
STEU 5803
STEU 4583
CMW 27801
CMW 27818
CMW 27820
CMW 26721
CMW 26716
CMW 27802
CMW 27817
CBS 116459
CBS 447.62
CBS 356.59
CBS 494.78
CMW 26162
CMW 26163
CBS 115812
CBS 116355
CBS 110492
CBS 118741
WAC 12539
WAC 12540
WAC 12535
WAC 12536
CBS 112555
CBS 119049
CBS 112553
CBS 230.30
0.94/56
1/75
0.96/100
0.99/98
0.93/55
0.99/85
1/100
0.95/87
0.86/100
0.98/97
0.98/100
1/100
0.76/63
1/100
0.98/84
1/100
1/90
1/99
0.52/68
0.78/86
0.53/55
1/100
1/100
1/100
0.66/100
1/100
0.98/100
1/100
0.99/100
1/99
1/100
N. batangarum
N. umdonicola
N. ribis
N. cordaticola
N. kwambonambiense
N. parvum
D. eucalypti
B. dothidea
L. theobromae
L. plurivora
L. mahajangana
L. pseudotheobromae
L. parva
L. margaritacea
L. gonubiensis
L. crassispora
L. venezuelensis
L. rubropurpurea
D. seriata
D.mutila
CMW 7063 G. philoprina
10 changes

Figure 3. One of the most parsimonious unrooted trees inferred from independent analyses of each data set (A = ITS; B = *tef* 1-α; C = *rpb2*; D = *β-tub*; E = BOTF15; F = combination of sequences of the five loci) in the *Neofusicoccum* spp. group of the Botryosphaeriaceae from *T. catappa*. Bootstrap support (%) from 1000 replications followed by Posterior probabilities are given on the branches (BS/PP). Isolates marked in bold represent those obtained from *T. catappa*.

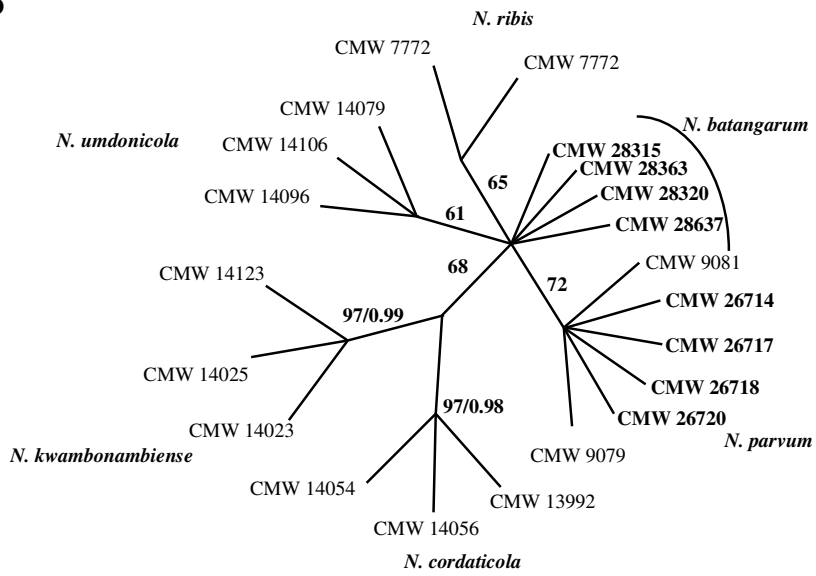

A
N. kwambonambiense
CMW 14023
CMW 14123
CMW 14025
CMW 9081
CMW 14054
N. cordaticola
CMW 26720
N. parvum
CMW 26718
88/1
CMW 13992
87/1
96/1
CMW 26717
CMW 14056
64
CMW 26714
CMW 9079
CMW 7773 N. ribis
CMW 7772 N. ribis
65/0.96
CMW 28315
63/0.97
CMW 14096
CMW 28363
N. batangarum
CMW 28320
CMW 14079
N. umdonicola
CMW 28637
CMW 14106


N. ribis
B
CMW 7054
CMW 7772
N. cordaticola
CMW 14056
N. batangarum
CMW 28315
CMW 13992
100/0.95
CMW 28363
CMW 14054
CMW 28320
CMW 28637
87/0.91
CMW 9079
CMW 14123
98/1
CMW 26714
CMW 26717
CMW 14025
CMW 26718
CMW 26720
N. parvum
CMW 14023
CMW 9081
N. kwambonambiense
CMW 14106
CMW 14096
CMW 14079
N. umdonicola

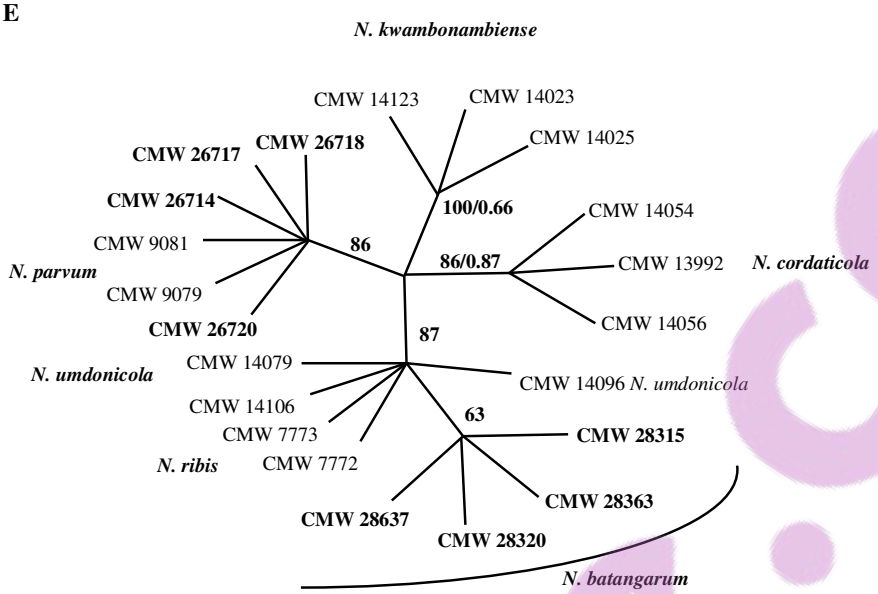
C


CMW 26720
CMW 26718
CMW 9081
N. parvum
CMW 14123
CMW 26717
CMW 14023
N. kwambonambiense
CMW 26714
96/1
98/1
CMW 14025
CMW 9079
CMW 7772 N. ribis
99/1
98/1
CMW 14054
CMW 28637
CMW 28320
CMW 13992
N. cordaticola
CMW 28363
N. batangarum
CMW 28315
CMW 14056
63/0.95
CMW 7773 N. ribis
CMW 14096
CMW 14106
CMW 14079
N. umdonicoola

D


N. ribis
CMW 7772
CMW 7772
CMW 14079
N. batangarum
N. umdonicola
CMW 14106
CMW 28315
CMW 28363
65
CMW 14096
CMW 28320
61
CMW 28637
68
CMW 9081
CMW 14123
72
CMW 26714
97/0.99
CMW 26717
CMW 14025
CMW 26718
97/0.98
CMW 26720
CMW 14023
N. parvum
N. kwambonambiense
CMW 9079
CMW 14054
CMW 13992
CMW 14056
N. cordaticola

E



F

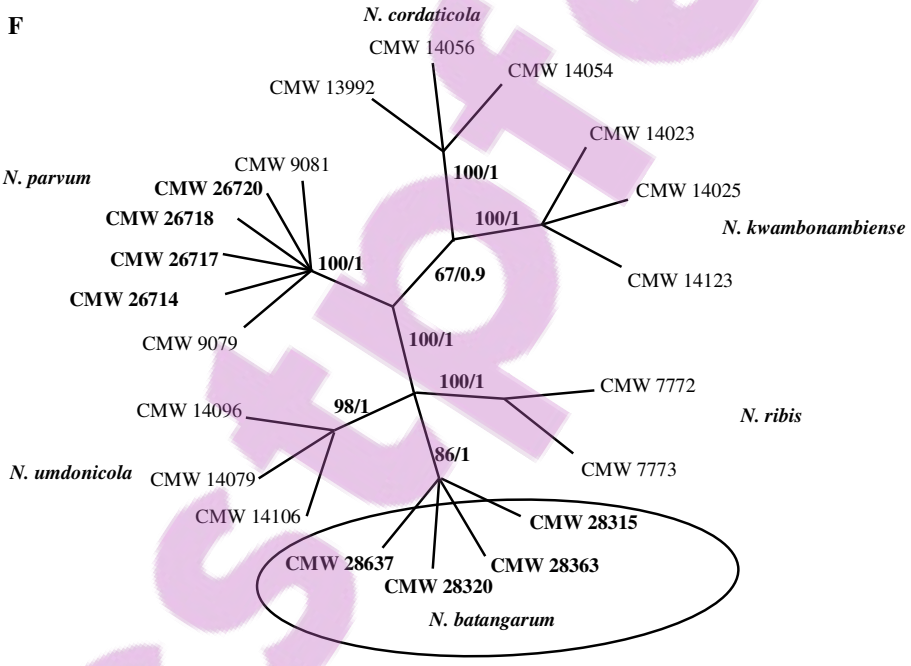
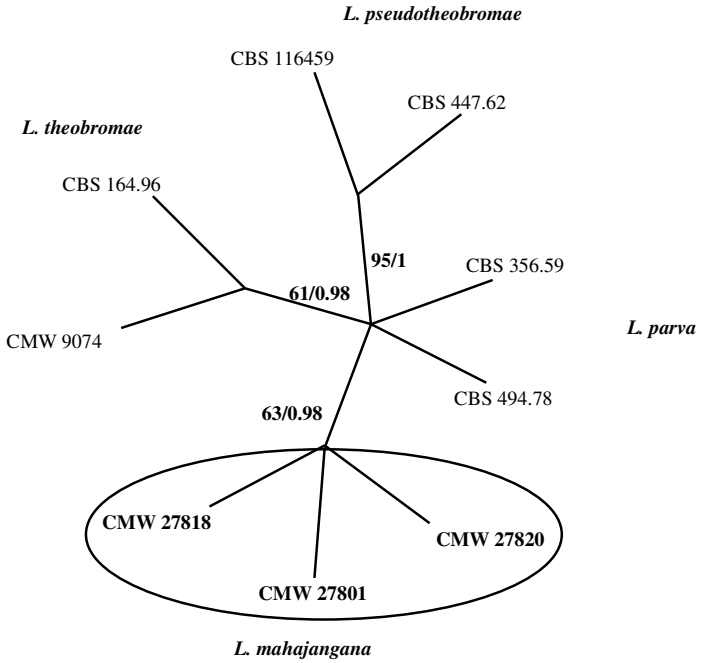
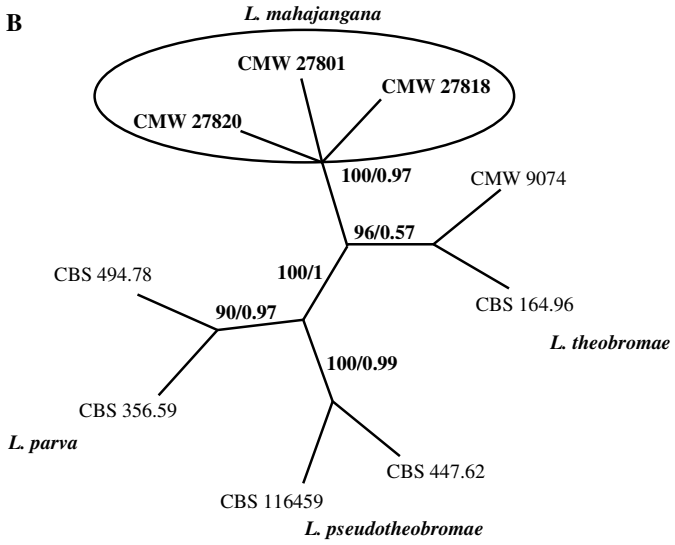


Figure 4. Most-parsimonious unrooted trees inferred from independent analyses of each data set (A = ITS; B = *tef* 1-α; C = *β-tub*; D = combination of sequences of the three loci) of the *Lasiodiplodia* spp. from *T. catappa* and related species. Bootstrap support (%) from 1000 replications followed by Posterior probabilities are given on the branch (BS/PP). Isolates marked in bold represent those obtained from *T. catappa*.

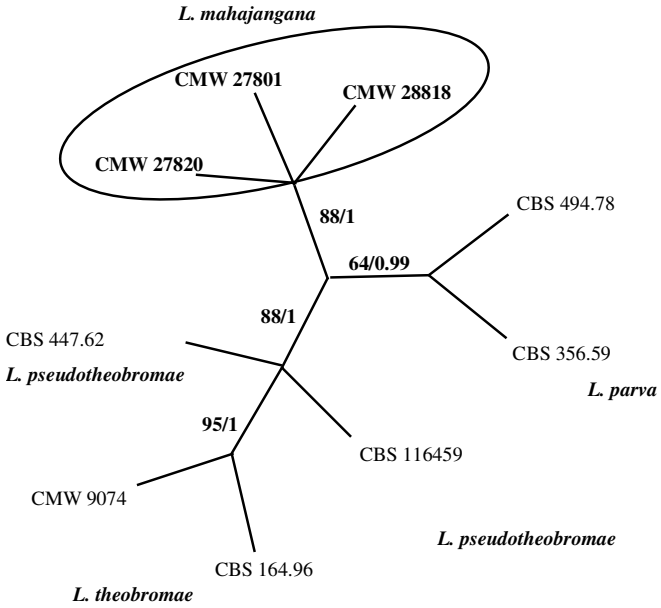
A



B



C


L. mahajangana
CMW 27801
CMW 28818
CMW 27820
88/1
CBS 494.78
64/0.99
88/1
CBS 447.62
L. pseudotheobromae
CBS 356.59
L. parva
95/1
CBS 116459
CMW 9074
L. pseudotheobromae
CBS 164.96
L. theobromae

D

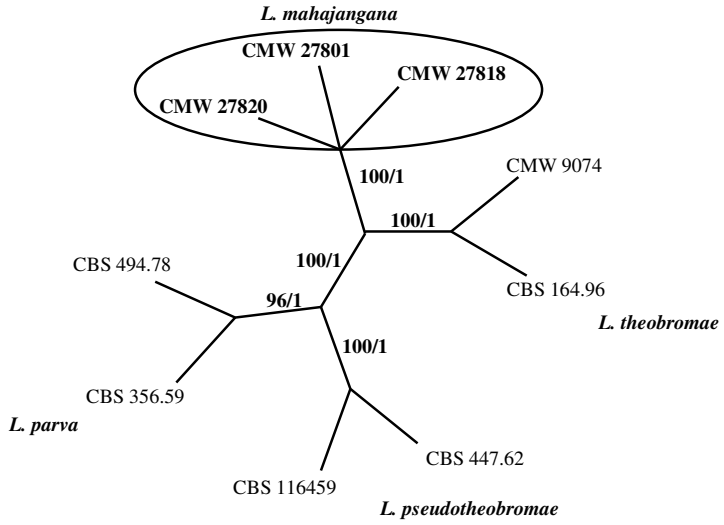

L. mahajangana
CMW 27801
CMW 27818
CMW 27820
100/1
CMW 9074
100/1
CBS 494.78
100/1
CBS 164.96
L. theobromae
96/1
100/1
CBS 356.59
L. parva
CBS 447.62
CBS 116459
L. pseudotheobromae

Figure 5. *Lasiodiplodia mahajangana.* (a) Pycnidium formed on pine needle in culture. (b) Paraphyses. (d) Conidiogenous cells with developing conidia. (d) conidia. (e) mature conidium showing septum. Bars: a = 500 µm; b, c, d, e = 10 µm.

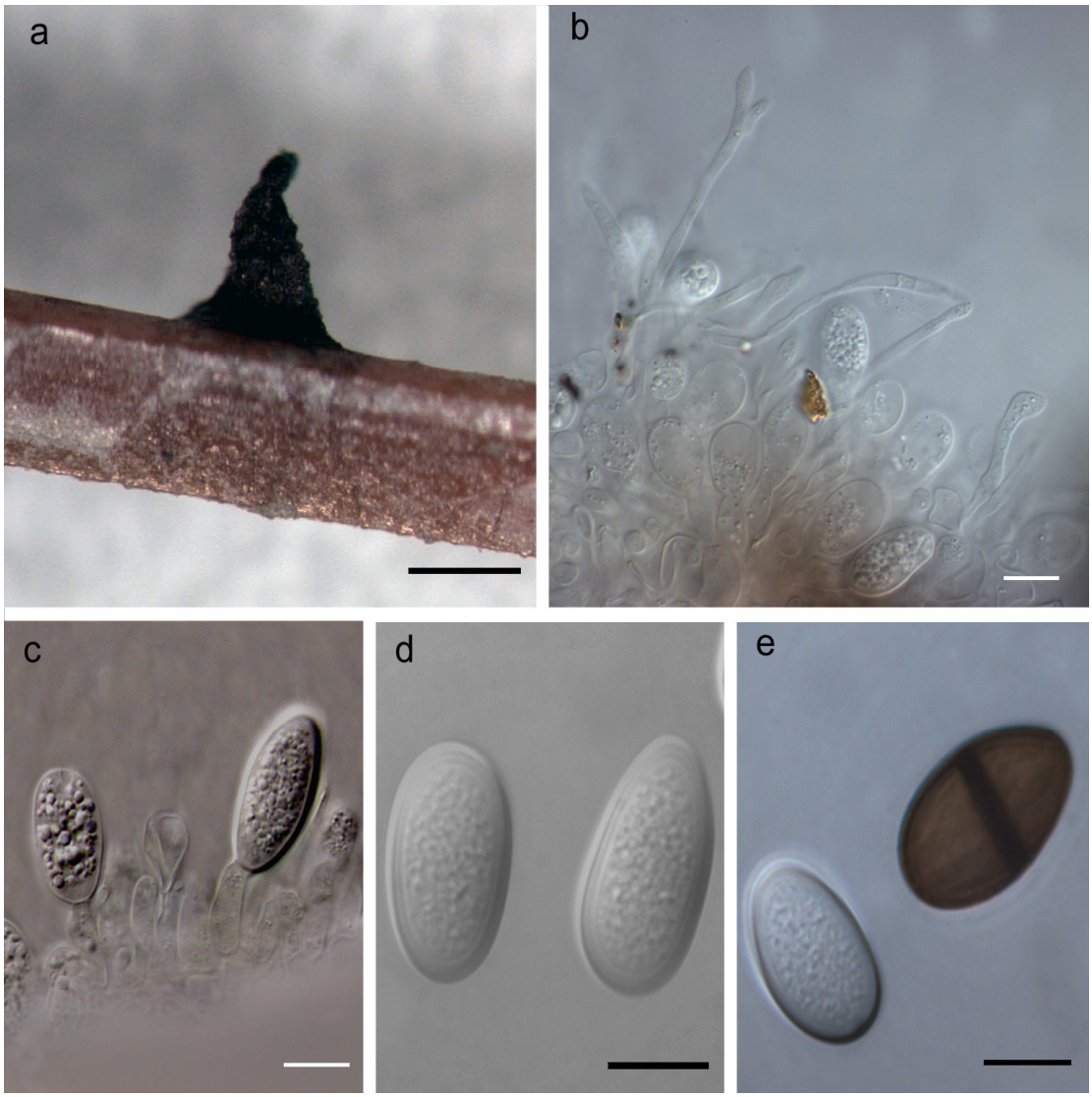

a
b
c
d
e

Figure 6. *Neofusicoccum batangarum.* (a) Pycnidium formed on pine needle in culture. (b, d) conidia. (c,e,f) Conidiogenous cells with developing conidia. Bars: a = 500 µm; b, c, d, e, f = 10 µm.

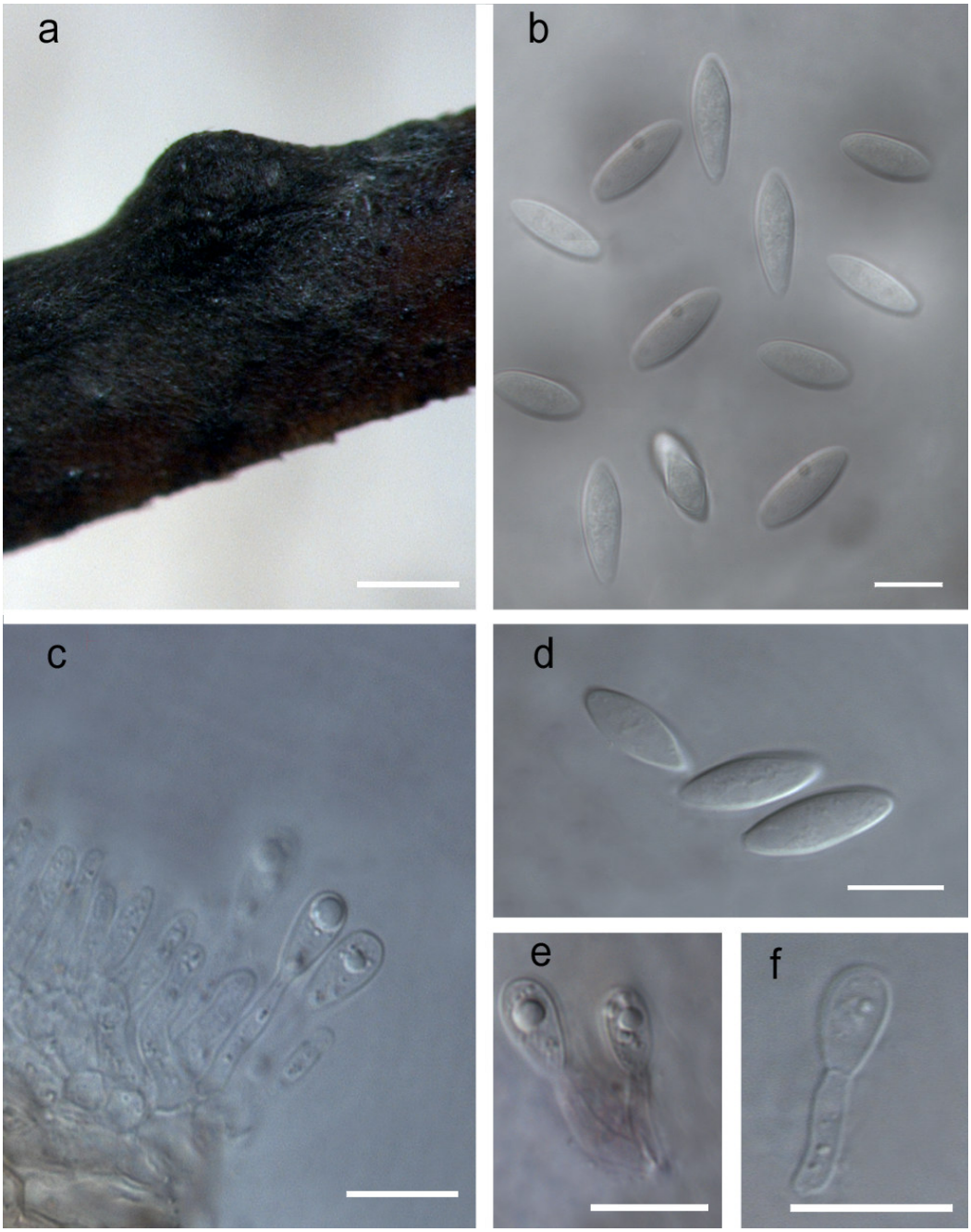

a
b
c
d
e
f

Figure 7. *Neofusicoccum batangarum* culture on MEA. (a) Front plate. (b) Reverse plate.

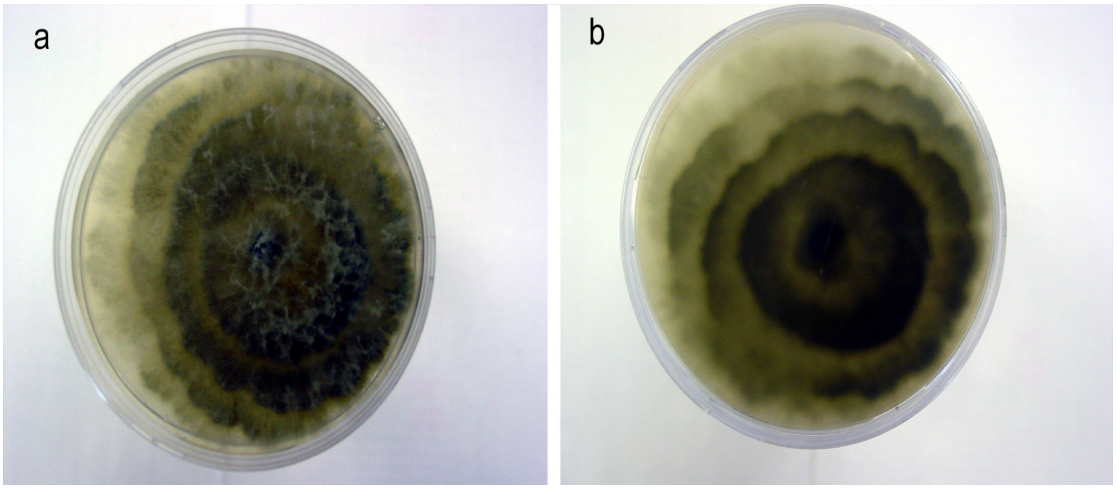

a
b

Figure 8. Distribution of Botryosphaeriaceae collected from *T. catappa* per locality.

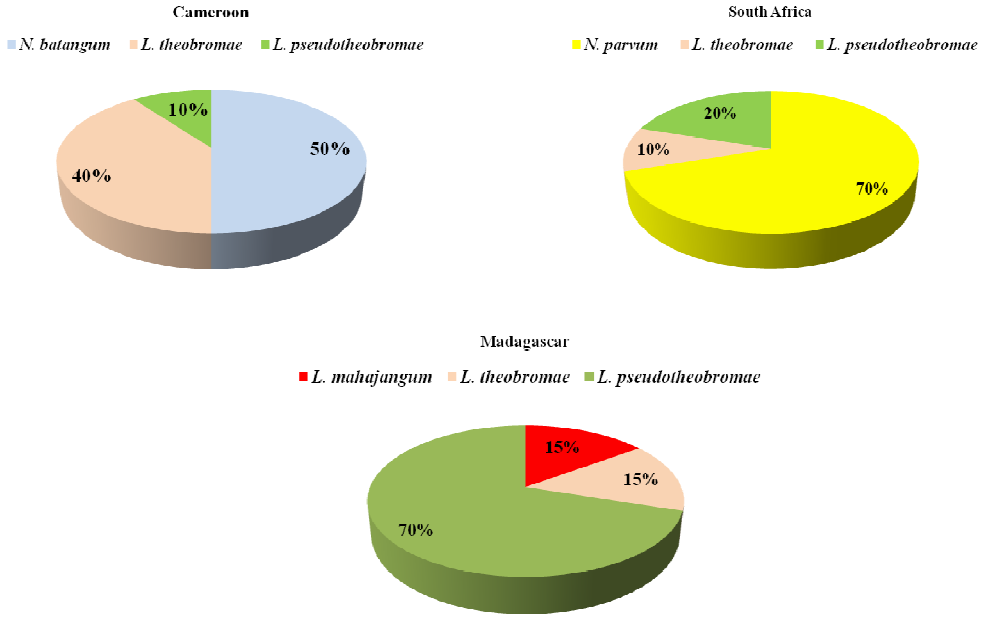

Cameroon
N. batangum
L. theobromae
L. pseudotheobromae
10%
50%
40%
South Africa
N. parvum
L. theobromae
L. pseudotheobromae
20%
10%
70%
Madagascar
L. mahajangum
L. theobromae
L. pseudotheobromae
15%
15%
70%

Figure 9. Mean lesion lengths (mm) of bark lesions for each Botryosphaeriaceae isolate six weeks after inoculation on *T. catappa* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *N. parvum* (NP), *L. theobromae* (LT), *L. mahajangana* (LM), *N. batangarum* (NB), Control.

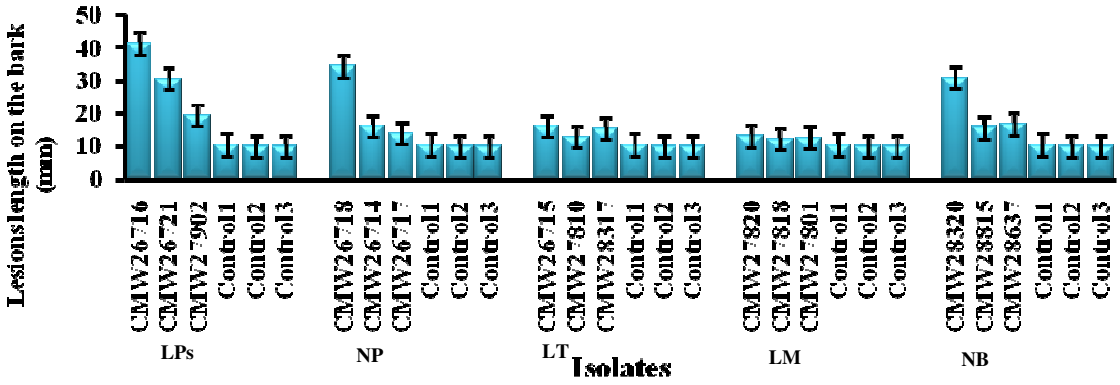
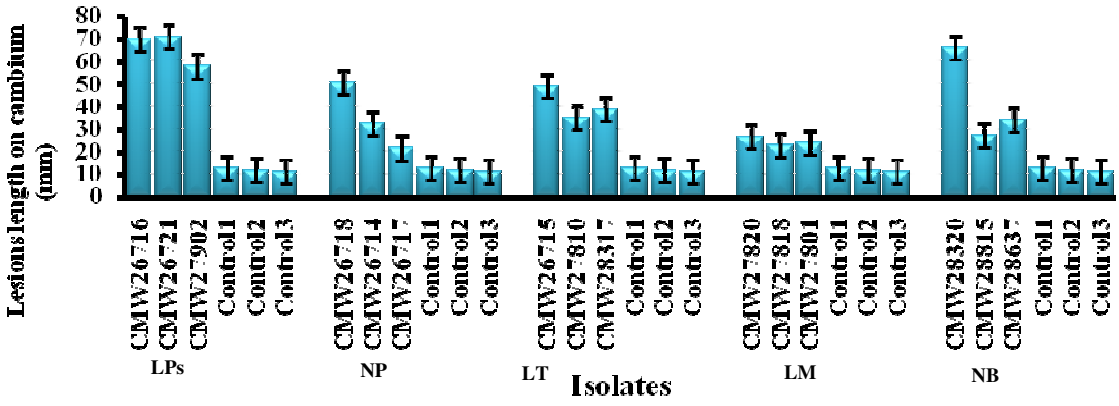

Lesionslength on the bark (mm)
50
40
30
20
10
0
CMW26716
CMW26721
CMW27902
Control1
Control2
Control3
CMW26718
CMW26714
CMW26717
Control1
Control2
Control3
CMW26715
CMW27810
CMW28317
Control1
Control2
Control3
CMW27820
CMW27818
CMW27801
Control1
Control2
Control3
CMW28320
CMW28815
CMW28637
Control1
Control2
Control3
LPs
NP
LT
LM
NB
Isolates

Figure 10. Mean lesion lengths (mm) on cambial lesions for each Botryosphaeriaceae isolate six weeks after inoculation on *T. catappa* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *N. parvum* (NP), *L. theobromae* (LT), *L. mahajangana* (LM), *N. batangarum* (NB), Control.


Lesions length on cambium (mm)
80
70
60
50
40
30
20
10
0
CMW26716
CMW26721
CMW27902
Control1
Control2
Control3
CMW26718
CMW26714
CMW26717
Control1
Control2
Control3
CMW26715
CMW27810
CMW28317
Control1
Control2
Control3
CMW27820
CMW27818
CMW27801
Control1
Control2
Control3
CMW28320
CMW28815
CMW28637
Control1
Control2
Control3
LPs
NP
LT
LM
NB
Isolates

Chapter 3

Botryosphaeriaceous fungi as endophytes on *Terminalia* species in Cameroon

This chapter has been submitted for publication as: Begoude BAD, Slippers B, Wingfield MJ, Roux J, 2009. Botryosphaeriaceous fungi as endophytes on *Terminalia* species in Cameroon. *Forest Pathology*

ABSTRACT

In Cameroon, native *Terminalia* spp. represent an important component of the forestry industry, but limited information is available regarding the fungal pathogens that affect them. The Botryosphaeriaceae are endophytic fungi and latent pathogens that can result in wood stain, cankers, die-back and death of trees, particularly when trees are under stress. The aim of this is study was, therefore, to identify and characterize the Botryosphaeriaceae occurring as endophytes on *Terminalia* spp. in Cameroon as part of a larger project to identify potential pathogens of these trees in the country. Samples were collected from three *Terminalia* spp. in the Central, Southern and Eastern Regions and the resultant Botryosphaeriaceae were identified using morphology and DNA sequence comparisons for the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials were conducted to consider the relative pathogenicity of the isolates collected. The majority of isolates (88 %) represented species of *Lasiodiplodia,* including *L. pseudotheobromae, L. theobromae* and *L. parva.* The remaining isolates were identified as *Endomelanconiopsis endophytica.* Pathogenicity trials on young *T. mantaly* and *T. catappa* trees revealed that *L. pseudotheobromae* was the most pathogenic species followed by *L. theobromae*.

1. INTRODUCTION

The forestry sector in Cameroon plays an important role in the national economy of the country. Timber is the second most exported product, after petroleum, with wood-based exports generating revenue of US $210 million in 2001 (Anonymous 2005a). The total forest area in Cameroon is estimated to represent ~12.8 million ha of natural forests and about 17000 ha of planted forests (Anonymous 2005b), made up of a variety of native trees such as *Terminalia* spp.

Species of *Terminalia* currently found in forest plantations in Cameroon include *T. ivorensis* and *T. superba*. These tree species have a well acknowledged commercial value with a total volume of exported logs representing 10 % of the national round wood production (Laird 1999). Besides their high commercial value, *Terminalia* spp. are commonly used in agriculture to establish a "taungya" agri-sylvicultural system in which they provide shade or improve soil fertility for crops (Norgrove and Hauser 2002). Furthermore, species such as *T. ivorensis* are important components for traditional medicine (Kamtchouing *et al.* 2006). Additional to native *Terminalia* spp., non-native species such as *T. mantaly* and *T. catappa* are frequently encountered as ornamentals in urban areas in Cameroon. The socio-economical importance of *Terminalia* spp. in Cameroon, coupled with their fast growth account for their extensive exploitation in national regeneration programs.

Among the potential threats to forest tree species are fungal pathogens belonging to the family Botryosphaeriaceae. Species in the Botryosphaeriaceae have a worldwide occurrence, causing a wide range of diseases, predominantly die-back, canker and blue stain, on numerous hosts, including trees (Brown and Britton 1986; Denman *et al.* 1999; 2000; Desprez-Laustaud *et al.* 2006). This group of fungi commonly exist as endophytes in healthy plant tissues (Smith *et al.* 1996; Swart *et al.* 2000; Slippers and Wingfield 2007). Disease symptoms typically appear only after stress caused by abiotic and biotic disturbances (Schoeneweiss 1981; Slippers and Wingfield, 2007). Their occurrence as endophytes makes them especially important in international trade, as they may be spread undetected from one area to another, causing potentially serious damage to hosts that might have no co-evolved resistance (Slippers and Wingfield 2007).

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Species of Botryosphaeriaceae contribute directly or indirectly to economic and environmental losses, although the impact of their diseases is difficult to assess in forestry. In South African pine plantations, for instance, up to 55 % loss of production have been

recorded after hail damage and die-back due to *Diplodia pinea* Fries (Zwolinski et al. 1990). In the United States of America (U.S.A.) several tree diseases associated with non-aggressive pathogens belonging to the Botryosphaeriaceae caused extensive mortality of Aspen during the 1930s (Schoeneweiss 1981). Moreover, other reports appear in literature recognizing severe decline of *Quercus* spp. due to species in the Botryosphaeriaceae in 1980 in the Mediterranean Basin (Sanchez et al. 2003).

In Africa, species of *Terminalia* occur in environments ranging from evergreen, primary and secondary forests to open woodlands or wooded savannahs (Carr 1994; Dale and Greenway 1961; Keay 1989; Lebrun and Stork 1991). Although these trees have tended to display natural resistance to pests and diseases (Groulez and Wood 1985; Lamb and Ntima 1971), their wide ecological distribution exposes them to highly variable climatic conditions, environmental stress and other negative factors such as human activities and diverse pests and diseases. These factors may play an important role in predisposing *Terminalia* spp. in Africa to infection by species of the Botryosphaeriaceae (Jurskis 2005).

The aim of this study was to identify species in the Botryosphaeriaceae that occur on *Terminalia* trees in Cameroon. This information will be valuable in the management of the health of these trees, because they are a key group of pathogens that generally affect forest trees, and especially given projections of changing weather patterns, that will negatively affect these trees. Identification were done using a combination of morphological and DNA sequence data of the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials using species of the Botryosphaeriaceae from *Terminalia* spp. were conducted to determine their relative pathogenicity.

2. MATERIALS AND METHODS

2.1. Sample collection and fungal isolation

Plant material was collected in 2007 and 2008 from two species of native and one non-native *Terminalia* in Cameroon. The tree species sampled were the non-native *T. mantaly* and native *T. ivorensis* and *T. superba.* Four sites, located in three regions, were chosen for sampling (Table 1). Depending on the availability of trees at each location, at least 15 trees per species were randomly chosen for sampling without considering either their size or age. In each area,

samples from healthy twigs or bark were collected and placed in paper bags and transferred to the laboratory where they were processed within a few days.

For each sample, two pieces of twig or bark (1 cm in length) were split longitudinally. Samples were surface disinfected by sequential soaking in 70 % ethanol (1min), undiluted bleach (3.5 % sodium hypochlorite for 1 min), 70 % ethanol (1 min), rinsed in sterile water and allowed to dry under sterile conditions. Three disinfected pieces were placed onto 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri plates were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, USA) and incubated at 20 °C under continuous near-UV light for one week. Single hyphal tips growing from the plant tissues were transferred to new Petri plates containing MEA. After two weeks of incubation under near UV-light, cultures resembling species of the Botryosphaeriaceae (fast growth, mycelium white originally, turning dark greening-grey or greyish within few days) were selected and transferred to new Petri dishes containing MEA.

2.2. Morphology and cultural characteristics

To encourage formation of fruiting structures, isolates were inoculated onto sterile pine needles on 1.5 % water agar (WA) (Biolab, S.A.) as described previously (Slippers et al. 2004). The plates were incubated at 25 °C under near UV-light for 4-6 weeks. Microscope slides of conidia from pycnidia formed on the pine needles were prepared in lactic acid for morphological observations. Conidial dimensions were taken from digital images using a HRc Axiocam digital camera and accompanying Axiovision 3.1 (Carl Zeiss Ltd., München, Germany) microscope. For each isolate, fifteen measurements of both conidial length and width were made. Colony appearance of cultures growing on 2 % MEA at 25 °C under near UV-light for two weeks was described and colours of the colonies were recorded. Cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.3. DNA extraction, PCR reactions and DNA sequencing

Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae were the same as those described in Begoude et al. (2009),

using two gene regions. The entire Internal Transcribed Spacer region (ITS) of the nrDNA, including the 5.8S operon, was amplified by PCR (polymerase chain reaction), for all isolates collected, using the primers ITS1 and ITS4 (White et al. 1990). A part of the Translation Elongation Factor-1α (*tef 1-α*) gene was amplified, for selected isolates, using the primers EF1F and EF1R (Jacobs et al. 2004).

2.4. Sequence Analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using MEGA version 4 (Tamura et al. 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in GenBank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005). The aligned sequences were transferred to PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2001) where a final manual alignment was made.

A phylogenetic analysis was run for separately each of the ITS and *tef 1-α* data sets, followed by combined analyses of these data sets for core isolates. A partition homogeneity test (Farris et al. 1995) was conducted in PAUP version 4.0b10 (Swofford 2001) to assess the possibility of combining the ITS and *tef 1-α* data sets. In all analyses, gaps were treated as fifth character and characters were unordered and of equal weight. The phylogenetic analyses for the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithm, and random taxon addition of sequences for the construction of maximum parsimonious trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. *Guignardia mangiferae* A.J. Roy was used as the outgroup in the analyses of ITS and *tef 1-α* data sets. The support for branches of the most parsimonious trees was assessed using a 1000 bootstrap replicates (Felsenstein 1985). Other measures considered were the tree length (TL), consistency index (CI), rescaled consistency index (RC), and retention index (RI) (Hillis and Huelsenbeck 1992).

Bayesian phylogenetic analyses using Markov Chain Monte Carlo (MCMC) were performed in MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001) for all three data sets described above. Version 2.2 of MrModeltest (Nylander 2004) was used to select the model that best fits each

of the partitions. The Likelihood settings from best-fit models, SYM+I+G and HKY+G, were selected based on the Akaike Information Criteria (AIC) for ITS and *tef 1-α* respectively. Bayesian analyses were performed for one million generations, with four independent chains, and sampled every 100th tree. The first 1000 trees were graphically identified as the burn-in and deleted when constructing consensus trees and calculating posterior probabilities. A total of 9001 trees were imported into MEGA version 4 to construct a 50% majority-rule consensus tree.

2.5. Pathogenicity

Plants of native species of *Terminalia* are rare and could not be obtained. Pathogenicity tests were consequently carried out on one-year-old non-native *T. mantaly* and *T. catappa* trees grown in the Yaoundé Urban Council nursery, Cameroon. These trials were conducted between October and December 2008. This period falls at the end of the rain season and the beginning of the dry season, with average day and night temperatures of 26 °C. The trees were maintained under shade in 15 cm diameter plastic bags and watered daily. At the time of inoculation, the stem diameters were approximately 10 mm and the trees varied from 15-30 cm in height. For inoculations, 14 isolates of the Botryosphaeriaceae, representing all the species identified in the study, were grown on 2 % MEA for 10 days prior to inoculation.

To inoculate trees, wounds were made on the stems, half way between the soil level and the first branch by removing the outer bark with a 5 mm diameter cork-borer. A 5 mm-diameter plug of each isolate was placed into each wound, with the mycelium facing the cambium, and wrapped with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum. The trees were divided into four separate blocks and within each block, six trees arranged in a completely randomized design, were used for inoculation with each isolate. The entire trial was repeated once. For the controls, sterile MEA plugs were used in place of the fungal cultures.

After six weeks, the lengths of the lesions produced in the cambium, including the inoculation point, were measured to obtain an indication of the virulence of the isolates tested. Furthermore, a small piece of necrotic tissue was cut from the edges of all lesions and placed on MEA for isolations to show that the inoculated fungus was associated with the lesions. As no significant differences were noticed between results obtained for the two inoculations (P >

0.05), the data for all isolates were pooled in a single dataset for analyses. Variations in the extent of the lesions were assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Isolates and morphology

A total of 115 trees were sampled at four localities. These included 35 *T. ivorensis* trees, 50 *T. superba* and 30 *T. mantaly* trees. Isolates of Botryosphaeriaceae were obtained from 55 of the 110 trees sampled. In total, 43 isolates were obtained from 37 *T. ivorensis* trees, 20 isolates from seven *T. superba* trees and 27 isolates from 11 *T. mantaly* trees. No sign of disease, caused by fungi in the Botryosphaeriaceae, was observed on any trees at the time of collection. It was thus assumed that all isolates were from healthy trees.

The isolates obtained were assigned to two groups based on colony and conidial morphology. The majority of isolates collected (82 isolates) produced aerial mycelium which was white at first, turning dark grey-green or grey after four to five days at 25 °C under near UV-light. These isolates produced thick-walled, hyaline conidia that turned dark with age (Figure 1). The conidia were aseptate when young, becoming uniseptate with age. Conidia were ovoid in shape and some developed longitudinal striations as they aged. These isolates were identified as belonging to species of *Lasiodiplodia* based on their conidial morphology. The second group of isolates (eight isolates) were characterized by dark grey or green to black mycelium, producing small dark brown conidia (Figure 1) and resembling species of *Endomelanconiopsis* (Rojas et al. 2008).

No sexual fruiting structures were produced on pine needles by any of the isolates from *Terminalia* spp. in Cameroon. The Botryosphaeriaceae occurring on *Terminalia* spp. in Cameroon were compared to similar species described in previous studies (Table 3). Isolates from the *Lasiodiplodia* group were found at all the localities sampled and from all three host species. Isolates residing in the second group were found only in three locations (Belabo, Nkoemvone and Mbalmayo) and only on *T. ivorensis* and *T. superba*.

3.2. DNA extraction and PCR amplification

A total of 55 isolates, each originating from a single *Terminalia* tree, were selected for sequencing of their ITS and 5.8S rDNA regions to obtain a broad indication of their identities and to select isolates for the data sets used in the final analyses. These were comprised of 51 isolates from the morphological group resembling *Lasiodiplodia* and four from the group resembling *Endomelanconiopsis.* Based on results of the ITS sequences, fourteen isolates were selected for sequencing of the *tef 1-α* gene region and considered in the final analyses. PCR fragments for the ITS and 5.8S gene regions were ~ 580 bp in size, and ~ 710 bp for the *tef 1-α* gene region, The *tef 1-α* sequences were larger than those retrieved from GenBank, which spanned 244 to 500 bp, and only the corresponding regions were used in the phylogenetic analyses.

3.3. Phylogenetic analyses

A BLAST search against the GenBank database, using ITS sequences obtained from *Terminalia* spp. in Cameroon, showed that isolates resembling species of *Lasiodiplodia* were most closely related to *L. theobromae* (Pat.) Griff. & Maubl. and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous. Isolates from the second group, with small dark brown conidia, were identified as *Endomelanconiopsis endophytica* Rojas & Samuels.

ITS phylogeny. The ITS dataset comprised 91 sequences of which 55 were for isolates from *Terminalia* spp. and 36 sequences were retrieved from GenBank. After alignment, the ITS sequence data set consisted of 575 characters of which 313 were constant, 112 were parsimony uninformative and 150 were parsimony informative. The MP analyses generated 100 trees with identical topologies with respect to the major clades (TL = 563, CI = 0.627, RI = 0.868, RC = 0.544).

Four main clades including isolates from *Terminalia* spp. in Cameroon emerged from the MP analyses of the ITS gene region. These clades represented the two genera *Lasiodiplodia* [Bootstrap support (BS) = 100 % and Bayesian posterior probabilities (BPP) = 0.99] and *Endomelanconiopsis* (BS = 100 % and BPP = 1) (Figure 2). In the *Endomelanconiopsis* group, except for two isolates where very little divergence (two to three base pairs) was observed, sequences from *Terminalia* spp. in Cameroon were identical to *E. endophytica* and

clustered with isolates from South America. The *Lasiodiplodia* group included most of the isolates obtained in this study and it was subdivided into three sub-clades with no significant Bootstrap support. The first sub-clade (20 isolates) consisted of isolates grouping with *L. theobromae*. Except for two isolates, no sequence variation was detected between isolates in this clade. The second sub-clade (25 isolates) accommodated isolates clustering with *L. pseudotheobromae*. Small sequence variations were observed in a few isolates of this group. The third group, consisting of five isolates from Cameroon was not clearly resolved and clustered close to *L. mahajangana* Begoude, Jol. Roux, Slippers. and *L. parva* A.J.L. Phillips, A. Alves & Crous. No statistical support was observed for any of these sub-clades. For this reason, representative isolates from the *Endomelaconiopsis* clade and the three sub-clades in the *Lasiodiplodia* group were selected for *tef 1-α* gene region sequencing.

Combined ITS and tef 1-α analyses. The partition homogeneity test indicated congruence between the ITS and *tef 1-α* partitions (P = 0.355) suggesting that the data sets could be combined. The combined dataset consisted of 48 isolates with 887 characters of which, 377 were constant, 146 were parsimony uninformative and 364 were parsimony informative. Gaps were treated as a fifth character. After heuristic searches, 42 most parsimonious trees were obtained (TL = 1068; CI = 0.738, RI = 0.914, RC = 0.674; TreeBase Accession No. SN4630) and one of them was chosen for representation (Figure 3). All 42 trees displayed the same topology with regard to the identified clades. The topology of the tree generated from the combined analyses with MP, as well as with the 50 % majority rule consensus tree from the trees obtained through Bayesian analysis, was congruent with the trees obtained with the individual analyses of ITS and *tef 1-α*, identifying the same clades.

All the isolates collected in this study grouped with previously described species of *Lasiodiplodia* and *Endomelanconiopsis*, strongly supported with Bootstrap and Bayesian posterior probability values (Figure 3). Similar to results obtained for the ITS gene region, isolates from Cameroon could be identified as *L. theobromae* (BS = 100 %, BPP = 1), *L. pseudotheobromae* (BS = 100 %; BPP = 1). The third group of *Lasiodiplodia* isolates clustered with *L. parva* (BS = 97 %; BPP = 1), but one base pair difference in the *tef 1-α* sequences was noticed among isolates in this later group. The fourth group of isolates consisted of *E. endophytica* from *Terminalia* spp. in Cameroon which formed a well supported clade (BS = 100 % and BPP = 1) with sequences from authentic isolates of this species from GenBank (Figure 3).

Isolates of Botryosphaeriaceae found on *Terminalia* spp. that were phylogenetically related to *L. parva* based on ITS and *tef 1-α* sequence comparisons, mostly conformed to previous morphological description of *L. parva*. However, important differences in conidial sizes were observed for isolates from Cameroon (Table 3), raising the question as to whether they represent a different and unique species. DNA sequence data for the ITS and *tef 1-α* gene regions, however, did not support the description of a discrete species for these isolates. Further sequences from additional gene regions (*β*-tubulin, LSU and SSU) not reported in this paper were found to be identical with those of original species of *L. parva* and, therefore, suggested that all these isolates represent the same species.

3.4. Pathogenicity

Pathogenicity trials conducted on *T. mantaly* using isolates of the Botryosphaeriaceae collected in this study revealed visible lesions within six weeks after inoculation (Figure 4). Trees inoculated with sterile MEA also produced small lesions that represented only wound reactions as no Botryosphaeriaceae could be isolated from them. All the isolates of Botryosphaeriaceae were successfully re-isolated from the lesions emerging from inoculations. Analysis of variance showed that the mean lengths of lesions produced by all of the isolates on *T. mantaly* differed significantly ($P < 0.0001$) from the controls (Figure 4). *L. pseudotheobromae* produced the longest lesions followed by *L. theobromae, L. parva* and *E. endophytica*.

On *T. catappa* trees, all isolates collected from *Terminalia* trees in Cameroon produced lesions significantly longer than those of the control inoculations (Figure 5). Similar to the situation on *T. mantaly*, control inoculations showed only small lesions. However, re-isolations did not yield any Botryosphaeriaceae from the controls, whereas the original Botryosphaeriaceae were re-isolated from all the trees inoculated with fungal cultures. Analysis of variance indicated that lesion lengths produced on the cambium by all the isolates were significantly different ($P < 0.0001$) to those associated with the controls (Figure 5). Isolates representing *L. pseudotheobromae* were most virulent and produced longer lesions than *L. theobromae* and *L. parva*. *E. endophytica* produced substantially smaller lesions than either *L. pseudotheobromae* or *L. theobromae*.

A positive correlation (R^2 = 77 %) was found between inoculations on *T. mantaly* and *T. catappa.* On both tree species, *L. pseudotheobromae* was most virulent. In general, the lesions observed in *T. catappa* were longer than those of *T. mantaly* for *L. pseudotheobromae* and *L. theobromae* isolates. In contrast, the lengths of lesions produced by isolates of *L. parva* and *E. endophytica* on *T. catappa* were smaller than those observed on *T. mantaly.* However, this difference in susceptibility between *T. catappa* and *T. mantaly* was not statistically significant.

4. DISCUSSION

This study represents the first attempt to identify the Botryosphaeriaceae on native *Terminalia* trees in Africa. Four species of the Botryosphaeriaceae were collected from *T. ivorensis* and *T. superba*, and three species were found on samples from the non-native *T. mantaly*. A combination of morphological characteristics and DNA sequence comparisons was used to identify these species as *L. theobromae, L. pseudotheobromae, L. parva* and *E. endophytica.* These fungi are reported on these hosts for the first time. Except for *E. endophytica,* which was isolated only from *T. superba* and *T. ivorensis*, *L. pseudotheobromae, L. theobromae* and *L. parva* were collected from all the tree species sampled in this study.

The majority of isolates obtained in this study represented species of *Lasiodiplodia* of which isolates were identified as *L. theobromae, L. pseudotheobromae* and *L. parva* based on sequence data of the ITS and *tef 1-α* gene regions. Until recently, most *Lasiodiplodia* spp. from tropical trees were treated as *L. theobromae* (Punithalingam 1976). However, application of DNA sequence comparisons for the ITS and *tef 1-α* gene regions has resulted in the description of 10 new *Lasiodiplodia* spp. (Pavlic et al. 2004; Burgess et al. 2006; Damm et al. 2007; Alves et al. 2008; Begoude et al. 2009; Pavlic et al. 2008). These species share similar morphological characteristics, such as slowly maturing conidia with thick walls that turn dark with age and develop longitudinal striations.

Lasiodiplodia theobromae has a wide geographic distribution and it has been found on more than 500 forest and agricultural plant species in tropical and subtropical areas (Punithalingam 1980). It is well known as an endophyte on healthy tropical trees (Suryanarayanan et al. 2002; Begoude et al. 2009). Furthermore, *L. theobromae* can act as a latent pathogen causing disease symptoms after onset of conditions unfavourable for the host (Schoeneweiss 1981;

Mullen et al. 1991; Slippers and Wingfield 2007). *L. theobromae* has previously been reported as an endophyte in the inner bark and twigs of healthy *T. arjuna* (Tejesvi et al. 2005), leaves of *T. tomentosa* and *T. bellerica* (Suryanarayanan et al. 2002) and the twigs and bark of healthy *T. catappa* (Begoude et al. 2009) in the tropics. On *Terminalia* spp., *L. theobromae* has mostly been recorded as the causal agent of blue stain of logs, soon after felling (Groulez and Wood 1985; Lamb and Ntima 1971; Apetorgbor et al. 2004). However, in Cameroon, *L. theobromae* is best known as the cause of die-back of cacao (*Theobromae cacao*) (Mbenoun et al. 2008). In the current study, *L. theobromae* was the second most abundant species identified on *Terminalia* spp. All isolates collected were from healthy trees, but pathogenicity trials on young *T. catappa* and *T. mantaly* showed that it is highly pathogenic to these trees. Pathogenicity tests on *T. ivorensis* and *T. superba* should, however, be conducted to determine whether it can cause disease on these important native trees.

Lasiodiplodia pseudotheobromae was the most commonly collected species of Botryosphaeriaceae, collected from all the species of *Terminalia* sampled in this study. This fungus was originally described from *Rosa* sp. in the Netherlands, *Gmelina arborea* and *Acacia mangium* in Costa Rica, *Coffea* sp. in Democratic Republic of Congo and *Citrus aurantium* in Suriname (Alves et al. 2008). In a recent study investigating the Botryosphaeriaceae on *T. catappa* in Cameroon, South Africa and Madagascar (Begoude et al. 2009), *L. pseudotheobromae* was also the most abundant species found in all the sampled areas. The information generated in the current study, which is supported by a previous one on *T. catappa*, suggests that *L. pseudotheobromae* has a worldwide distribution. In pathogenicity trials *L. pseudotheobromae* was found to be the most virulent species tested. This was also the case in a study of *T. catappa* (Begoude et al. 2009). *L. pseudotheobromae* is, therefore, the most likely species of Botryosphaeriaceae to cause health problems on *Terminalia* trees in Africa where they are subjected to stressful conditions.

Lasiodiplodia parva was only recently described and was previously treated as *L. theobromae*, together with *L. pseudotheobromae* (Alves et al. 2008). Isolates collected from *Terminalia* spp. in this study, however, differed in their conidial sizes from descriptions for the type specimen. The conidia of isolates from Cameroon were larger than those previously described for *L. parva*. DNA sequence data for both ITS and *tef 1-α, β*-tubulin, LSU and SSU, however, confirmed that isolates from Cameroon represent *L. parva*, despite minor differences for two nucleotides in ITS sequences and a single nucleotide in *tef 1-α* sequences.

Our results thus show that some isolates of *L. parva* can produce conidia as large as those produced by other closely related species, such as *L. pseudotheobromae* and *L. theobromae*. This emphasizes the importance of considering multiple criteria for species identification when treating species of the Botryosphaeriaceae.

Prior to this study, *L. parva* was known only to occur in agricultural field soil and crops in Latin America (Alves et al. 2008). Although *L. parva* was the least abundant *Lasiodiplodia* sp. isolated from *Terminalia* spp., its occurrence on these trees in Cameroon has substantially broadened its host range and geographic distribution. Previously, the only plant host from which *L. parva* was known was *Theobroma cacao* in Colombia (Alves et al. 2008) and no information concerning its pathogenicity to this tree is available. In the current study, assessment of its pathogenicity on *T. mantaly* and *T. catappa* trees showed that *L. parva* consistently produced lesions on both hosts. However, in comparison to *L. theobromae* and *L. pseudotheobromae*, *L. parva* was only mildly pathogenic, suggesting that this fungus is unlikely to emerge as an important pathogen on these trees.

Endomelanconiopsis endophytica is a recently described species found as an endophyte in leaves of *T. cacao* and associated native woody hosts in the same environment (Rojas et al. 2008). Isolates of *E. endophytica* found in the present study were shown to group with the South American isolates of the fungus. The Cameroonian isolates were obtained from *T. ivorensis* and *T. superba*. These tree species are commonly used in cacao farms to establish a "taungya" agri-sylvicultural system in which they provide shade or improve soil fertility (Norgrove and Hauser 2002). It would not, therefore, be surprising to obtain further isolates of this fungus on hosts such as cocoa trees in Cameroon. The collection of *E. endophytica* from native *Terminalia* spp. in Cameroon adds to previous records of the fungus from plants in South America (Rojas et al. 2008). Even though very few isolates representing *E. endophytica* were found in this study, its presence on tropical species of *Terminalia* is particularly interesting as this could indicate a possible tropical origin of the fungus.

Two distinct genera of Botryosphaeriaceae, *Lasiodiplodia* and *Endomelanconiopsis,* were found associated with species of *Terminalia* in Cameroon. Although little information related to the ecology of the genus *Endomelanconiopsis* is available, both *Lasiodiplodia* and *Endomelanconiopsis* appear to be tropical species. Apart from *E. endophytica,* which was isolated only from *T. superba* and *T. ivorensis*, no evidence of host specialization was

observed for species of *Lasiodiplodia* identified in this study. This is characteristic of many species of Botryosphaeriaceae (Slippers and Wingfield, 2007) and contributes to their potential to cause diseases on trees. Although this study focussed exclusively on healthy tree tissue, the common occurrence of generalist species such as *L. theobromae* and *L. pseudotheobromae* which are reputed virulent pathogens on a wide range of hosts (Punithalingam 1980; Slippers and Wingfield 2007; Begoude et al. 2009) suggests that they could be pathogens if unfavourable conditions for the host occur.

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Table 1. Locations and characteristics of sites from where *Terminalia* trees were sampled.

Site		**GPS coordinates**	**Tree species sampled**
Region	Locality		
Central	Mbalmayo	N3 26.034 E11 29.344	*T. superba, T. ivorensis*
South	Nkoemvone	N2 49.045 E11 07.577	*T. superba*
	Kribi	N2 58.064 E9 54.904	*T. mantaly*
Eastern	Belabo	N4 57.376 E13 19.433	*T. superba, T. ivorensis, T. mantaly*

Table 2. Botryosphaeriaceae used in phylogenetic analyses in this study.

Species	Culture number	Origin	Host	Collectors	Genbank Accession No.				
					ITS	*tef* 1-α	*β-tub*	LSU	SSU
Botryosphaeria dothidea	CMW7999	Switzerland	*Ostrya* sp.	B. Slippers	AY236948	AY236897			
	CMW8000	Switzerland	*Prunus* sp.	B. Slippers	AY236949	AY236898			
Diplodia mutila	CBS112553	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259093				
	CBS230.30	USA	*P. dactylifera*	L.L. Huilllier	DQ458886				
Diplodia. seriata	CMW7774	USA	*Ribes* sp.	B. Slippers/G.Hudler	EF445343	EF445382			
	CMW7775	USA	*Ribes* sp	B. Slippers/G.Hudler	EF445344	EF445383			
Endomelanconiopsis endophytica	**CMW28618**	Cameroon	*Terminalia ivorensis*	D. Begoude	GQ469966	GQ469906			
	CMW28551	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469967	GQ469907			
	CMW28552	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469968	GQ569908			
	CMW28563	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469965				
	CBS120397	Panama	*Theobroma cacao*	E. Rojas/L.Mejia/Z. Maynard	EU633656	EU683637			
	CBS122546	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683661	EU683642			
	CBS122550	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683664	EU683645			
Endomelanconiopsis microspora	CBS353.97	Panama	Soil	E. Rojas/L.Mejia/Z. Maynard	EU683655	EU683636			
Guignardia mangiferae	1095	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683671	EU683652			
Lasiodiplodia crassispora	WAC12533	Venezuela	*Eucalyptusurophylla*	S. Mohali	DQ103552	DQ10355			
	WAC12534	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103550	DQ103557			
	WAC12535	Australia	*S. album*	T.I. Burgess/B. Dell	DQ103551	DQ103558			

Lasiodiplodia gonubiensis	CBS115812	South Africa	*S. cordatum*	D. Pavlic	DQ458892	DQ458877			
	CBS116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567			
Lasiodiplodia mahajangana	CMW27801	Madagascar	*T. catappa*	J. Roux	FJ900595	FJ900641			
	CMW27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642			
	CMW27820	Madagascar	*T. catappa*	J. Roux	FJ900597	FJ900643			
Lasiodiplodia margaritacea	CMW26162	Australia	*A. gibbosa*	D. Pavlic	EU144050	EU144065			
	CMW26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066			
Lasiodiplodia parva	CBS356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF622082	EF622062			
	CBS494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064			
	CMW28333	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469961	GQ469903	GQ469892	GQ469909	GQ469912
	CMW28309	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469962	GQ469904	GQ469894	GQ469911	GQ469914
	CMW28292	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469963	GQ469905	GQ469893	GQ469910	GQ469913
	CMW28295	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469964				
	CMW28628	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469960				
Lasiodiplodia plurivora	STEU-5803	South Africa	*P. salicina*	U.Damm	EF445362	EF445395			
	STEU-4583	South Africa	*V. vinifera*	F.Halleen	AY343482	EF445396			
Lasiodiplodia pseudotheobromae	**CMW28297**	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469937	GQ469899			
	CMW28300	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469939	GQ469900			
	CMW28574	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469947	GQ469901			
	CMW28624	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469956	GQ469902			
	CMW28328	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469935				
	CMW28330	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469936				
	CMW28299	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469938				
	CMW28301	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469940				
	CMW28332	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469941				
	CMW28566	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469942				
	CMW28314	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469943				
	CMW28568	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469944				
	CMW28569	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469945				
	CMW28633	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469946				
	CMW28632	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469948				

	CMW28310	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469949	
	CMW28557	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469950	
	CMW28558	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469951	
	CMW28313	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469952	
	CMW28560	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469953	
	CMW28561	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469954	
	CMW28562	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469955	
	CMW28298	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469957	
	CMW28627	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469958	
	CMW28622	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469959	
	CBS116459	Costa Rica	*Gmelinea arborea*	J.Carranza/Velásquez	EF622077	EF622057
	CBS447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060
Lasiodiplodia rubropupurea	WAC12535	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103553	DQ103571
	WAC12536	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103554	DQ103572
Lasiodiplodia theobromae	**CMW28550**	Cameroon	*T. mantaly*	D. Begoude/ J.Roux	GQ469921	GQ469895
	CMW28570	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469923	GQ469896
	CMW26571	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469924	GQ469897
	CMW27311	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469932	GQ469898
	CMW28326	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469915	
	CMW28327	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469916	
	CMW28329	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469918	
	CMW28547	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469919	
	CMW28548	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469920	
	CMW28573	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469925	
	CMW28575	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469926	
	CMW28308	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469927	
	CMW28312	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469928	
	CMW28554	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469929	
	CMW28555	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469930	
	CMW28556	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469931	
	CMW28625	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469933	
	CMW28626	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469934	
	CMW9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054
	CBS164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258

Lasiodiplodia venezuelensis	WAC12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568
	WAC12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569
Neofusicoccum parvum	CMW9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888
	CMW9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885
Neofusicoccum ribis	CMW7772	USA	*Ribes* sp.	B. Slippers/G.Hudler	AY236935	AY236877
	CMW7773	USA	*Ribes* sp.	B. Slippers/G Hudler	AY236936	AY236878

Table 3. Conidial dimensions of the Botryosphaeriaceae from *Terminalia* spp. and comparison with those reported in previous studies.

Species	**Conidial size (µm)**		**Source of data**
	This study	**Previous studies**	
L. pseudotheobromae	(20.5-)23.5-27.5(-31.5) x (10.5-)12.0-14.0(-16.5)	(22.5-)23.5-32(-33) x (13.3-)14-18(-20)	Alves *et al.* 2008.
L. theobromae	(17.5-)21.5-27.5(-31.0) x (10.5-)12.0-14.0(-16.5)	(19-)21-31(-32.5) x (12-)13-15.5(-18.5)	Alves *et al.* 2008.
L. parva	(24.5-)26.5-29.5(-33.5) x (11.0-)12-14.5(-17.5)	(15.5-)16-23.5(-24.5) x (10-)10.5-13(-14.5)	Alves *et al.* 2008
E. endophytica	(5.5-)6.0-7.5(-8) x (3.0-)3.5-4.0(-4.5)	(4.7-)5.5-7.5(-10.0) x (3.0-)3.5-4.5(-6.2)	Rojas *et al.* 2008

Figure 1. Conidial morphology of species of the Botryosphaeriaceae from *Terminalia* spp. (a) young hyaline thick-walled conidia of *Lasiodiplodia theobromae,* (b) *L. pseudotheobromae,* (c) *L. parva* (d) dark brown conidia of *E. endophytica.* Bars: a, b, c, d = 10 µm.

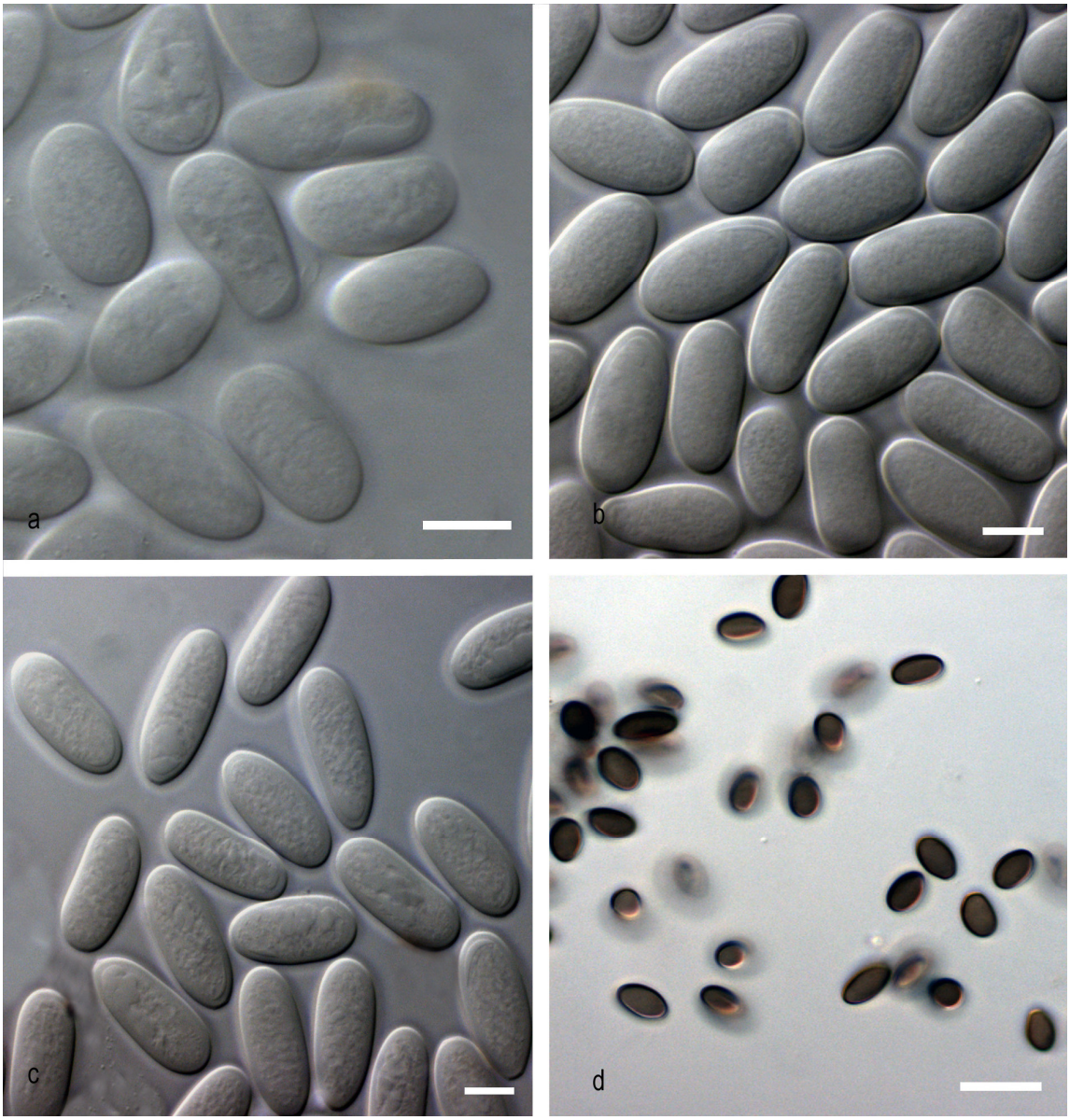
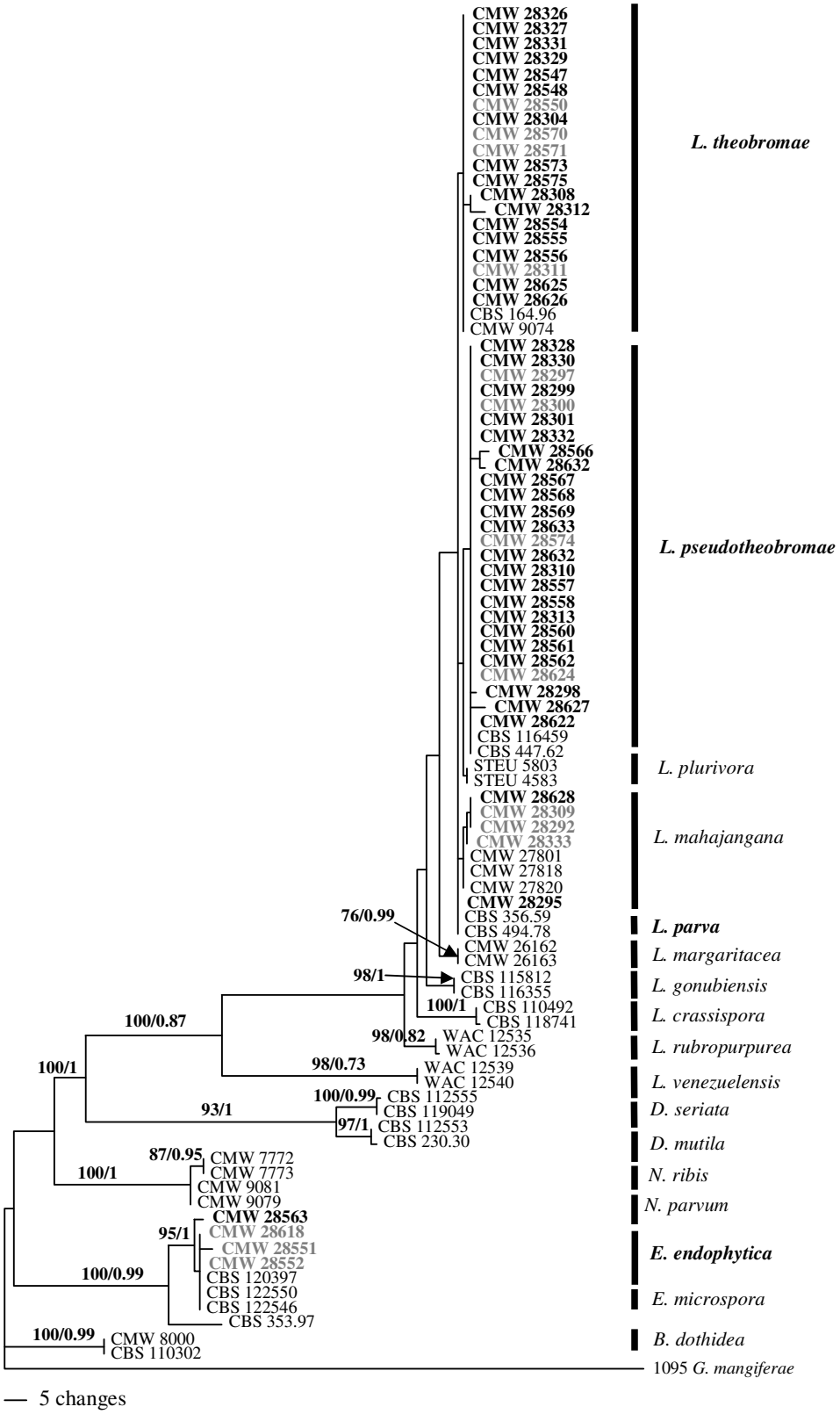

a
b
c
d

Figure 2. One of the most parsimonious trees obtained from analyses of the ITS sequence data of the Botryosphaeriaceae from *Terminalia* spp. Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/ PP). Isolates marked in bold represent those obtained from *Terminalia* spp. in Cameroon. Isolates marked in grey were selected for *tef 1-α* sequencing.


CMW 28326
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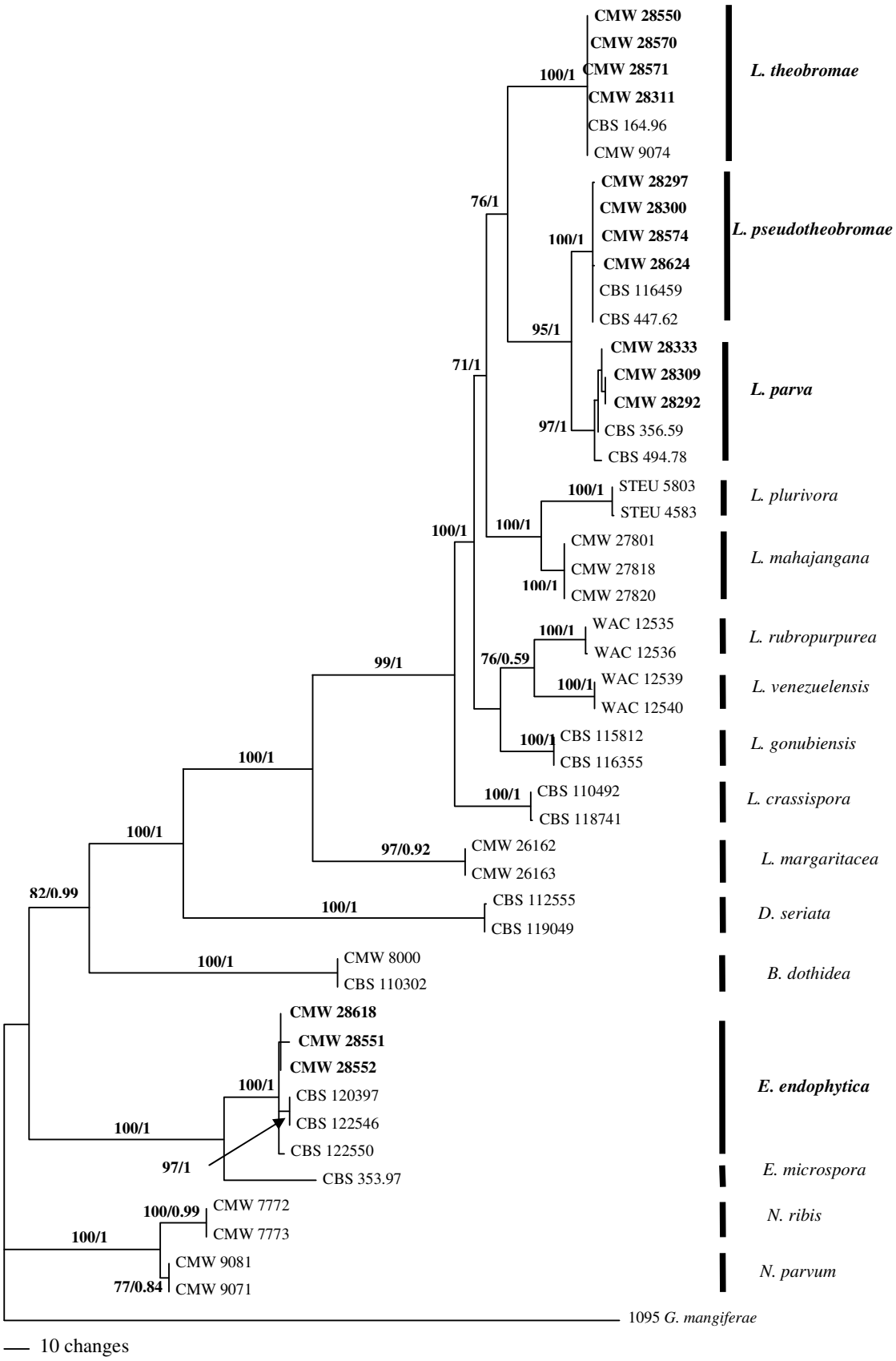
CMW 28331
CMW 28329
CMW 28547
CMW 28548
CMW 28550
CMW 28304
CMW 28570
CMW 28571
CMW 28573
CMW 28575
CMW 28308
CMW 28312
CMW 28554
CMW 28555
CMW 28556
CMW 28311
CMW 28625
CMW 28626
CBS 164.96
CMW 9074
L. theobromae
CMW 28328
CMW 28330
CMW 28297
CMW 28299
CMW 28300
CMW 28301
CMW 28332
CMW 28566
CMW 28632
CMW 28567
CMW 28568
CMW 28569
CMW 28633
CMW 28574
CMW 28632
CMW 28310
CMW 28557
CMW 28558
CMW 28313
CMW 28560
CMW 28561
CMW 28562
CMW 28624
CMW 28298
CMW 28627
CMW 28622
CBS 116459
CBS 447.62
L. pseudotheobromae
STEU 5803
STEU 4583
L. plurivora
CMW 28628
CMW 28309
CMW 28292
CMW 28333
CMW 27801
CMW 27818
CMW 27820
L. mahajangana
CMW 28295
CBS 356.59
CBS 494.78
L. parva
CMW 26162
CMW 26163
L. margaritacea
CBS 115812
CBS 116355
L. gonubiensis
CBS 110492
CBS 118741
L. crassispora
WAC 12535
WAC 12536
L. rubropurpurea
WAC 12539
WAC 12540
L. venezuelensis
CBS 112555
CBS 119049
D. seriata
CBS 112553
CBS 230.30
D. mutila
CMW 7772
CMW 7773
CMW 9081
CMW 9079
N. ribis
N. parvum
CMW 28563
CMW 28618
CMW 28551
CMW 28552
CBS 120397
CBS 122550
CBS 122546
CBS 353.97
E. endophytica
E. microspora
CMW 8000
CBS 110302
B. dothidea
1095 G. mangiferae
76/0.99
98/1
100/1
100/0.87
98/0.82
98/0.73
100/1
100/0.99
93/1
97/1
87/0.95
100/1
95/1
100/0.99
100/0.99
— 5 changes

Figure 3. One of the most parsimonious trees obtained from analyses of the combined ITS and *tef 1-α* sequence data of the Botryosphaeriaceae from *Terminalia* spp. Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/ PP). Isolates marked in bold represent those obtained from *Terminalia* spp. in Cameroon.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CMW 28571
CMW 28311
CBS 164.96
CMW 9074
L. theobromae
CMW 28297
CMW 28300
CMW 28574
CMW 28624
CBS 116459
CBS 447.62
L. pseudotheobromae
CMW 28333
CMW 28309
CMW 28292
CBS 356.59
CBS 494.78
L. parva
STEU 5803
STEU 4583
L. plurivora
CMW 27801
CMW 27818
CMW 27820
L. mahajangana
WAC 12535
WAC 12536
L. rubropurpurea
WAC 12539
WAC 12540
L. venezuelensis
CBS 115812
CBS 116355
L. gonubiensis
CBS 110492
CBS 118741
L. crassispora
CMW 26162
CMW 26163
L. margaritacea
CBS 112555
CBS 119049
D. seriata
CMW 8000
CBS 110302
B. dothidea
CMW 28618
CMW 28551
CMW 28552
CBS 120397
CBS 122546
CBS 122550
E. endophytica
CBS 353.97
E. microspora
CMW 7772
CMW 7773
N. ribis
CMW 9081
CMW 9071
N. parvum
1095 G. mangiferae
10 changes

Figure 4. Mean lesion lengths (mm) on cambium for each Botryosphaeriaceae isolate six weeks after inoculation on *T. mantaly* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control.

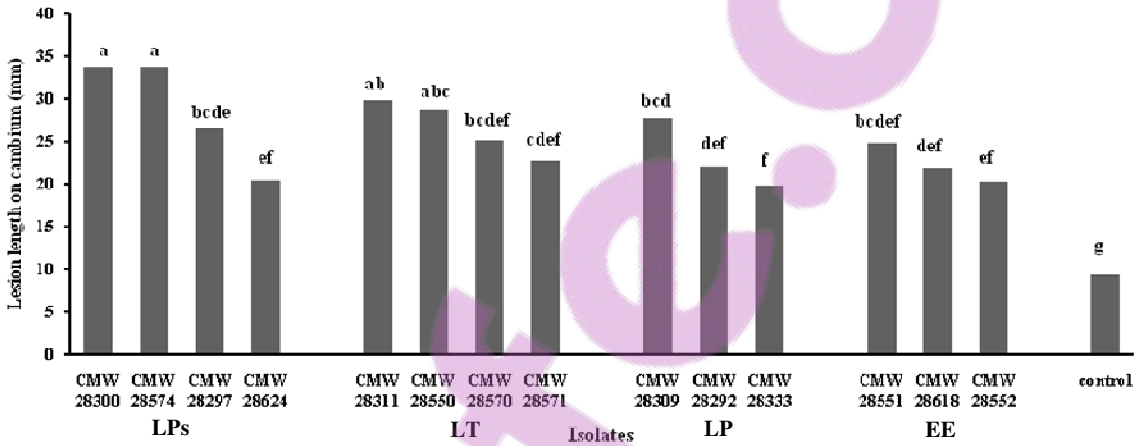
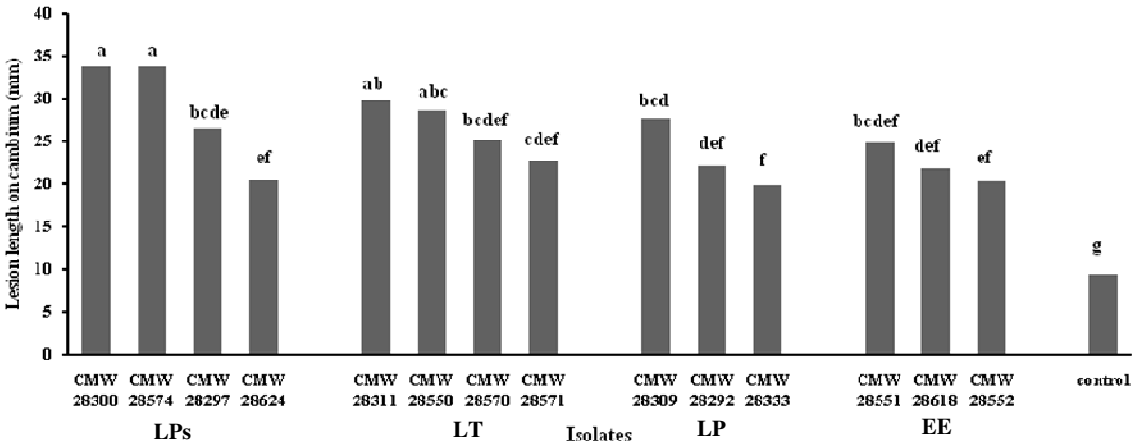

Lesion length on cambium (mm)
40
35
30
25
20
15
10
5
0
a
a
bcde
ef
ab
abc
bcdef
cdef
bcd
def
f
bcdef
def
ef
g
CMW 28300
CMW 28574
CMW 28297
CMW 28624
CMW 28311
CMW 28550
CMW 28570
CMW 28571
CMW 28309
CMW 28292
CMW 28333
CMW 28551
CMW 28618
CMW 28552
control
LPs
LT
LP
EE
Isolates

Figure 5. Mean lesion lengths (mm) on cambium for each Botryosphaeriaceae isolate six weeks after inoculation on *T. catappa* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control.



Chapter 4

Phenotypic and molecular characterization of the *Botryosphaeriaceae* associated with native *Terminalia* spp. in Southern Africa

ABSTRACT

As part of a broad investigation considering fungal host jumps between native and non-native trees in Africa, this study considers the diversity of Botryosphaeriaceae from healthy native *Terminalia* spp. in Southern Africa. A combination of morphological characteristics, DNA sequence data for the ITS, *tef 1-α* and *β-tub* gene regions were used to identify species. From a total of 135 isolates obtained from 232 *T. sericea* and *T. sambesiaca* trees sampled in Southern Africa, nine species were identified. These included seven known species, *Lasiodiplodia crassispora, L. pseudotheobromae, Diplodia alatafructa, Pseudofusicoccum olivaceum, Neofusicoccum parvum, N. kwambonambiense* and *N. vitifusiforme,* as well as two taxa described here as *L. cryptotheobromae* sp. nov. and *N. terminaliae* sp. nov. Other than *L. pseudotheobromae* and *N. parvum,* all are recorded on *Terminalia* spp. for the first time. Inoculation trials on branches of healthy *T. sericea* trees showed that all species are capable of causing lesions on these trees. *N. vitifusiforme, D. alatafructa* and *P. olivaceum* were mildly pathogenic and *L. pseudotheobromae* was the most virulent.

1. INTRODUCTION

The flora of Southern Africa is remarkably diverse with ~1700 indigenous tree species belonging to more than 90 families, including the Combretaceae (Palmer and Pitman 1972; Pooley 1999). The Combretaceae includes 20 genera and about 475 species (Thiombiano *et al.* 2006). Of these about 200 belong to the genus *Terminalia,* making it the second largest genus in the family (McGaw *et al.* 2001). At least 13 indigenous species of *Terminalia* are found in Southern Africa, of which the most abundant are *T. sericea* Burch. ex DC., *T. prunoïdes* Lawson. and *T. sambesiaca* Engl. & Diels (Coastes-Palgrave 1988). In Southern Africa, species of *Terminalia* are small shrubs to medium sized trees, found in open woodlands and wooded savannahs (Dale and Greenway 1961; Carr 1994). Indigenous species of *Terminalia* provide various benefits such as food for animals, improved soil fertility, control of soil erosion, wood for various social activities, fodder and shelter for livestock, human and veterinary medicines (Schmidt *et al*. 2002; Smith *et al.* 2004; Singh *et al*. 2002; Masoko *et al*. 2005; Katjiua and Ward 2006). A number of *Terminalia* spp. are also grown in plantations for commercial purposes, especially in Central and Eastern Africa (Groulez and Wood 1985; Lamb and Ntima 1971).

In Southern Africa, very few indigenous tree species are suitable for timber and pulp production (Immelman *et al.* 1973). For this reason non-native tree species have been introduced in many countries to meet the domestic needs for wood and simultaneously to relieve pressure on native forests (Immelman *et al.* 1973). Consequently, increasing land areas are being afforested and plantations of non-native tree species are reshaping the natural vegetation. Strips of native trees, such as *Terminalia* spp., thus occur adjacent to, and intermixed, with trees such as *Eucalyptus* or *Pinus* spp., introduced for commercial forestry. Many introduced, commercially propagated trees are related to native woody plants and there is growing evidence that pathogens are able to move between them (Wingfield 2003; Slippers *et al.* 2005; Slippers and Wingfield 2007). Knowledge of potentially pathogenic fungi on native trees contributes to assessments of the vulnerability of both native forests and plantations of non-native trees.

Diseases affecting forest tree species are of increasing concern to countries in the Southern African region. Both native pathogens that have undergone host shifts (Slippers *et al.* 2005)

from indigenous trees as well as non-native pathogens that have been accidentally introduced with germplasm of non-indigenous tree species are responsible for these diseases (Wingfield 2003; Wingfield *et al.* 2008). As forest plantations commonly adjoin native communities of trees, this close association can constitute a risk as both plant communities can act as sources of pests and pathogens (Slippers *et al.* 2005; Strauss 2001). For example, *Phytophthora cinnamomi* Rands., an important pathogen of *Eucalyptus* spp. in Australia, has been introduced into South Africa, where it seriously affects native Proteaceae and *Ocotea bullata* (Birch) Baill trees (Von Broembsen & Kruger 1985; Linde *et al.* 1999). In contrast, *Ceratocystis albifundus* M. J. Wingf., De Beer & M. J. Morris, a native African fungus (Barnes *et al.* 2005; Roux *et al.* 2007), has spread from native tree species to plantation grown Australian *Acacia mearnsii* De Wild., resulting in disease and death of these economically important trees (Roux *et al*. 2007).

The Botryosphaeriaceae represents a cosmopolitan group of fungi with a wide host range. Members of this family include important canker and dieback pathogens of numerous tree species (Denman *et al.* 1999; 2000; Slippers and Wingfield 2007). These fungi have the ability to live in healthy plant organs as symptomless endophytes making their detection difficult (Smith *et al.* 1996; Swart *et al.* 2000). This facilitates their ability to spread into new environments where they can infect new hosts. A number of recent studies have shown the occurrence of species of Botryosphaeriaceae on both native and introduced hosts in Southern Africa. *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips. has, for example, been found commonly on non-native *Eucalyptus* spp. and *T. catappa* L. trees, and on native *Syzygium cordatum* Hochst (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a). The common occurrence and wide host range of *N. parvum* on native trees in South Africa has led to the suggestion that this fungus is native in southern Africa. This hypothesis is further supported by the fact that it causes disease on non-native *Eucalyptus* spp. and *T. catappa*, but not on native *S. cordatum* (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a;). In contrast, *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips. a native pathogen of *Eucalyptus* spp. in Australia has been found on both non-native *Eucalyptus* spp. and native *S. cordatum* in South Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007; Maleme 2008).

Fungi belonging to the Botryosphaeriaceae constitute interesting model organisms to study the movement of fungal pathogens between native and introduced hosts. Not only can they be spread unnoticed, but increasing knowledge on the taxonomy and ecology of these fungi makes them ideal to study broader scale aspects of fungal movement and ecology. Following this view, the aim of the present study was to investigate the diversity of the Botryosphaeriaceae associated with native *Terminalia* spp. in Southern Africa. Isolates obtained in the study were characterized based on morphology, DNA sequence data and their pathogenicity to a *Terminalia* sp.

2. MATERIALS AND METHODS

2.1. Sample collection and fungal isolation

Plant material was collected in 2007 from native *T. sericea* and *T. sambesiaca* in Namibia, Botswana, South Africa and Tanzania (Figure 1). In each area, five to 20 trees were randomly chosen for sampling without considering either their size or age. A single healthy branch was collected from each tree, placed in a paper bag and transported to the laboratory for processing.

From each branch, two one centimetre long segments, were cut and split vertically. Samples were surface sterilized by immersing them in 96 % ethanol for 1 min, followed by 1 min in undiluted 3.5 % sodium hypochlorite and 1 min in 70 % ethanol, before rinsing in sterile distilled water and allowing them to dry under sterile conditions. The four disinfected branch pieces from each tree were plated on 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri dishes were sealed with Parafilm and incubated at 20 °C under continuous near-Ultra Violet (UV) light. One week later, filamentous fungi growing from the plant tissue and resembling the Botryosphaeriaceae were transferred to new Petri dishes containing fresh MEA.

All cultures used in this study were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representatives of all species were also deposited with the Centraalbureau voor

Schimmelcultures (CBS, Utrecht, Netherlands). Herbarium materials for previously undescribed species were deposited at the National Fungal Collection (PREM), Pretoria, South Africa.

2.2. Morphology and culture characteristics

Fungal isolates were grown in Petri plates containing 1.5 % water agar (Biolab, S.A.) overlaid with three pine needles that had been autoclaved twice, and incubated at 25 °C under near UV-light for two to six weeks to induce the formation of fruiting bodies. Morphological features of the resultant fruiting bodies were observed using a HRc Axiocam and accompanying Axiovision 3.1 camera (Carl Zeiss Ltd., München, Germany) and measurements of all relevant characters were made. These measurements are presented in descriptions as the extremes in brackets and the ranges calculated as the mean of the overall measurements plus or minus the standard deviation.

The morphology of fungal colonies growing on 2 % MEA at 25 °C under near UV-light for two weeks was described and colony colours (upper and reverse surfaces) of the isolates were recorded using the colour notations of Rayner (1970). Growth rates of cultures on 2 % MEA in the dark was determined at 5 °C temperature intervals from 10 to 35 °C. For growth rates, evaluations of five plates were used for each isolate at each temperature. Two measurements, perpendicular to each other, were made after three days for each plate resulting in 10 measurements for each isolate at each temperature. The experiment was repeated once.

2.3. DNA extraction, PCR reactions and DNA sequencing

Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae were as described in Begoude *et al.* (2009a) using three gene regions. The entire Internal Transcribed Spacer region (ITS) of the nrDNA, including the 5.8S operon was amplified by PCR (polymerase chain reaction) using the primers ITS1 and ITS4 (White *et al*. 1990). A portion of the Translation Elongation Factor-1α (*tef 1-α*) gene was amplified using the primers EF1F and EF1R (Jacobs *et al*. 2004) and part of the β-tubulin (*β-tub*) gene region was amplified with primers Bt2a and Bt2b (Glass and Donaldson, 1995).

2.4. DNA Sequence Analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using MEGA version 4 (Tamura *et al.* 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in GenBank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh *et al.* 2005). The aligned sequences were transferred to PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 1998) where a final manual alignment was made.

A single gene phylogenetic analysis was done for the dataset from each locus, followed by a combined analysis of ITS and *tef 1-α* sequence data. Additional analyses were also done with combined data from the ITS, *tef 1-α* and *β-tub* for a cryptic species in the *Lasiodiplodia* clade. In the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition of 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition sequences for the construction of maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. *Guignardia mangiferae* A.J. Roy was used as outgroup in analyses of ITS and *tef 1-α* data sets. The support for branches of the most parsimonious trees was assessed with a 1000 bootstrap replications (Felsenstein 1985). Other measures considered were the tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992). A partition homogeneity test (Farris *et al.* 1995) was conducted in PAUP to assess the possibility of combining the ITS and *tef 1- α* data sets in analyses of all the isolates. Incongruence Length Difference (Farris *et al.* 1995) was used in combined analyses for *Lasiodiplodia* isolates.

Both ITS and *tef 1-α* data sets were subjected to Bayesian phylogenetic analyses employing Markov Chain Monte Carlo (MCMC) implemented in MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001). MrModeltest version 2.2 (Nylander 2004) was used to estimate separately the model that best fit each of the partitions. The Likelihood settings from best-fit models

[(GTR+I+G) and (HKY+G)] were selected based on the Akaike Information Criteria (AIC) for the ITS and *tef 1- α* data sets, respectively. In the additional analyses, aiming to clarify the relationship among species close to *L. theobromae,* the K80 model was selected for the ITS data set whilst HKY was chosen for the *tef 1- α* and *β-tub* data sets, respectively. Bayesian analyses were performed for one million generations, with four independent chains, and sampled every 100th tree. The first 1000 trees were graphically identified as the burn-in and deleted when constructing consensus trees and calculation of posterior probabilities. A total of 9001 trees were imported into MEGA version 4 to construct a 50 % majority-rule consensus tree.

2.5. Pathogenicity

Pathogenicity tests were performed on branches of healthy seven to 12-year-old field grown *T. sericea* trees with branch diameters ranging from 10-20 mm. For inoculations, two isolates per species of the Botryosphaeriaceae identified in the study (Table 1) were grown on 2 % MEA for 10 days prior to inoculation. For each isolate, as well as the control, 10 trees were used. The method described by Begoude *et al.* (2009a) was used to inoculate branches and obtain an indication of the pathogenicity of the isolates tested. Data were analysed using the statistical analyses software programme SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Isolation, morphology and culture characteristics

In total, 135 isolates of the Botryosphaeriaceae were obtained from 232 trees sampled in Southern Africa. A total of 82 isolates were obtained from 134 *T. sericea* trees in the Moloto, Makhado, Musina, Pongola and Sudwala Caves areas in South Africa, 26 isolates from 58 *T. sericea* trees in Namibia from Katima Mulilo and Bagani, three isolates from 30 *T. sericea* trees in the Nata area in Botswana and 24 isolates from 10 *T. sambesiaca* trees sampled in the Ifakara area of Tanzania.

All isolates from *Terminalia* spp. derived from single conidia could be grouped into two categories based on conidial morphology. The isolates in the first category produced hyaline, elongated, thin-walled, *Fusicoccum*-like conidia. Isolates in this group represented two

different morphological forms. One group of isolates (Group 1) produced hyaline, bacilliform, aseptate and thin-walled conidia, and the other (Group 2) had hyaline, elongate, thin-walled, fusoid conidia. The second category contained isolates with pigmented, broad, thick-walled, *Diplodia*-like conidia. This category could also be sub-divided into two groups. Several isolates were characterized by thick-walled conidia; hyaline when immature and turning brown with age, aseptate and one-septate, sometimes exhibiting longitudinal striations (Group 3). A small set of three isolates (Group 4), differed from Group 3 isolates in that they had dark, thick-walled conidia. All the isolates produced only anamorph structures on pine needles in culture. Conidial dimensions of species of the Botryosphaeriaceae occurring on *Terminalia* spp. in Southern Africa were compared with those of similar species described in previous studies (Table 1).

All of the groups of isolates had similar culture morphology. Isolates on MEA grew fast, covering the surfaces of the Petri dishes within five days. The aerial mycelium was initially white, turning dark greenish or greyish after four to five days at 25 °C under near UV-light. It was not possible to identify the isolates to species level based on conidial or culture morphology.

3.2. DNA extraction and PCR amplification

A total of 47 isolates were selected for ITS sequencing to represent the four groups identified based on conidial morphology, hosts and areas sampled. These comprised 27 from Groups 1 and 2, and 20 from Groups 3 and 4. After ITS characterization, 27 isolates were selected for *tef 1- α* sequencing and three isolates for *β-tub* sequencing. DNA extraction and PCR was conducted successfully for all gene regions selected. PCR fragments for the ITS were ~ 580 bp, for *tef 1- α* they were 710 bp and for the *β-tub* gene region they were 440 bp.

3.3. Phylogenetic analyses

Combined ITS and tef 1- α analyses. The partition homogeneity test showed no significant conflict between the ITS and *tef 1-α* partitions (P = 0.103) suggesting that the data sets could be combined. The combined dataset consisted of 72 isolates with 900 characters of which, 388 characters were constant, 146 characters were parsimony uninformative and 366 characters were parsimony informative. Gaps were treated as a fifth character. After heuristic

searches, 42 most parsimonious trees were obtained (Tree length (TL) = 1053; Consistency index (CI) = 0.752, Retention index (RI) = 0.963, Rescaled consistency index (RC) = 0.724 TreeBase Accession No. SN4634) and one of these was chosen for presentation (Figure 2). All 42 trees displayed the same overall topology, with slightly different arrangements of isolates within the terminal clades. The topology of the tree generated from the combined analyses with MP, as well as with the 50 % majority rule consensus tree, was congruent with the trees obtained with the individual analyses of *tef 1-α* and ITS (data not shown).

Isolates from *Terminalia* spp. grouped with seven previously described species corresponding to four genera, namely *Neofusicoccum, Lasiodiplodia, Pseudofusicoccum* and *Diplodia*. All these clades were strongly supported with Bootstrap and Bayesian posterior probability values (Figure 2).

The *Neofusicoccum* clade included isolates obtained from *Terminalia* spp. in South Africa only and represented four sub-clades (Figure 2). Three of these sub-clades represented the previously described species *N. parvum, N. kwambonambiense* Pavlic, Slippers, & M.J. Wingf. and *N. vitifusiforme* (Niekerk & Crous) Crous, Slippers & A.J.L. Phillips. The isolates residing in the fourth clade (BS = 87 % and BPP = 0.99), did not group with any known *Neofusicoccum* sp., suggesting that it represented an undescribed species. This clade clustered close to *N. ursorum* Maleme, Pavlic, Slippers. with three and two fixed nucleotide differences in their ITS and *tef 1- α* sequences, respectively (Table 3). No sequence variation was observed between isolates from *Terminalia* spp. and those from GenBank in any group other than in the *N. parvum* cluster, where a single fixed nucleotide difference was seen in the ITS sequence.

Three main clades within *Lasiodiplodia* accommodated isolates from *Terminalia* spp. These were *L. mahajangana* Begoude, Jol. Roux, Slippers., *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous and *L. crassispora* Burgess, Barber. No sequence variation was observed in the ITS region for isolates from *Terminalia* spp. and isolates from GenBank for any of these groups. However, based on *tef 1-α* sequence data, and despite the high statistical values supporting the group containing isolates of *L. pseudotheobromae,* a sub-structure made up of two isolates was observed in the clade. This had two base pairs separating these isolates from other isolates of *L. pseudotheobromae*. Another clade (BS = 78 % and BPP = 0.93)

accommodating an apparently undescribed species, nested alone between *L. mahajangana* and *L. theobromae.* Ten and six nucleotide modifications respectively were observed between isolates from *Terminalia* spp. and representatives of *L. mahajangana* and *L. theobromae* (Table 4).

The remaining isolates from *Terminalia* spp. formed single clades with *D. alatafructa* J.W.M. Mehl & B. Slippers (BS = 100 % and BPP = 0.96) and *P. olivaceum* J.W.M. Mehl & B. Slippers (BS = 99 % and BPP = 0.97), respectively. The isolates included in the *Diplodia* clade originated from South Africa only, whereas those included in the *Pseudofusicoccum* clade originated from South Africa and Namibia. Pairwise sequence comparisons revealed no divergence in the ITS sequence and minor divergence in the *tef 1-α* sequence (one to two nucleotides) between isolates.

Additional analyses: To resolve uncertainties in the relationships between the seven taxa in the clade containing *L. mahajangana, L. theobromae* and isolates from *Terminalia* spp. (CMW26709, CMW26699, CMW26710) (Figure 2), individual and collective analyses were conducted using part of the ITS, *tef 1- α* and *β-tub* gene regions (Table 4). The tree topologies obtained from MP analyses were identical to those obtained in Bayesian analyses for each partition separately, as well as in combination. While no differences were observed in ITS sequences between isolates from *Terminalia* spp. and those representing *L. mahajangana* (Table 4) (Figure 3a), the separation of the apparently undescribed isolates in this clade was consistently observed in individual partitions of both *tef 1- α* and *β-tub* (Figure 3b, 3c). These regions contained two unique fixed polymorphisms each. Furthermore, a better resolution in the relationship of these isolates was provided after combining the data from each partition into one phylogenetic analysis.

The Incongruence Length Difference (Farris *et al.* 1995) calculated for all the data, related to all the isolates included in the clade (I = 0), indicated that the gene phylogenies were congruent (BS = 89 %; BPP = 0.90) for the consensus tree obtained from both MP and Bayesian analyses (Figure 3d) of the combined data set. These analyses clearly showed that isolates from *Terminalia* spp. consistently formed a sub-clade distinct from *L. theobromae* and *L. mahajangana,* confirming previous observations from the *tef 1-α* gene region.

3.4. Taxonomy

DNA sequence data for the ITS, *tef 1-α* and *β-tub* gene regions distinguished two previously undescribed species of Botryosphaeriaceae from amongst the isolates collected from *T. sericea* and *T. sambesiaca* in this study. The morphology of these isolates was studied and this confirmed that they represent previously undescribed species, for which we provide the following descriptions:

Lasiodiplodia cryptotheobromae Begoude, Jol. Roux, Slippers, sp. nov. MB515130 FIGURE 4.

Etymology: the name refers to the fact that this is a cryptic species closely related to *L. theobromae*.

Conidiomata pycnidialia usque ad 420.0 µm diametro, in foliis pini in MEA in 14 diebus facta, nigra solitaria mycelio tecta subimmersa conica. *Paraphyses* inter cellulas conidiogenas, cylindricae hyalinae. *Cellulae conidiogenae* holoblasticae discretae hyalinae cylindricae percurrente proliferantes ita incrassationem periclinalem formantes. *Conidia* primo non septata, hyalina ellipsoidea vel ovoidea, parietibus crassis (< 2.5 µm), contentis granularibus, liberata semel septata, colorata, dum matura strias verticales visas, 24.86 x 14.02 µm.

Conidiomata: pycnidial (up to 420.0 µm diam.), produced on sterilized pine needles on MEA within 14 days, black, solitary and covered by mycelium, semi-immersed, conical. *Paraphyses*: formed among conidiogenous cell, cylindrical, hyaline, 1-2 septate (38.5-) 49.0-73.5 (-66.0) x (2.0-) 2.5-3.0 (-3.5) µm, (average 50 paraphyses 61.29 x 2.61 µm), rounded at the tips, unbranched. *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, discrete, hyaline, cylindrical, proliferating percurrently to form a periclinal thickening (14.0-) 15.5-23.0 (-28.0) x (2.5-) 3.0-4.5 (-5.0) µm (average 50 conidiogenous cells 19.04 x 3.76 µm). *Conidia*: initially aseptate, hyaline, ellipsoid to ovoid, thick-walled (< 2.5 µm), granular content, becoming one-septate and pigmented after release, vertical striations observed at maturity, (17.0-) 22.5-27.5 (-29.0) x (10.5-) 13.0-15.0 (-16.0) µm (average 50 conidia 24.86 x 14.02 µm, l/w 1.7). *Culture characteristics*: white fluffy and

abundant aerial mycelium, becoming pale olivaceous grey (23’’’’’f) after 4 days, with the reverse sides of the colonies olivaceous grey (23’’’’’b). *Optimum temperature for growth* 30 °C, with a growth rate of 30 mm/day on MEA in the dark, no growth observed at 10 °C.
Teleomorph: not observed
Host: *Terminalia sambesiaca, Terminalia sericea.*
Distribution: Ifakara, Tanzania; Bagani, Namibia.
Specimen examined: TANZANIA, IFAKARA: isolated from healthy branches of *Terminalia sambesiaca*, April 2007, collected by J. Roux, Holotype (PREM 60319) a dried culture of isolate CMW26699 = CBS125262 on pine needles; ex-Holotype (PREM 60315) representing dried culture of isolate CMW26710 = CBS125267.
Additional specimens: TANZANIA, IFAKARA: isolated from healthy branches of *Terminalia sambesiaca*, April 2007, collected by J. Roux, Paratype (PREM 60318) representing a dried culture of isolate CMW26709 = CBS125266.

Neofusicoccum terminaliae Begoude, Jol. Roux, Slippers, sp. nov. MB515131
FIGURE 5.

Etymology: The name refers to the host from which the type specimen was isolated.

Conidiomata pycnidialia in foliis pini in 14 diebus facta, solitaria vel aggregata mycelio tecta primo immersa, matura ¾ erumpentia, papillata nigra usque ad 2100.0 µm longa 1300.0 µm diametro. *Paraphyses* abundantes cylindricae hyalinae non septatae. *Conidiophorae* ad cellulas conidiogenas reductae. *Cellulae conidiogenae* holoblasticae hyalinae percurrente proliferantes interdum incrassationem periclinalem formantes, laeves conidium unicum facientes. *Conidia* non vel ad ter septata, hyalina laevia fusiformia parietibus tenuibus apice rotundata 20.02 x 6.84 µm.

Conidiomata: pycnidial produced on sterilized pine needles within 14 days, solitary to aggregate and covered by mycelium, initially embedded, ¾ erumpant through the pine needles at maturity, papillate, black, up to 2100.0 µm in length and 1300.0 µm diam. *Paraphyses*: abundant, cylindrical, hyaline, aseptate (27.0-) 32.5-52.5 (-64.0) x (1.5-) 2.0-2.5 (-3.0) µm, (average 50 paraphyses 42.61 x 2.37 µm). *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, hyaline, cylindrical, proliferating

percurrently, sometimes forming a periclinal thickening, smooth producing a single conidium, (11.5-) 15.5-24.0 (-30.0) x (1.5-) 2.0-2.5 (-3.0) µm (average of 50 conidiogenous cells (19.6 x 2.4 µm). *Conidia*: aseptate to 3-septate, hyaline, smooth, fusiform, thin-walled, round at apex (14.5-) 18.0-22.5 (-25.0) x (3.5-) 6.0-7.5 (-8.5) µm (average 50 conidia 20.02 x 6.84 µm, l/w 2.9). *Culture characteristics*: colonies initially white, becoming olivaceous grey (21''''b) from the centre after seven days. Aerial mycelium dense, fluffy, edge smooth to crenalate. *Optimum temperature for growth* 25 °C, with a growth rate of 22.5 mm/day on MEA in the dark, little growth observed at 10 °C.

Teleomorph: not observed

Host: *Terminalia sericea*

Distribution: Moloto, Gauteng Province, South Africa.

Specimen examined: SOUTH AFRICA, MOLOTO: isolated from healthy branches of *Terminalia sericea*, Jan 2007, collected by D. Begoude and J. Roux, Holotype (PREM 60316), a dried culture of isolate CMW26683 = CBS125264 on pine needles; ex-Holotype (PREM 60314), representing dried culture of isolate CMW26679; ex-paratype culture CMW26679 = CBS125263.

Additional specimens: SOUTH AFRICA, MOLOTO: isolated from healthy branches of *Terminalia sericea*, Feb 2007, collected by D. Begoude and J. Roux. ex-paratype culture CMW26687; CMW26685 = CBS125265.

3.5. Pathogenicity

All inoculations with isolates of Botryosphaeriaceae collected in this study resulted in visible lesions on the bark and cambium of *T. sericea* trees after six weeks. Analysis of variance showed that there were significant differences in the level of pathogenicity between species ($P < 0.0001$). *L. pseudotheobromae, N. parvum, L. cryptotheobromae*, *L. crassispora* and *N. terminaliae* produced the longest lesions in both the bark and cambium, whereas *N. vitifusiforme, D. alatafructa* and *P. olivaceum* produced the smallest lesions (Figure 6). Considerable variation in levels of pathogenicity was also observed among isolates of the same species. There was a positive correlation (R^2 = 89 %) between lesions produced on the bark and those in the cambium. Isolations from lesions on the inoculated trees resulted in recovery of the inoculated fungi, confirming that they were the cause of the lesions.

4. DISCUSSION

This study represents the first attempt to characterize species of the Botryosphaeriaceae on native *Terminalia* spp. in Southern Africa. Nine species, corresponding to four genera, were identified from *T. sericea* and *T. sambesiaca*. Seven of these, *L. crassispora, L. pseudotheobromae, D. alatafructa, P. olivaceum, N. parvum, N. kwambonambiense* and *N. vitifusiforme,* represent previously described species and two, *L. cryptotheobromae* and *N. terminaliae,* were described as new. *L. pseudotheobromae* and *N. parvum* have previously been reported from *Terminalia* spp. (Begoude *et al*. 2009a,b) and the remaining species are recorded on *Terminalia* spp. for the first time.

Three species of *Lasiodiplodia* were identified in this study. These are *L. pseudotheobromae*, *L. crassispora* and the newly described *L. cryptotheobromae*. The taxonomy of species in *Lasiodiplodia* has undergone considerable change in recent years. The best known species, *L. theobromae,* was originally described as *Botryodiplodia theobromae* Pat. from cacao fruit in Ecuador (Patouillard and Lagerheim 1892). Later, Griffon and Maublanc (1909) renamed this species in *Lasiodiplodia* (Goos *et al*. 1961). Subsequently, all collections of fungi in the group with dark conidia, becoming striated with age, were treated as *L. theobromae*. This resulted in *L. theobromae* representing a pleomorphic, plurivorous group of fungi with a global geographic distribution and a very wide host range (Punithalingam 1976). The advent of DNA sequence comparisons to define species boundaries has shown that *L. theobromae* represents a relatively large group of distinct species. To date, 10 species, previously grouped with *L. theobromae*, have been described using DNA sequence comparisons (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Begoude *et al.* 2009a). In the current study, it was thus necessary to use DNA sequence data for multiple gene regions to distinguish *Lasiodiplodia* spp. collected from *Terminalia* spp.

Lasiodiplodia cryptotheobromae is described here based primarily on evidence from DNA sequence comparisons. *L. cryptotheobromae* is phylogenetically most closely related to *L. theobromae* and *L. mahajangana*. Although ITS sequences for both *L. cryptotheobromae* and *L. mahajangana* were identical, considerable sequence variation was observed across *tef 1-α* and *β-tub* gene regions, providing support for their distinction as distinct species. Eighteen fixed, unique, single nucleotide polymorphisms (SNPs) out of 23 informative characters

across the three gene regions differentiated these species. Morphologically, conidial measurements of *L. crytotheobromae* overlapped with those of *L. theobromae*, but they were larger than those of *L. mahajangana*. Moreover, septate paraphyses were observed in *L. cryptotheobromae,* similar to *L. theobromae*. It is thus not possible to distinguish *L. cryptotheobromae* from *L. theobromae* based only on morphology.

Isolates of *L. cryptotheobromae* were found as endophytes in twigs of healthy *T. sericea* and *T. sambesiaca* collected in Namibia and Tanzania. However, pathogenicity tests on branches of *T. sericea* trees showed that this fungus was among the most pathogenic species. This suggests that it could be a pathogen of these trees, for example if it were introduced into an area where it does not occur naturally, or when the trees are under stress. This is consistent with the fact that a number of species in the Botryosphaeriaceae are known as virulent pathogens of trees under environmental stress (Schoeneweiss 1981; Mullen *et al.* 1991; Smith *et al.* 1994; Ahimera *et al.* 2003).

Four isolates of *L. crassispora* were found on healthy twigs of *T. sericea* and *T. sambesiaca* collected in South Africa, Botswana and Tanzania. This fungus was first reported from *Santalum album* and *Eucalyptus urophylla* in Australia and Venezuela (Burgess *et al.* 2006). Its range has remained restricted to these hosts and localities until recently, when it was found on *Pterocarpus angolensis*. in South Africa (Mehl *et al*. 2009). Results of the present study have, therefore, substantially increased the host range and geographic distribution of *L. crassispora*. Conidial dimensions of isolates from *Terminalia* spp. were very similar to those of the type isolate. Judging from the length of lesions produced on branches of *T. sericea* trees, *L. crassispora* could be considered as a potential pathogen of these trees.

Lasiodiplodia pseudotheobromae was commonly collected from healthy twigs of *T. sericea* and *T. sambesiaca* in Namibia and Tanzania. Previously, it has been reported from *T. catappa* in Madagascar, South Africa and Cameroon (Begoude *et al.* 2009a), *T. ivorensis, T. superba* and *T. mantaly* in Cameroon (Begoude *et al.* 2009b) as well as on various genera of woody plants in other localities (Alves *et al.* 2008). Isolates from Southern Africa displayed slight divergences in *tef 1-α* sequences and clustered as a sub-clade within the larger *L. pseudotheobromae* clade. However, these minor differences were considered to

represent normal variation within the species, similar to that described previously for *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (Lazzizera *et al*. 2008a). Pathogenicity trials showed that *L. pseudotheobromae* was consistently the most virulent species among those Botryosphaeriaceae tested in this study. This is similar to results of other studies, such as those on *P. angolensis* (Mehl *et al*. 2009) and *T. catappa* and *T. mantaly* (Begoude *et al.* 2009a,b), where *L. pseudotheobromae* was found to be highly virulent. Even though these results stemmed from artificial inoculations, the consistently high levels of pathogenicity of *L. pseudotheobromae* on different hosts and in different locations add to the view that this is an important pathogen of trees.

Neofusicoccum species represented the most abundant and the most diverse group of fungi identified from *Terminalia* spp. in Southern Africa. Four species were collected from *T. sericea* and *T. sambesiaca*. These included the previously undescribed *N. terminaliae*, which was found from asymptomatic *T. sericea*. Based on the phylogeny inferred from MP analyses of individual and combined ITS and *tef 1-α* gene regions, isolates representing *N. terminaliae* consistently resided in a single and well supported clade. The morphological features of this species are consistent with those described for other members of the genus, such as fusoid, hyaline, elongate and thin-walled conidia (Crous *et al.* 2006). The closest relative of *N. terminaliae* is the newly described species *N. ursorum* (Maleme 2008) with which three and two nucleotide differences in the ITS and *tef 1-α* sequences were respectively observed. Morphologically, *N. terminaliae* can be distinguished from *N. ursorum* by its wider pycnidia, the presence of paraphyses and up to 3-septate conidia. Similar to many members of the Botryosphaeriaceae, the presence of *N. terminaliae* on healthy tissue confirmed its endophytic nature. However, *N. terminaliae* produced lesions on branches of *T. sericea* in pathogenicity trials showing that it has the capacity to cause disease.

Two species were identified in this study in what is known as the *N. parvum / N. ribis* complex. This complex, which originally consisted of two species, has recently been amended based on multiple gene genealogies, and now includes six species (Begoude *et al.* 2009a; Pavlic *et al.* 2009a,b). The most commonly collected species from *Terminalia* spp. in this study was *N. parvum*, which accounted for 21 % of the total number of isolates. *N. parvum* was, however, collected only from healthy twigs of *T. sericea* in South Africa and not

from any of the other areas surveyed. *N. parvum* has been found abundantly on non-native *Eucalyptus* trees and on native *S. cordatum* in previous studies conducted in South Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007). Very recently, the fungus was also reported from non-native *T. catappa* in South Africa (Begoude *et al.* 2009a). The high levels of occurrence on both introduced and native hosts in South Africa has led to suggestions that *N. parvum* might be native to the country (Pavlic *et al.* 2007; 2009b; Begoude *et al.* 2009a) and the results of this study add credence to this view. In this study *N. parvum* was able to produce lesions in pathogenicity trials. The pathogenicity of *N. parvum* has previously been recognized on native *S. cordatum* and non-native *T. catappa* and *Eucalyptus* trees in Southern Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a). This remarkable association of *N. parvum* with native and non-native hosts in South Africa supports evidence for movement of the pathogen between native and non-native tree hosts in this area (Strauss 2001; Slippers *et al.* 2005). Further investigations on populations of *N. parvum* from native and non-native hosts are needed to clarify the question of origin of this fungus.

A small number of isolates from *T. sericea* in South Africa were identified as *N. kwambonambiense*. This fungus was one of the first phylogenetic species described in the Botryosphaeriaceae using sequence differences as the most important defining characteristics (Pavlic *et al.* 2009b). In that study, it was sister to *N. ribis* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips that was found on native *S. cordatum* in South Africa. In the present study, isolates representing *N. kwambonambiense* were isolated as endophytes from native *T. sericea* in South Africa and this represents the second record of this fungus on a native South African tree species. Its presence in this niche suggests that the fungus is most likely native to South Africa.

Neofusicoccum vitifusiforme, collected in this study from a native tree, was first described from South Africa when it was discovered on *Vitis vinifera* (van Niekerk *et al.* 2004). Interestingly, it has not previously been found on a forest tree host and other reports of the fungus are from *Prunus salicina* Lindell. and *P. persica* trees in South Africa (Damm *et al.* 2007) and on olive drupes in Italy (Lazzizera *et al.* 2008b). In their study, Lazzizera *et al.* (2008b) found that isolates of *Botryosphaeria* studied by Barber *et al.* (2005) clustered with ex-type cultures of *N. vitifusiforme* and subsequently concluded that *Dichomera*

eucalypti is the synanamorph of *N. vitifusiforme.* At the time of its identification, *N. vitifusiforme* was considered as a weak pathogen restricted to *V. vinifera* (van Niekerk *et al.* 2004). Our consideration of its pathogenicity on branches of *T. sericea* trees also showed that it is not a pathogen.

Three isolates found in this study clustered with each of *D. alatafructa* and *P. olivaceum.* Both fungi were previously isolated from native *P. angolensis* trees in South Africa, *P. olivaceum* from asymptomatic branches and *D. alatafructa* from branch lesions on this host (Mehl *et al.* 2009). Of particular interest is the fact that some of the isolates obtained from *T. sericea* in South Africa were found at the same location as the reference strains. The fact that *D. alatafructa* and *P. olivaceum* occurred on two native hosts in the same area suggests that these species are most likely native to South Africa. They do not appear to be pathogenic and are currently of little concern in terms of the health of *T. sericea* in South Africa.

The inoculation trials conducted on branches of healthy *T. sericea* trees in this study tested the ability of the mycelium of the Botryosphaeriaceae to infect living plant tissue. In terms of pathogenicity, the species tested fell into one of two groups. These accommodated the potentially pathogenic species that produced distinct lesions on the branches. These were, in order of decreasing virulence, *L. pseudotheobromae, N. parvum, L. cryptotheobromae*, *L. crassispora* and *N. terminaliae*. The second group included species with low levels of pathogenicity, including *N. vitifusiforme, D. alatafructa* and *P. olivaceum.* There was considerable variation in the levels of pathogenicity for different isolates of some species. For example, in the case of *L. pseudotheobromae* and *L. crassispora*, isolates CMW26702 and CMW26688 produced lesions that were three times as large as those produced by the other isolates of these species. Similar variation in pathogenicity amongst isolates of the same species has previously been reported for *N. australe* and *Diplodia seriata* on grapevine (Larignon *et al.* 2001; van Niekerk *et al.* 2004; Taylor *et al.* 2005) and for *B. dothidea* on apple fruit (Latorre and Toledo 1984; Parker and Sutton 1993). This suggests that pathogenicity data for the Botryosphaeriaceae must be interpreted with some circumspection. In addition, this group of fungi are known to be associated with conditions of stress (Smith *et al.* 1994; Ahimera *et al.* 2003) and the impact of the environment of the trees being studied should also be considered.

A large number of taxonomic lineages of the Botryosphaeriaceae were found on two species of native *Terminalia* in Southern Africa. This number was as high as previous surveys of the Botryosphaeriaceae on other native hosts in subtropical and temperate areas in Southern Africa and Australia (Pavlic *et al.* 2008; Mehl *et al.* 2009; Taylor *et al.* 2009; van der Walt 2008).The high diversity of species found on native trees supports the views of Taylor *et al.* (2009) that there is a need to extend knowledge regarding fungi on native trees. Furthermore, some of these fungi could be important pathogens of economically important crops with the native trees serving as a source of inoculum (Pavlic *et al.* 2009b). It is, therefore, important to promote studies in natural ecosystems to better understand the diversity of these environments.

5. REFERENCES

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Table 1. Conidial measurements of species of the Botryosphaeriaceae isolated from *Terminalia* spp. in Southern Africa and comparison with those reported in previous studies.

	Conidial size (µm)		Source of data
Species	This study	Previous studies	
N. parvum	(15.5-)16.5-20.5(-21.5) x (4.5-)6.0-7.5(-8.0)	(12-)15-19(-24) x 4-6	Slippers *et al.* 2004
N. kwambonambiense	(16.0-)20.5-21.0(-22.0) x (5.0-)4.5-7.5	16-28 x 5-8	Pavlic *et al.* 2009
N. terminaliae	(14.5-)18.0-22.5(-25.0) x (3.5-)6.0-7.5(-8.5)		This study
N. vitifusiforme	(14.5-)18.0-21.0(-24.5) x (4.0-)4.5-8.5	(18-)19-21(-22) x (4.5-)5.5-6.5(-8)	van Niekerk *et al.* 2004
P. olivaceum	(20.0-)22.0-25.5(-27.5) x (5.0-)5.5-7.0(-8.0)	(17.9-)19.9-25.7(-30.4) x (5.9-)6.3-7.7(-8.9)	Mehl *et al.* 2009
L. crassispora	(19.5-)23.0-28.5(31.5) x (15.0-)16.0-18.5(-20.5)	27-30(-33) x 14-17	Burgess *et al.* 2006
L. cryptotheobromae	(17.0-)22.5-27.5(-29.0) x (10.5-)13.0-15.0(-16.0)		This study
L. pseudotheobromae	(21.5-)24.5-27.0(30.5) x (12.0-)13.5-15.5(-16.5)	(22.5-)23.5-31(-32.5) x (12-) 13-15.5(-18.5)	Alves *et al.* 2008
D. alatafructa	(20.0-)22.0-25.5(-27.5) x (5.0-)5.5-7.0(-8.0)	(22.4-)24.6-29.2(-32.9) x (9.3-)11-13.8(-15.8)	Mehl *et al.* 2009

Table 2. Botryosphaeriaceae used for phylogenetic analyses in this study.

Species	Culture number	Origin	Host	Collectors	Genbank Accession No.		
					ITS	*tef* 1-α	*β-tub*
Diplodia alatafructa	CMW 22627	South Africa	*Pterocarpus angolenssis*	J. Mehl/J. Roux	FJ888460	FJ888444	
	CMW 22721	South Africa	*P. angolensis*	J. Mehl/J. Roux	FJ888478	FJ888446	
	CMW 26854	South Africa	*Terminalia sericea*	D. Begoude	GQ471835	GQ471793	
	CMW 26840	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471834	GQ471795	
	CMW 26838	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471833	GQ471794	
D. seriata	CMW 7774	USA	*Ribes* sp.	B. Slippers/G. Hudler	EF445343	EF445382	
	CMW 7775	USA	*Ribes* sp.	B. Slippers/G. Hudler	EF445344	EF445383	
Guignardia mangiferae	1095	Panama	*Theobroma cacao*	E. Rojas/L. Mejia/Z. Maynard	EU683671	EU683652	
Lasiodiplodia crassispora	CBS 118741	Venezuela	*Eucalyptus urophylla*	S. Mohali	DQ103552	DQ103556	
	CBS 110492	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103551	DQ103558	
	CMW 26688	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471822	GQ471775	
	CMW 26698	Botswana	*T. sericea*	J. Roux	GQ471823	GQ471776	
L. cryptotheobromae	**CMW 26699**	Tanzania	*Terminalia sambesiaca*	J. Roux	GQ471812	GQ471778	GQ471773
	CMW 26709	Tanzania	*T. sambesiaca*	J. Roux	GQ471814	GQ471777	GQ471772
	CMW 26710	Tanzania	*T. sambesiaca*	J. Roux	GQ471813	GQ471779	GQ471774
L. gonubiensis	CBS 115812	South Africa	*Syzygium cordatum*	D. Pavlic	DQ458892	DQ458877	
	CBS 116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567	
L. mahajangana	CMW 27801	Madagascar	*T. catappa*	J. Roux	FJ900595	FJ900641	FJ900630
	CMW 27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642	FJ900631
L. margaritacea	CMW 26162	Australia	*Adansonia gibbosa*	D. Pavlic	EU144050	EU144065	
	CMW 26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066	

L. parva	CBS 356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF622082	EF622062	
	CBS 494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064	
L. plurivora	STEU-5803	South Africa	*Prunus salicina*	U. Damm	EF445362	EF445395	
	STEU-4583	South Africa	*Vitis vinifera*	F. Halleen	AY343482	EF445396	
L. pseudotheobromae	**CMW 26702**	Tanzania	*T. sambesiaca*	J. Roux	GQ471830	GQ471796	
	CMW 26724	Namibia	*T. sericea*	J. Roux	GQ471832	GQ471797	
	CMW 26695	Namibia	*T. sericea*	J. Roux	GQ471831	GQ471798	
	CBS 116459	Costa Rica	*Gmelina arborea*	J. Carranza/Velásquez	EF622077	EF622057	
	CBS 447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060	
L. rubropupurea	WAC 12535	Australia	*E. grandis*	T.I. Burgess/G. Pegg	DQ103553	DQ103571	
	WAC 12536	Australia	*E. grandis*	T.I. Burgess/G. Pegg	DQ103554	DQ103572	
L. theobromae	CMW 9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054	AY236930
	CBS 164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258	EU673110
L. venezuelensis	WAC 12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568	
	WAC 12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569	
Neofusicoccum batangarum	CMW 28315	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900606	FJ900652	
	CMW 28363	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900607	FJ900653	
N. cordaticola	CMW 13992	South Africa	*S. cordatum*	D. Pavlic	EU821898	EU821868	
	CMW 14056	South Africa	*S. cordatum*	D. Pavlic	EU821903	EU821873	
N. kwambonambiense	CMW 14023	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870	
	CMW 14025	South Africa	*S. cordatum*	D. Pavlic	EU821901	EU821871	
	CMW 26850	South Africa	*T. sericea*	D. Begoude	GQ471846	GQ471799	
	CMW 26856	South Africa	*T. sericea*	D. Begoude	GQ471843	GQ471800	
	CMW 26865	South Africa	*T. sericea*	D. Begoude	GQ471845	GQ471801	
N. parvum	CMW 9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888	
	CMW 9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885	
	CMW 26690	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471815	GQ471787	
	CMW 26851	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471817	GQ471788	

	CMW 26844	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471819	GQ471789
N. ribis	CMW 7772	USA	*Ribes* sp.	B. Slippers/G. Hudler	AY236935	AY236877
	CMW 7773	USA	*Ribes* sp.	B. Slippers/G. Hudler	AY236936	AY236878
N. terminaliae	**CMW 26679**	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471802	GQ471780
	CMW 26687	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471803	GQ471781
	CMW 26683	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471804	GQ471782
	CMW 26685	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471805	GQ471783
N. umdonicola	CMW 14106	South Africa	*S. cordatum*	D. Pavlic	EU821899	EU821869
	CMW 14058	South Africa	*S. cordatum*	D. Pavlic	EU821904	EU821874
N. ursorum	CMW 23790	South Africa	*Eucalyptus* sp.	H. M. Maleme	FJ752745	FJ752708
	CMW 24480	South Africa	*Eucalyptus* sp.	H. M. Maleme	FJ752746	FJ752709
N. vitifusiforme	STE-U 5252	South Africa	*V. vinifera*	J. M. van Niekerk	AY343383	AY343343
	STE-U 5820	South Africa	*Prunus salicina*	U. Damm	EF445347	EF445389
	CMW 26689	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471838	GQ471790
	CMW 26676	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471841	GQ471791
	CMW 26686	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471840	GQ471792
Pseudofusicoccum olivaceum	CMW 20881	South Africa	*P. angolensis*	J. Roux	FJ888459	FJ888437
	CMW 22637	South Africa	*P. angolensis*	J. Mehl/ J. Roux	FJ888462	FJ888438
	CMW 26673	South Africa	*T. sericea*	D. Begoude	GQ471806	GQ471784
	CMW 26824	South Africa	*T. sericea*	J. Roux	GQ471807	GQ471785
	CMW 26836	South Africa	*T. sericea*	J. Roux	GQ471808	GQ471786
P. stromaticum	CBS 117448	Venezuela	*Eucalyptus hybrid*	S. Mohali	AY693974	AY693975
	CBS 117449	Venezuela	*Eucalyptus hybrid*	S. Mohali	DQ436935	DQ436936

Table 3. Polymorphic nucleotides from sequence data of the ITS and *tef* 1-α gene regions for isolates representing two closely related *Neofusicoccum* spp.

Identity	**Culture number**	**ITS**			***tef* 1-α**	
		103	**132**	**141**	**61**	**78**
N. ursorum	CMW23790	T	G	T	T	T
	CMW24480	T	G	T	T	T
N. terminaliae	CMW26679	C	A	C	C	G
	CMW26687	C	A	C	C	G
	CMW26683	C	A	C	C	G
	CMW26685	C	A	C	C	G

Table 4. Polymorphic nucleotides from sequence data of the ITS, *tef* 1-α and *β-tub* gene regions for isolates of three *Lasiodiplodia* spp. Unique, fixed polymorphisms in each species are shaded.

Identity	**Culture number**	**ITS**		***tef* 1-α**								***β-tub***						
		46	**98**	**36**	**44**	**59**	**60**	**68**	**82**	**99**	**261**	**85**	**124**	**143**	**157**	**177**	**189**	**244**
L. theobromae	CBS164.96	C	C	C	A	C	1	A	C	C	G	C	C	G	T	T	A	C
	CMW9074	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. mahajangana	CMW27801	T	T	G	T	-	0	G	T	T	.	T	T	C	C	C	G	A
	CMW27818	T	T	G	T	-	0	G	T	T	.	T	T	C	C	C	G	A
L. cryptotheobromae	CMW26709	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.
	CMW26699	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.
	CMW26710	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.

Table 5. Sequence characteristics and phylogenetic information for ITS, *tef 1-α* and *β-tub* and combined data sets of *Lasiodiplodia* spp.

Data set	Sequence range	No. of variable sites	No. of informative sites	No. of most parsimonious trees	Tree length	Consistency index	Retention index
ITS	461	4	2	1	4	1	1
***tef* 1-α**	270	14	14	1	15	1	1
β-tub	422	7	7	1	7	1	1
Combined data	1153	25	23	1	26	1	1

Figure 1. Collection sites and distribution of species of the Botryosphaeriaceae collected from *Terminalia* spp. in Southern Africa

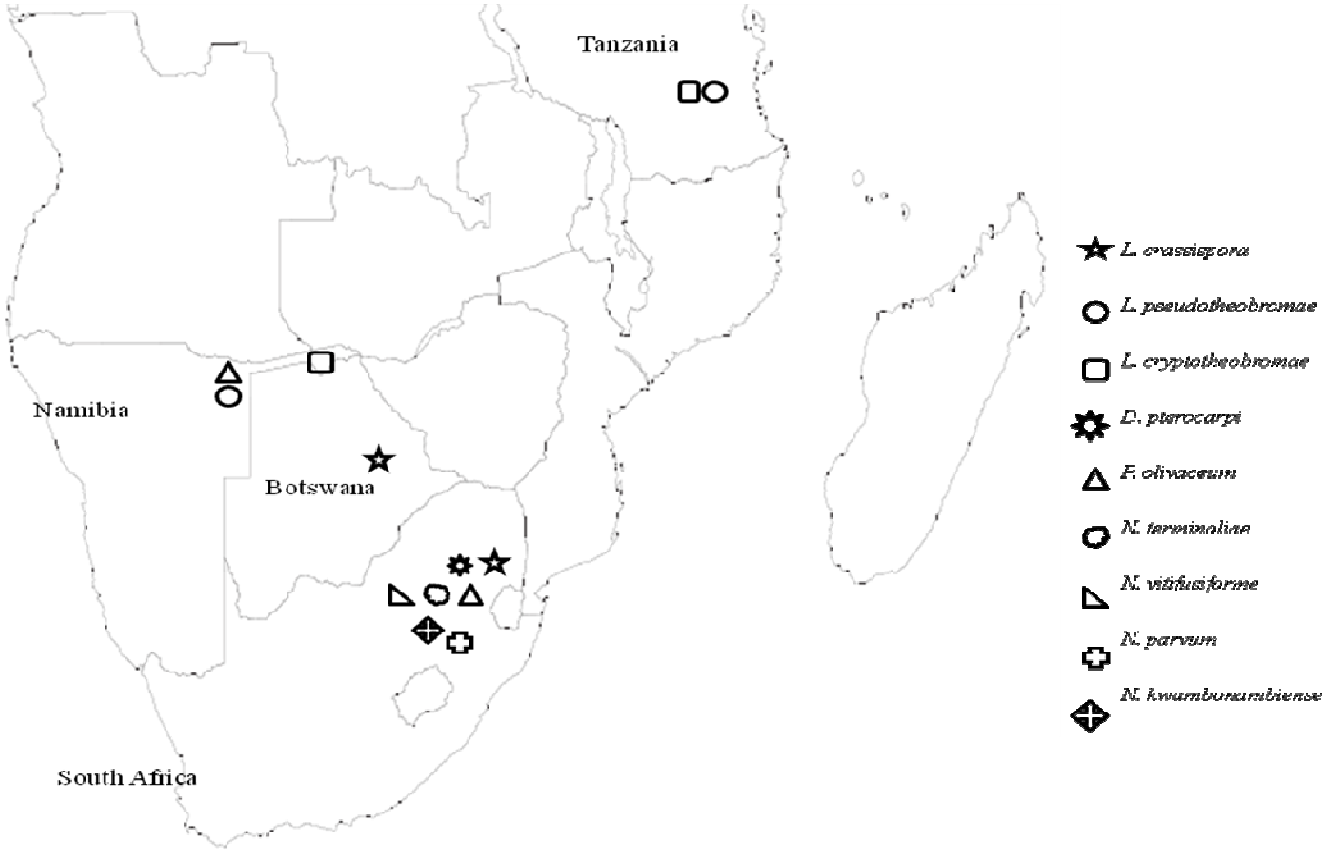

Tanzania
Namibia
Botswana
South Africa
L. ovassiepora
L. pseudotheobromae
L. cryptotheobromae
D. pterocarpi
F. olivaceum
N. terminalina
N. vitifusiforme
N. parvum
N. kwambonambiense

Figure 2. One of the most parsimonious trees obtained from MP analyses of the combined ITS and *tef* 1-α sequence data of the Botryosphaeriaceae from *Terminalia* spp. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (PP/BS). Isolates marked in bold represent those obtained from *Terminalia* spp.

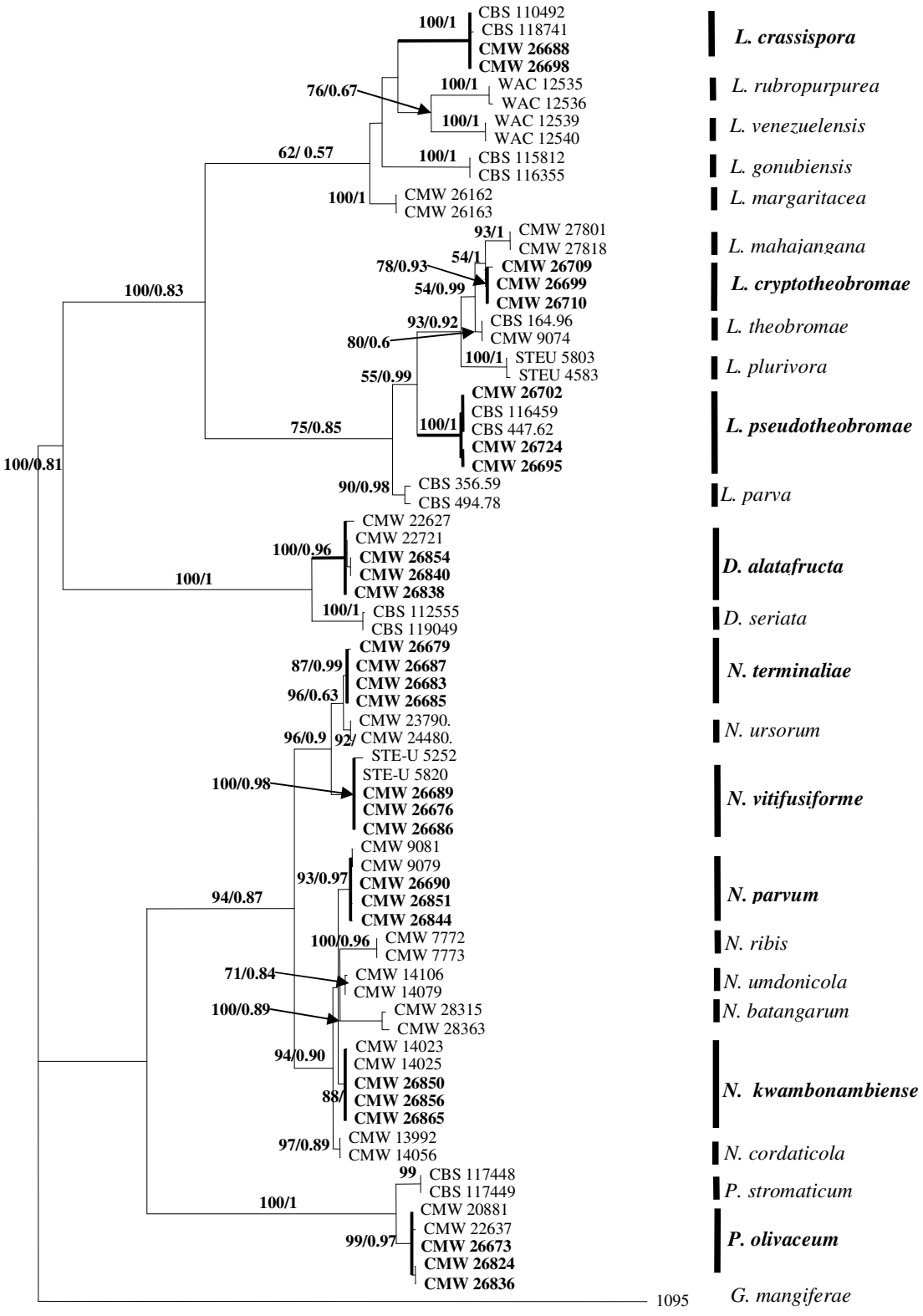
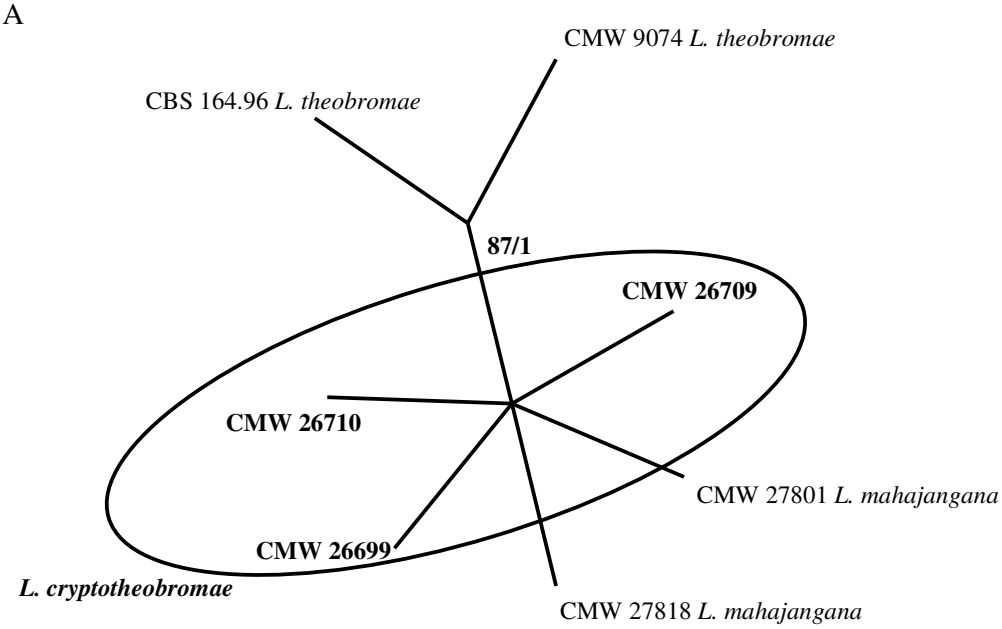
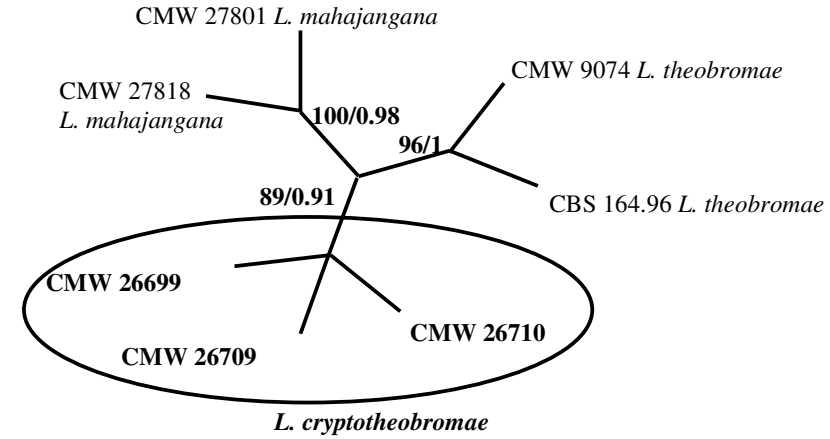

100/1
CBS 110492
CBS 118741
CMW 26688
CMW 26698
L. crassispora
76/0.67
100/1
WAC 12535
WAC 12536
L. rubropurpurea
100/1
WAC 12539
WAC 12540
L. venezuelensis
62/ 0.57
100/1
CBS 115812
CBS 116355
L. gonubiensis
100/1
CMW 26162
CMW 26163
L. margaritacea
93/1
CMW 27801
CMW 27818
L. mahajangana
54/1
78/0.93
CMW 26709
CMW 26699
CMW 26710
L. cryptotheobromae
100/0.83
54/0.99
93/0.92
CBS 164.96
CMW 9074
L. theobromae
80/0.6
100/1
STEU 5803
STEU 4583
L. plurivora
55/0.99
CMW 26702
CBS 116459
CBS 447.62
CMW 26724
CMW 26695
L. pseudotheobromae
75/0.85
100/1
100/0.81
90/0.98
CBS 356.59
CBS 494.78
L. parva
CMW 22627
CMW 22721
100/0.96
CMW 26854
CMW 26840
CMW 26838
D. alatafructa
100/1
100/1
CBS 112555
CBS 119049
D. seriata
CMW 26679
87/0.99
CMW 26687
CMW 26683
96/0.63
CMW 26685
N. terminaliae
CMW 23790.
96/0.9
92/
CMW 24480.
N. ursorum
STE-U 5252
100/0.98
STE-U 5820
CMW 26689
CMW 26676
CMW 26686
N. vitifusiforme
CMW 9081
CMW 9079
93/0.97
CMW 26690
CMW 26851
CMW 26844
N. parvum
94/0.87
100/0.96
CMW 7772
CMW 7773
N. ribis
71/0.84
CMW 14106
CMW 14079
N. umdonicola
100/0.89
CMW 28315
CMW 28363
N. batangarum
CMW 14023
94/0.90
CMW 14025
CMW 26850
88/
CMW 26856
CMW 26865
N. kwambonambiense
97/0.89
CMW 13992
CMW 14056
N. cordaticola
99
CBS 117448
CBS 117449
P. stromaticum
100/1
CMW 20881
CMW 22637
99/0.97
CMW 26673
CMW 26824
CMW 26836
P. olivaceum
1095
G. mangiferae
– 5 changes

Figure 3. Most-parsimonious unrooted trees inferred from independent analyses of each data set (A=ITS; B=*tef 1-α*; C=*β-tub*; D= combination of sequences of the three loci) of the isolates representing *L. theobromae, L. mahajangana* and *L. cryptotheobromae*. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (PP/BS).

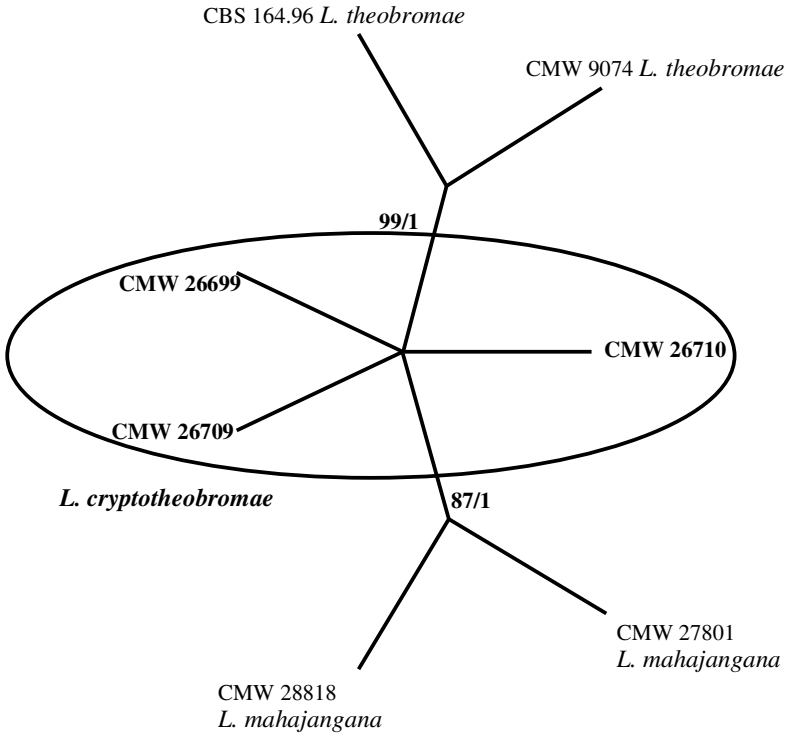
A


CMW 9074 L. theobromae
CBS 164.96 L. theobromae
87/1
CMW 26709
CMW 26710
CMW 27801 L. mahajangana
CMW 26699
L. cryptotheobromae
CMW 27818 L. mahajangana

B


CMW 27801 L. mahajangana
CMW 9074 L. theobromae
CMW 27818
L. mahajangana
100/0.98
96/1
89/0.91
CBS 164.96 L. theobromae
CMW 26699
CMW 26710
CMW 26709
L. cryptotheobromae

C


CBS 164.96 L. theobromae
CMW 9074 L. theobromae
99/1
CMW 26699
CMW 26710
CMW 26709
L. cryptotheobromae
87/1
CMW 27801
L. mahajangana
CMW 28818
L. mahajangana

D

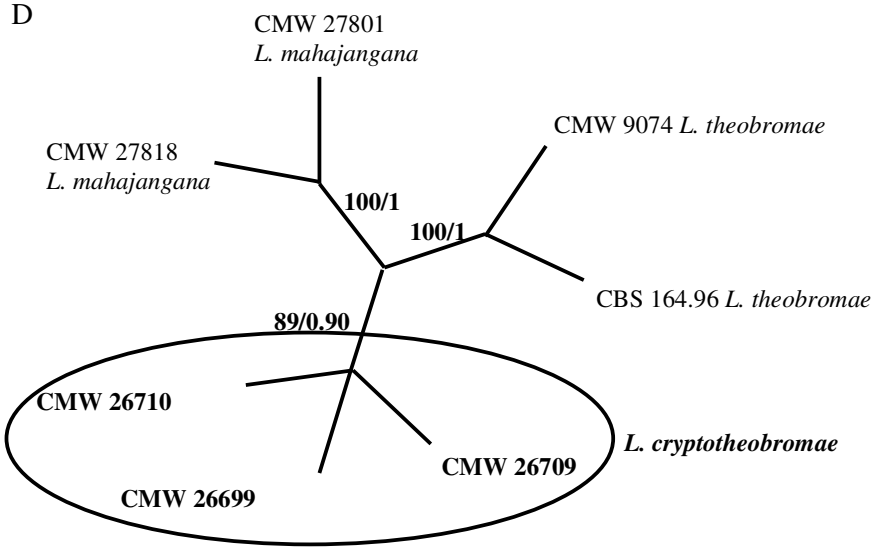

CMW 27801
L. mahajangana
CMW 9074 L. theobromae
CMW 27818
L. mahajangana
100/1
100/1
CBS 164.96 L. theobromae
89/0.90
CMW 26710
CMW 26709
L. cryptotheobromae
CMW 26699

Figure 4. *Lasiodiplodia cryptotheobromae.* (a) Pycnidium formed on pine needle in culture, (b, c) conidiogenous cells with developing conidia, (d) paraphyses, (e) young and mature conidia and (f) mature conidium showing striations. Bars: a = 200 µm; b, c, d, e, f = 10 µm.

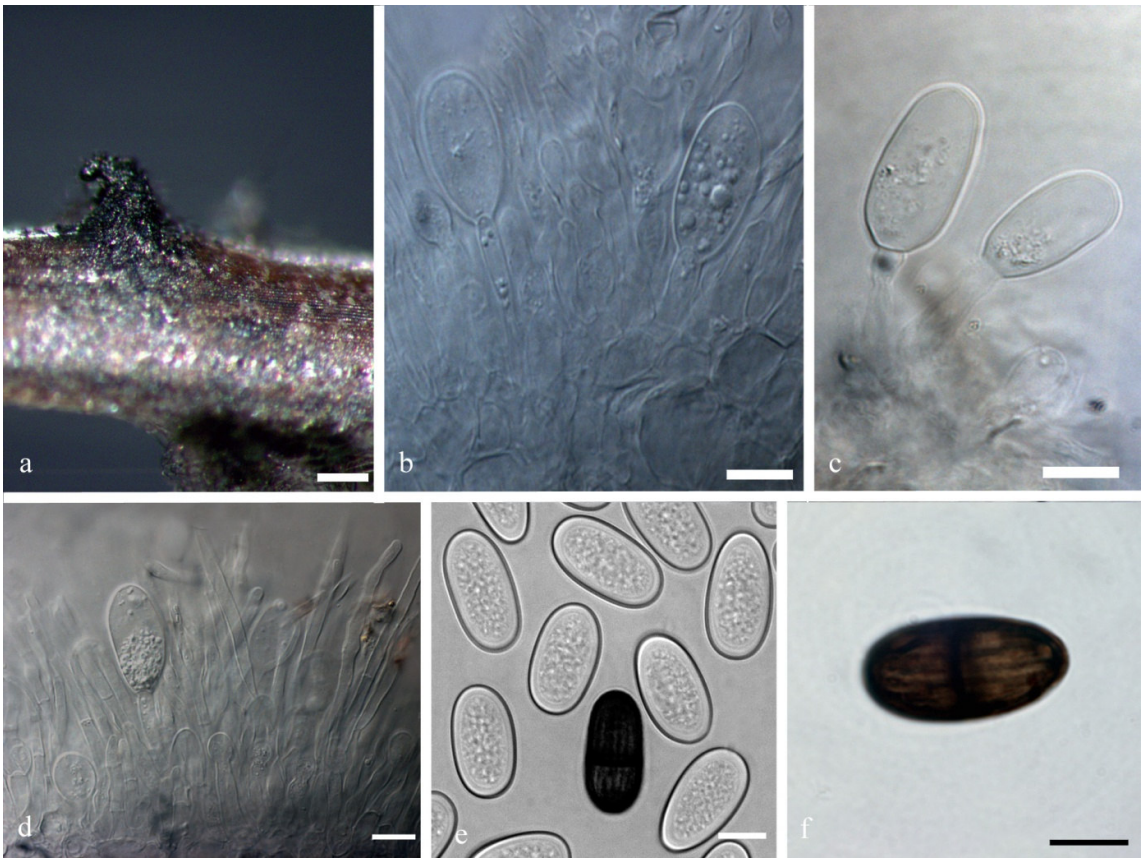

a
b
c
d
e
f

Figure 5. *Neofusicoccum terminaliae.* (a) Pycnidium formed on pine needles in culture, (b, c) conidiogenous cells with developing conidia, (d) paraphyses, (e) aseptate conidia and (f) 3-septate conidium. Bars: a = 200 µm; b, c, d, e, f = 10 µm.

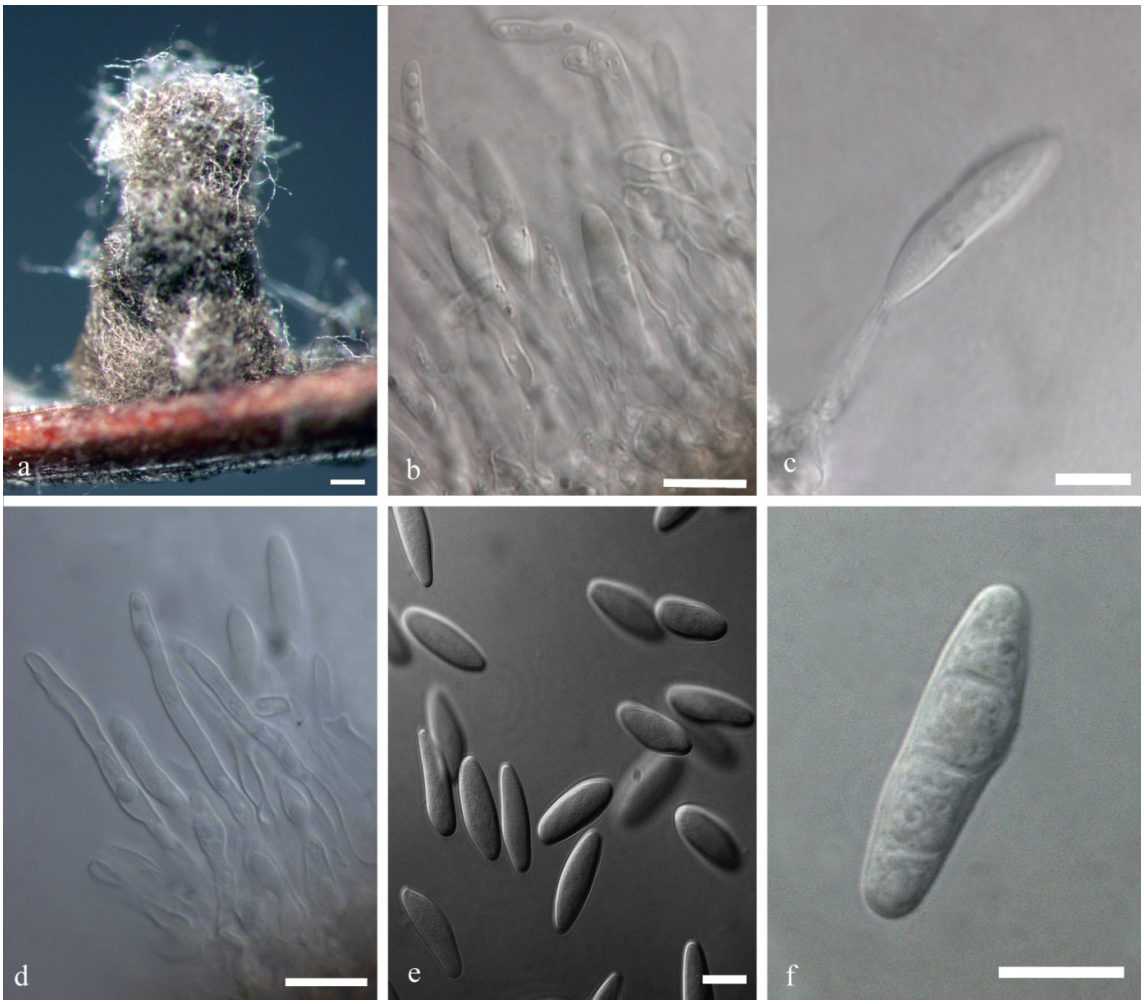
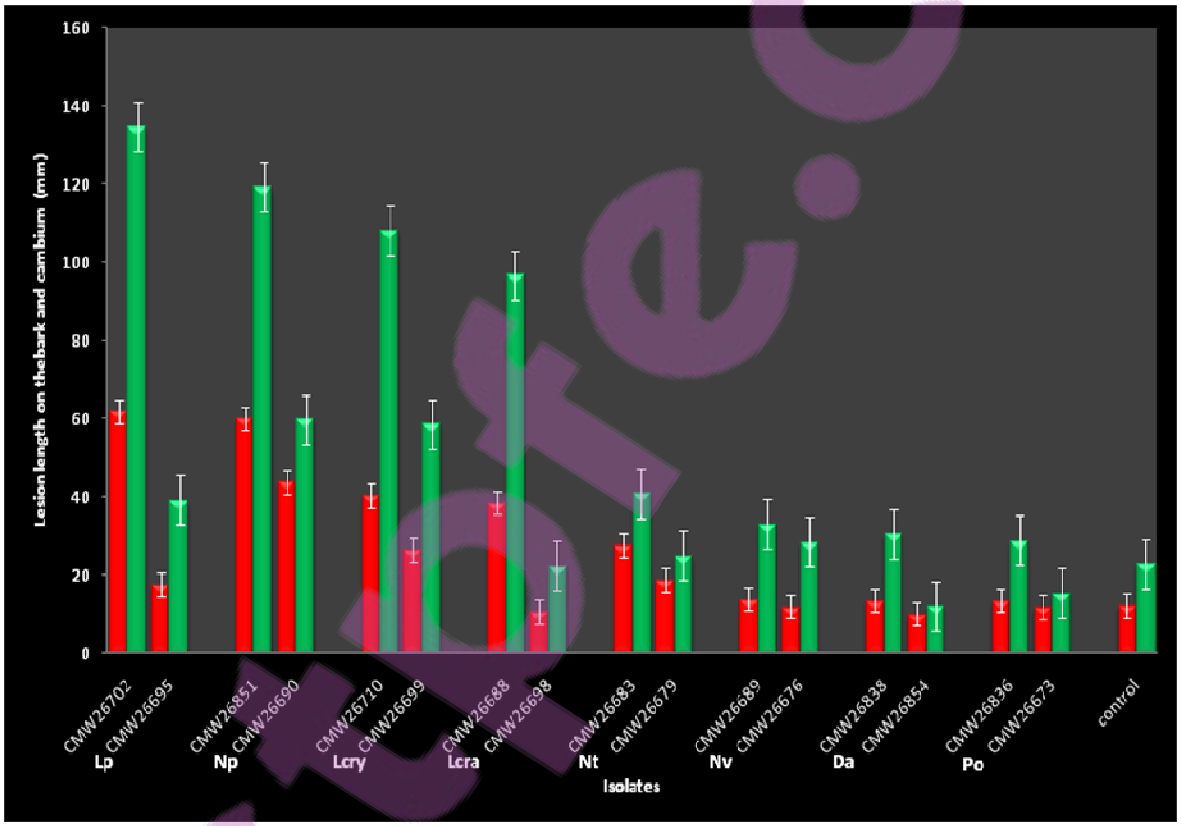

a
b
c
d
e
f

Figure 6. Mean lesion lengths (mm) on bark (red) and cambium (green) for each Botryosphaeriaceae isolate six weeks after inoculation on branches of *T. sericea* ($P < 0.0001$). *L. pseudotheobromae* (Lp), *N. parvum* (Np), *L. cryptotheobromae* (Lcry), *L. crassipora* (Lcra), *N. terminaliae* (Nt), *N. vitifusiforme* (Nv), *D. alatafructa* (Da), *P. olivaceum* (Po) and the control.


Lesion length on the bark and cambium (mm)
160
140
120
100
80
60
40
20
0
CMW26702
CMW26695
CMW26851
CMW26690
CMW26710
CMW26699
CMW26688
CMW26698
CMW26683
CMW26679
CMW26689
CMW26676
CMW26838
CMW26854
CMW26836
CMW26673
control
Lp
Np
Lcry
Lcra
Nt
Nv
Da
Po
Isolates

Chapter 5

Genetic structure of *Lasiodiplodia theobromae* and *L. pseudotheobromae* from native and non-native hosts in Cameroon

ABSTRACT

Lasiodiplodia theobromae and *L. pseudotheobromae* are common colonists of native *Terminalia* spp. and non-native *Theobromae cacao*, grown in a taungya intercropping system, in Cameroon. Studies have shown that these fungi are capable of causing disease on both hosts. These sister species are very closely related, but their populations have not been studied in the same geographic area, where introgression would be possible. In this study we determined the genetic diversity and structure of 126 isolates representing both *L. theobromae* and *L. pseudotheobromae,* collected from *T. cacao* and *Terminalia* spp. in Cameroon, using SSR markers. The objectives were to assess the integrity of the species boundaries, determine the population structure of the fungi, the level of gene flow between isolates from *Terminalia* spp. and *T. cacao* and the mode of reproduction of both species. Analysis of the SSR alleles showed clear genetic distinction between *L. theobromae* and *L. pseudotheobromae*, supporting their earlier distinction as sister species. Both *L. theobromae* and *L. pseudotheobromae* populations from Cameroon had high levels of gene diversity, moderate degrees of genotypic diversity, and high levels of gene flow between isolates from *T. cacao* and *Terminalia* spp. There was no evidence for geographic substructure in these populations. These *Lasiodiplodia* spp. are either native to the region, or have been introduced repeatedly or in large numbers. Despite evidence for abundant asexual reproduction, based on fruiting bodies and in repeated SSR genotypes, the SSR alleles were randomly associated in both species, suggesting outcrossing.

1. INTRODUCTION

Lasiodiplodia theobromae (Pat) Griffon and Maubl., the type species of the genus *Lasiodiplodia,* was first described from *Theobromae cacao* fruit in Ecuador in the late 1800s (Patouillard and Lagerheim 1892). It is recognized as an important plant pathogen of many woody and herbaceous hosts, especially in the tropics and subtropics (Punithalingam 1980; Sinclair and Lyon 2005). Symptoms of diseases associated with *L. theobromae* include dieback, canker, gummosis, leaf blight, root and collar rot of woody plants and agricultural crops (Punithalingam 1980; Sinclair and Lyon 2005). There are also reports of *L. theobromae* causing timber stain after felling (Apetorgbor *et al.* 2004). *L. theobromae* is predominantly a latent pathogen, frequently found as an endophyte in healthy plant tissue, but that can become a virulent pathogen when the host is weakened or stressed (Mullen *et al.*1991; Sinclair and Lyon 2005).

Subsequent to the description of *L. theobromae,* the taxonomy of species in *Lasiodiplodia* has undergone considerable change. Two routes were initially followed in the taxonomy of *Lasiodiplodia* spp. with dark, striate conidia. On the one hand isolates resembling this fungus, but originating from different hosts, were described as separate species (Punithalingam 1976). Alternatively, all isolates resembling *L. theobromae* based on colony and conidial morphology, were lumped under the name *L. theobromae* (Punithalingam 1976). Both these strategies lead to significant errors in the systematics of this plant pathogen. More recently, DNA sequence comparisons have shown that *L. theobromae* represents several unique lineages, for which names have been applied. Thus, 10 *Lasiodiplodia* species are presently recognised based on morphology and sequence data (Pavlic *et al.* 2004; Burgess *et al.* 2006a; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Begoude *et al.* 2009a).

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous. emerged from a recent separation of cryptic species originally identified as *L. theobromae* (Alves *et al.* 2008). The species is known from Africa, Europe and Latin America, where it has been described from forest and fruit trees. Growing evidence suggests that *L. pseudotheobromae,* like *L. theobromae,* has a worldwide distribution and a wide host range (Begoude *et al*. 2009a,b; Mehl *et al.* 2009; van der Walt 2008).

Both *L. theobromae* and *L. pseudotheobromae* have been reported from trees in Cameroon (Mbenoun *et al.* 2008; Begoude *et al*. 2009a,b). *L. theobromae* has been reported to cause die-back and death of *T. cacao* in the country (Mbenoun *et al.* 2008). Together with *L. pseudotheobromae* and many other species in the Botryosphaeriaceae, it was also recently reported from native and introduced *Terminalia* spp. in Africa (Begoude *et al*. 2009a,b). In both studies, *L. theobromae* and *L. pseudotheobromae* were common on *Terminalia* spp. and they were highly pathogenic in inoculation trials.

Theobromae cacao is native to South America (Purseglove 1968) and was introduced into West Africa towards the end of the 19th century (Havinden 1970). It has become one of the most important cash crops in Cameroon and other West African countries (Havinden 1970). Traditionally, *T. cacao* is planted in the shade of forest trees. Various timber and fruit trees are also intercropped with *T. cacao*. In Cameroon, some of the most popular timber trees planted as a shade crop for *T. cacao* include *T. ivorensis* and *T. superba*. These native tree species are used to establish a "taungya" agrisylvicultural system where the production of timber is combined with that of *T. cacao* (Lawson 1995; Norgrove and Hauser 2002).

Insect pests and pathogens are recognised as important constraints to the productivity of agroforestry systems (Epila 1986; Rao *et al.* 2000). Schroch *et al.* (2000), provided an extensive review of pests and diseases in agroforestry systems in the humid tropics and raised the fact that latent pathogens of one crop could move to other crops grown in association with it. The Botryosphaeriaceae provide an excellent example of latent pathogens of woody plants that move between hosts (Slippers and Wingfield 2007). Because native *Terminalia* spp. and non-native *T. cacao* trees occur in close association in plantations, it is possible that pathogens such as the Botryosphaeriaceae can move between these trees.

Very few studies have been conducted on the population genetics of fungi in the Botryosphaeriaceae (Burgess *et al.* 2004; Mohali *et al.* 2005; Burgess *et al.* 2006b). None of these studies have considered the structure of two closely related species from two different hosts occurring sympatrically. Because *L. theobromae* and *L. pseudotheobromae* are closely related, and occur on closely associated hosts, studying the genetic structure of these species in a taungya system will help to better understand the ecology of the interacting partners.

The aim of this study was to analyse the genetic diversity and structure of populations of *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp.

in Cameroon using polymorphic microsatellite DNA markers previously developed for *L. theobromae* by Burgess *et al*. (2003). The specific objectives were to: (i) test the integrity of species boundaries between *L. theobromae* and *L. pseudotheobromae,* (ii) determine the population structure of *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp. in Cameroon, (iii) determine the level of gene flow between isolates of these species from different hosts and (iv) determine the mode of reproduction of *L. theobromae* and *L. pseudotheobromae*.

2. MATERIALS AND METHODS

2.1. Fungal isolates

A total of 126 *L. theobromae* and *L. pseudotheobromae* isolates, collected from two different regions in Cameroon (Table 1), were used for population analyses in this study. Of these, 42 isolates were previously obtained from asymptomatic bark and branches of *Terminalia* spp. in December 2007 and January 2008 (Begoude *et al.* 2009b). The remaining 84 isolates were collected in November 2008 from *T. cacao* trees showing symptoms of dieback. All the collection sites occurred within an area of 250 km^2. One isolate per tree was selected to be used in the population genetic studies. For isolation of fungi from *T. cacao*, the technique described in Begoude *et al*. (2009a) was used. Single conidial cultures were prepared for all isolates and duplicates of these cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.2. DNA extraction, PCR reactions and DNA sequencing

To identify isolates collected from *T. cacao*, conidial morphology and DNA sequence data for the Internal Transcribed Spacer regions (ITS) of the nrDNA, including the 5.8S operon, were used. Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae from *T. cacao* were as described in Begoude *et al.* (2009a). The identity of isolates representing *L. theobromae* and *L. pseudotheobromae* was confirmed by comparing the ITS sequences of isolates obtained in

this study with corresponding sequences in GenBank for isolates CBS 164.96 and CMW 9074, representing *L. theobromae,* and isolates CBS 116459 and CBS 447.62, representing *L. pseudotheobromae.* The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition of 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition for the construction of maximum parsimonious trees. The support for branches of the most parsimonious trees was assessed with a 1000 bootstrap replication (Felsenstein 1985).

2.3. Simple sequence repeat (SSR)-PCR and GENESCAN analyses

Thirteen PCR-based SSR microsatellite markers (Burgess *et al.* 2003) were employed to study the population structure of *L. theobromae* and *L. pseudotheobromae* isolates. The PCR reactions and conditions were the same as those described by Burgess *et al.* (2003). The DNA concentration of the PCR products were measured visually against the intensity of a 100 bp marker (Roche Molecular Biochemicals) on 2 % agarose gels, exposed to Ultra-violet (UV) illumination.

PCR products were multiplexed for GENESCAN analysis based on the approximate sizes of the PCR products and type of fluorescent label attached to the primer. Each sample mix contained 1 µl of combined DNA, 0.14 µl 1x loading buffer and 1 µl internal standard GENESCAN-500 LIZ (Applied Biosystems, Warrington, UK). Fluorescent-labelled SSR-PCR products were separated on an ABI Prism 3100 sequencer (Applied Biosystems). Allele sizes were determined by comparing the mobility of the SSR products with that of the LIZ internal size standard using a combination of the GENESCAN 2.1 analysis software (Applied Biosystems) and GENOMAPPER V3.5 (Applied Biosystems).

2.4. Statistical analyses

Isolates that contained the same alleles at each locus potentially represented clones. The inclusion of clonal multicopies can strongly distort estimates of population genetic

parameters (Frantz *et al.* 2006). Therefore, duplicates of each multilocus genotype were discarded from the analyses to provide a clone-corrected dataset.

2.4.1. Bayesian clustering analyses

The software programme Structure version 2.2 (Pritchard *et al*. 2000; Falush *et al*. 2003) was used to infer the population structure of all isolates, without any *a priori* knowledge of population subdivision, using a Bayesian model-clustering algorithm. This algorithm assumes a model in which there are *K* populations or clusters, in which a set of allele frequencies at each locus characterized each population. Individuals in the same sample are probabilistically distributed to *K* clusters, or jointly to two or more clusters if their genotypes indicate that they are admixed, regardless of their region or host origin. Parameter estimation considers that populations are at Hardy-Weinberg equilibrium and that the marker loci are unlinked and at linkage equilibrium. The model with admixture was applied in all simulations as this model is recommended for situations where little is known about the existence of admixture (Falush *et al.* 2003). Priors were assumed uniform for the vectors of proportions q_i of the individual *i*'s genome deriving from each cluster. Iteration parameters were set to 950 000 Monte Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 50 000 iterations and 20 independent simulations were performed to test for the consistency of the results. The number of clusters, *K,* was varied from 1 to 10. Individuals were assigned to a single cluster when their proportion of ancestry in that cluster was greater than 80 %. This threshold was determined after analyzing the distribution of mean ancestry coefficients for each *K*.

2.4.2. Gene and genotypic diversity

Single alleles were assigned a different letter for each of the loci. For each isolate, a data matrix of multistate characters, each state corresponding to a different locus, was compiled with the polymorphic loci, thus providing each isolate with a haplotype. A number was assigned to each haplotype and the equation $\hat{G} = 1/\Sigma p_i^2$, developed by Stoddart and Taylor (1988), was applied to estimate the genotypic diversity ($\hat{G}$). In this equation, p_i stands for the observed frequency of the i^{th} phenotype. The maximum percentage of genotypic diversity (G_{max}), obtained from the equation $G_{max} = \hat{G}/N \times 100$ (where N is the population size), was used to compare the genotypic diversities between populations (Chen *et al*. 1994). Allelic

frequency, as well as the number of alleles at each locus, was calculated and gene diversity determined, using the program POPGENE version 1.31 (Yeh *et al.* 1999) based on the equation $H = 1-\Sigma x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973). Chi-square tests for differences in allele frequencies were calculated for each locus across clone-corrected collections. The free software Programme Multilocus version 1.2 (Agapow and Burt 2000) available at http://www.agapow.net/software/multilocus/ was used to plot the genotypic diversity against the number of loci with 1000 resampling repetitions, in order to determine whether the isolates and microsatellite markers used were sufficient to recover the maximum genotypic diversity.

2.4.3. Genetic differentiation and gene flow

The genetic differentiation among populations was assessed in POPGENE, using Nei's (1973) G_{ST} statistic, which varies between zero and one. POPGENE was also used to estimate the number of migrants (N_m) exchanged among the populations for each generation from the estimate of G_{ST} where $N_m = 0.5(1-G_{ST})/G_{ST}$ (McDermott and McDonald, 1993). Populations that are completely genetically isolated would have N_m values of zero and G_{ST} values tending towards one (Hartl and Clark 1989). The software programme GENALEX 6.2 (Peakall and Smouse 2006) was used to analyse the molecular variance (AMOVA) among populations of *Lasiodiplodia* spp. from *T. cacao* and *Terminalia* spp. at different locations.

2.4.4. Linkage disequilibrium

The multilocus linkage disequilibrium for each clone-corrected population was tested with the Index of Association (I_A) (Maynard-Smith *et al.* 1993). The Index of Association provides information related to whether two different individuals which possess the same allele at one locus, will more likely possess the same allele at another locus (Fournier and Giraud 2008). The tests were performed on a data matrix of multistate characters using the program Multilocus (Agapow and Burt 2000). For any pair of individuals, the number of loci at which they differ was calculated and its variance was compared with that expected ($I_A = 0$). There is no linkage disequilibrium when the observed data fall within the distribution range of the recombined data and the population is most likely clonal if the observed data falls outside the distribution range with a significant P value ($P < 0.05$).

3. RESULTS

3.1. Fungal isolates

A total of 16 *L. theobromae* isolates and 26 *L. pseudotheobromae* isolates were obtained from *T. ivorensis* and *T. superba* in a previous study (Begoude *et al*. 2009b). An additional 84 isolates resembling species of *Lasiodiplodia* were collected from *T. cacao* in the present study.

Lasiodiplodia isolates from *T. cacao* were identified to species level using DNA sequence data for the ITS and 5.8S gene regions. The ITS dataset comprised 114 sequences of which 97 originated from *Terminalia* spp. and *T. cacao* and 17 sequences were retrieved from GenBank. Of the 486 characters present in the ITS sequence data set, 34 were parsimony informative. The MP analyses generated two trees with identical topology (Tree length (TL) = 129, Consistency index (CI) = 0.698, Retention index (RI) = 0.839, Rescaled consistency index (RC) = 0.585). These analyses revealed that all 97 isolates from *Terminalia* spp. and *T. cacao* belonged to the clades accommodating either *L. theobromae* (Bootstrap support (BS) = 55 %) or *L. pseudotheobromae* (BS = 77 %) (Figure 1). Of the isolates from *T. cacao*, 54 represented *L. theobromae* and 33 *L. pseudotheobromae*.

3.2. Microsatellite PCR amplification

Eleven of the 13 microsatellite primer pairs developed previously for *L. theobromae* by Burgess *et al*. (2003) successfully amplified DNA markers for *L. theobromae* from Cameroon. Primers las13&14 and las31&32 produced multiple bands even after numerous attempts to optimize them. Among the primers that yielded successful PCR products, six were monomorphic for *L. theobromae* in Cameroon.

Nine of the 13 microsatellites primers previously developed for *L. theobromae* by Burgess *et al*. (2003) successfully amplified DNA markers in Cameroonian *L. pseudotheobromae*. Primers las13&14, las31&32 and las37&38 produced multiple bands, whereas primers las23&24 failed to amplify a product from isolates of *L. pseudotheobromae*. Among the primers that yielded successful PCR products, four were monomorphic for *L. pseudotheobromae*.

Products of two monomorphic primer pairs, las17&18 and las33&34 had identical alleles (249 and 276, respectively) among isolates of both fungal species. The product amplified with primers las3&4 was monomorphic within the species, but distinct between them (351 and 361 for isolates of *L. pseudotheobromae* and *L. theobromae*, respectively). Products from primer pair las35&36 was monomorphic among isolates of *L. pseudotheobromae* (387), but contained two alleles (376 and 379) amongst isolates of *L. theobromae*. PCR products from primer pairs las15&16, las27&28 and las29&30, which were monomorphic among isolates of *L. theobromae*, were polymorphic among isolates of *L. pseudotheobromae*. Overall, seven primers were polymorphic among isolates of both species (Figure 2) and five primers were polymorphic among isolates of only one of the species (Table 2 & 3).

3.3. Statistical analyses

3.3.1. Bayesian clustering analyses

The Bayesian inference of the population structure was performed with 21 unique haplotypes representing all the multilocus genotypes inferred with seven polymorphic loci among isolates of *L. theobromae* and *L. pseudotheobromae*. These samples included isolates from *Terminalia* spp. and *T. cacao* from all the locations sampled. The distribution of the maximum likelihood was the highest for $K = 2$ with an assignment rate value of 98.8 % (Figure 3). The first cluster included all the genotypes of isolates representing *L. pseudotheobromae* while the second cluster consisted of genotypes of *L. theobromae*. There was no subdivision in the population according to either host or location. Separate investigation of the population structure within each species, showed that the distribution of the maximum likelihood was the highest for $K = 1$, suggesting a high degree of admixture which suggests that neither the host nor the geographic location considered influenced the population structure within the species. This analysis confirmed the classification of *L. theobromae* and *L. pseudotheobromae* as distinct sister species, but did not show any differentiation within species according to host or geographic location.

3.3.2. Gene diversity

Sixty-nine isolates of *L. theobromae* from *Terminalia* spp. and *T. cacao* were analyzed at five polymorphic loci (Table 2). The number of alleles ranged from two to four per locus. A total of 13 alleles were produced across populations from *Terminalia* spp. and *T. cacao*, of which nine alleles were observed across isolates from *Terminalia* spp. whereas all 13 alleles were observed among isolates from *T. cacao*. Four unique alleles, with low frequency (8-24 %), were observed in isolates from *T. cacao*. The mean total gene diversity (*H*), calculated using the allele frequencies across all isolates of *L. theobromae* was 0.46, which was similar to the gene diversity observed in isolates from *T. cacao* and higher that the gene diversity observed in isolates from *Terminalia* spp. (Table 2).

Fifty three isolates of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* were analyzed at five polymorphic loci after clone correction of populations (Table 3). The number of alleles ranged from two to six per locus. A total of 14 alleles were identified across isolates from both hosts of which 13 alleles were observed among isolates from *Terminalia* spp. and 12 alleles were observed in isolates from *T. cacao*. Two unique alleles, with low frequency (16 %), were observed in isolates from *Terminalia* spp. and only one unique allele, with low frequency (12 %), was observed in isolates originating from *T. cacao*. The mean total gene diversity across all isolates of *L. pseudotheobromae* was 0.445, which was similar to the gene diversity observed in isolates from each host (Table 3).

3.3.3. Genotypic diversity

Among the 69 isolates of *L. theobromae,* 26 different multilocus genotypes were discriminated. Among these genotypes, 19 were unique to the sampled localities (three in Nkong, six in Mbalmayo and ten in Nkoemvone) whereas seven genotypes representing 60.9 % of the isolates collected were shared among the three localities (Figure 4). Where *Terminalia* spp. and *T. cacao* occurred in the same area, such as in Mbalmayo, of 14 genotypes found in the area, three genotypes representing 65.6 % of the isolates collected were shared between both hosts.

When considering isolates of *L. theobromae* from different hosts separately, six genotypes were found in isolates from *Terminalia* spp. and 25 genotypes were found in isolates from *T.*

cacao (Table 4). Among these genotypes, only one genotype (33.3 %) was unique to *Terminalia* spp. whereas 16 genotypes (64 %) were unique to the *T. cacao* population. The percentage of shared genotypes between *Terminalia* spp. and *T. cacao* represented 19 % of all the genotypes observed. The most common genotypes accounted for 14.5 % of the population and the genotypes occurring only once were very abundant in isolates from *T. cacao* (Table 4). Overall, moderate values were generated for the genotypic diversities in each population, ranging from 28.57 % on the *Terminalia* spp. to 32.12 % for isolates from *T. cacao*. The moderate value of genotypic diversity obtained from these populations was probably due to the occurrence of only a few single isolate genotypes (12 and 23.2 %, respectively for each host).

Ten different multilocus genotypes were detected amongst the 57 isolates of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao*. Among these genotypes, three were unique to the localities of Mbalmayo (one) and Nkoemvone (two), whereas seven genotypes representing 87.7 % of the isolates collected were shared among the three sampled locations (Figure 4). Where isolates were collected from both *Terminalia* spp. and *T. cacao* in the same locality, such as Mbalmayo and Nkoemvone, of seven genotypes obtained in Mbalmayo, four were shared between both hosts and represented 90 % of the total isolates collected in Mbalmayo. Two out of nine genotypes obtained in Nkoemvone, representing 65.2 % of the isolates collected in the area, were shared between *Terminalia* spp. and *T. cacao.*

When considering isolates from different hosts, regardless of their locality of origin, six different genotypes of *L. pseudotheobromae* were found in isolates from *Terminalia* spp. and eight genotypes were found in isolates from *T. cacao* (Table 5). Among these genotypes, two genotypes (33.3 %) were unique to *Terminalia* spp. whereas four genotypes (50 %) were unique to the *T. cacao* population. The percentage of shared genotypes between *Terminalia* spp. and *T. cacao* represented 40 % of all the genotypes observed for *L. pseudotheobromae*. The most common genotypes accounted for 30.2 % of the population and the genotypes occurring only once were very few in both the *Terminalia* spp. and the *T. cacao* populations (Table 5). The overall genotypic diversities calculated for each population were low, ranging from 17.57 % on *Terminalia* spp., to 15.97 % for the *T. cacao* population. This is most probably due to the occurrence of few single isolate genotypes (7.6 and 6.5 %, respectively for each host genus).

In both *L. theobromae* and *L. pseudotheobromae* populations, from single hosts as well as those that occur on both *Terminalia* spp. and *T. cacao*, the genotypic diversity was plotted against the number of loci. The graph obtained reached a plateau in the case of *L. pseudotheobromae,* whereas the graph approached a plateau in the case of *L. theobromae* (Figure not shown). This suggested that the genotypic diversity calculated from the isolates and microsatellite markers used in this study was sufficient to characterize the populations of both species.

3.3.4. Genetic differentiation and gene flow

The measure of genetic differentiation between populations of *L. theobromae* from *Terminalia* spp. and *T. cacao* reflected a lack of substructuring in the *L. theobromae* population. The values obtained for χ^2 tests revealed no significant differences ($P > 0.05$) in allele frequencies at any loci for populations from either the *Terminalia* spp. or *T. cacao* (Table 6). These results were further confirmed by very low G_{ST} values corresponding to 0.046, indicating that most of the gene diversity is found within the subpopulations (*Terminalia* spp. and *T. cacao*). This was also true when comparing populations of *L. theobromae* from different hosts at different locations. Consequently, a low level of differentiation exists in populations of *L. theobromae* from *Terminalia* spp. and *T. cacao*. The number of migrants (N_m) exchanged between populations for each generation was estimated at 10.47.

Similar to *L. theobromae,* the measure of genetic differentiation between populations of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* showed a lack of substructuring. The values obtained for χ^2 tests revealed no significant differences ($P > 0.05$) in allele frequencies at any loci for either the *Terminalia* spp. or *T. cacao* populations of *L. pseudotheobromae* (Table 6). There was only 3.5 % genetic diversity distributed between populations from *Terminalia* spp. and *T. cacao* and no difference was observed after comparing populations of *L. pseudotheobromae* from different hosts at different locations. This indicated that most of the genetic variation is distributed within each subpopulation. Therefore, a low level of differentiation also exists in populations of *L. pseudotheobromae* from *Terminalia* spp. and *T.*

cacao. The number of migrants (N_m) exchanged between populations for each generation was estimated at 13.83.

3.3.5. Linkage disequilibrium

The Index of association (I_A) calculated for populations of *L. theobromae* and *L. pseudotheobromae* were -0.153 ($P = 0.99$) and 0.069 ($P = 0.4$), respectively. These values did not significantly deviate from the expected value when there is no linkage disequilibrium. This suggests that alleles are randomly associated, as would be expected for outcrossing populations.

4. DISCUSSION

Genetic diversity analyses for *L. theobromae* and *L. pseudotheobromae* in Cameroon showed that there is no gene flow between these recently diverged sister species. This supports the previous segregation of *L. pseudotheobromae* from *L. theobromae* based on divergence in sequences of their nuclear genes, ITS and *tef 1-*α (Alves *et al.* 2008). Both *L. theobromae* and *L. pseudotheobromae* have a worldwide distribution and they share similar biological and ecological characteristics. Since their description as cryptic species, *L. pseudotheobromae* and *L. theobromae* have not been studied in areas where they occur on the same host or environment, which is where possible hybridization of two such closely related fungal genets might be expected (Shardl and Craven 2003).

A number of lines of evidence supported the distinction of *L. theobromae* and *L. pseudotheobromae*. Of the 22 alleles that were detected, 11 and six were unique to populations of *L. pseudotheobromae* and *L. theobromae*, respectively. A number of these were fixed in either species, and any recombination between them would have shared these alleles. The five alleles that were shared between these species occurred in significantly different frequencies. The alleles shared between populations of *L. theobromae* and *L. pseudotheobromae* in this study possibly remain from an ancestral species, which would be expected in such closely related species (Carbone and Kohn 2004). The structure inferred from a Bayesian clustering algorithm confirmed the most likely number of clusters amongst

all isolates to be two. All the individuals representing *L. theobromae* and *L. pseudotheobromae* clustered separately in either of these two clusters.

Delimitating boundaries between sister species with low levels of genetic divergence is challenging. Two methods are used in species delimitation, one of which encompasses tree-based approaches that delimit species as historical lineages (Goldstein and DeSalle 2000). The other method includes non tree-based analyses where information regarding the level of gene flow is the main basis to determine boundaries between species (Sites and Marshall 2003; 2004). Application of microsatellite markers, which fall within the latter category, represents a powerful tool to demarcate barriers to gene flow between individuals of closely related fungal species (Fisher *et al.* 2000; 2002). Using microsatellites, it is possible to screen multilocus genotypes for many individuals rapidly. Consequently, the significant genetic distinction between *L. theobromae* and *L. pseudotheobromae* using a non tree-based method with microsatellite markers, provides strong additional support for their previous separation based on phylogenetic analyses of ITS and *tef 1-α* sequence data (Alves *et al.* 2008).

No evidence of host or geography linked population structure was observed for either *L. theobromae* or *L. pseudotheobromae* in Cameroon. Agro-ecology in Cameroon is subdivided into five zones based on vegetation and climatic conditions (http://www.irad-cameroon.org/carte_us.php). The collection sites in this study occurred within zone five, characterized by humid forests with bimodal rainfall. The maximum distance between collection sites was ~200 km. Consequently, there is no effective barrier that could restrict dispersal of fungal propagules between locations. The hosts are fairly continuous over the distances studied, providing a possible explanation for the connectedness of the populations. Endophytic Botryosphaeriaceae infections of woody hosts are thought to develop over time by horizontal transmission through wind- or water-dispersed spores (Arnold *et al.* 2003a,b; Slippers and Wingfield 2007). Movement of infected material would provide another explanation of genetic similarity between populations. When establishing cacao in the shade of thinned forest trees, farmers obtain seedlings from a centralized seedling distributor (Sonwa 2002). This could have contributed to the spread of the pathogens as endophytes over large areas. The population of *L. theobromae* and *L. pseudotheobromae* on *T. cacao* and native *Terminalia* spp. appeared to be totally integrated, suggesting that the movement of

these pathogens between the hosts may be symmetrical (Hayden *et al.* 2007; Fournier and Giraud 2008).

We detected no restriction in the movement of *L. theobromae* and *L. pseudotheobromae* on *Terminalia* spp. and *T. cacao*. Most alleles were found on both hosts, and in similar frequencies. Both *L. theobromae* and *L. pseudotheobromae* commonly occur in the tropics and subtropics on a wide diversity of hosts with no observed specialization (Mohali *et al.* 2005; Alves *et al.* 2008), so this result is not unexpected. Many cases of shared genotypes were also observed on both *Terminalia* spp. and *T. cacao*, possibly representing clonal lineages. These occurred either in the same field within the location or among locations (over scales of a few metres to 200 km). Both populations displayed a high allelic diversity and almost all of them were shared between isolates from *T. cacao* and *Terminalia* spp. As explained above, this could reflect natural spread through asexual conidia in a step-wise manner, or by direct transport of infected material from a central location. The non-limited genetic exchange between populations on *T. cacao* and *Terminalia* spp. could increase the gene diversity because gene flow introduces new alleles into populations that are part of the same genetic neighborhood (McDonald and Linde 2002a).

Our results showed high genetic diversity of microsatellite loci in populations of both *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp. in Cameroon. In the *L. theobromae* population, the number of alleles and genotypes observed in isolates from *T. cacao* was higher than that found in isolates from native *Terminalia* spp., resulting in a higher genetic diversity for isolates from *T. cacao*. This could, however, be explained by the larger number of isolates collected from *T. cacao*. Native populations or populations closest to their centre of origin generally have high levels of genetic diversity while introduced populations often exhibit lower levels of diversity (McDonald and Linde 2002a; Stukenbrock *et al.* 2006). At the time of its description, *L. pseudotheobromae,* was known from few hosts and a very limited geographic distribution (Alves *et al.* 2008). This distribution is expanding markedly and is beginning to reflect the worldwide distribution of *L. theobromae* (Begoude *et al*. 2009a,b; Mehl *et al.* 2009; van der Walt 2008). The wide distribution, both geographically and in host relationships, of *L. theobromae* and *L. pseudotheobromae* complicates speculation regarding their likely origin since multiple introductions through anthropological action and movement between various hosts could strongly influence their genetic diversity.

Analyses of the mode of linkage disequilibrium amongst alleles at the SSR loci in populations of *L. theobromae* and *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* suggest that both species undergo regular sexual reproduction (Milgroom 1996). However, despite this evidence of sexual reproduction, sexual states for this group of fungi are rarely seen. *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx [which is no longer a valid name following Crous *et al.* (2006)] is frequently reported as the sexual state of *L. theobromae.* However, since Stevens (1925; 1926) determined *B. rhodina* as the teleomorph of *L. theobromae*, this connection has not been confirmed. The recent description of a number of species that were previously confused with *L. theobromae* sheds doubt on the accuracy of those discoveries. Although the purported sexual state might be known for *L. theobromae*, there is no evidence for such a state in *L. pseudotheobromae.*

Both *L. theobromae* and *L. pseudotheobromae* exhibit a genetic structure with a moderate degree of genotypic diversity. The level of genotypic diversity obtained for *L. theobromae* isolates from both *Terminalia* spp. and *T. cacao*, suggests that a high level of recombination takes place in this fungus. These results are different from those of Mohali *et al.* (2005) who found very low levels of recombination for *L. theobromae* from *Pinus* sp., *Acacia* sp. and *Eucalyptus* sp. in Venezuela. Populations of *L. pseudotheobromae* were characterized based on a small number of single isolate genotypes. Indeed, 60 % of the total genotypes occurred more than once and the proportion of the most common genotype (30.2 %) was high, resulting in a clonal fraction of 82.4 %. These results indicate the presence of a high proportion of widely distributed clonal genotypes across both *Terminalia* spp. and *T. cacao,* despite some evidence of recombination. Although similar observations were made for *L. theobromae,* the frequency of recombination was higher than that in *L. pseudotheobromae*.

Fungi with both sexual and asexual reproduction pose the highest risk of pathogen evolution because they receive benefits from both modes of reproduction (McDonald and Linde 2002a,b). Thus, the fit combination of alleles generated through regular recombination with the highest level of fitness could be increased rapidly through asexual reproduction (Ciampi *et al.* 2008). This risk is increased in cases where there is high gene flow over large areas (as we observed here), because fitter genotypes can quickly spread. Therefore, *L. theobromae* and *L. pseudotheobromae* correspond to the highest category of evolutionary risk as defined by McDonald and Linde (2002a,b). Together with data from previous studies showing the pathogenicity of these fungi, they clearly pose a significant threat to both native *Terminalia*

spp., and introduced *T. cacao*. Given their wide host and geographic ranges, this is most likely also true for other native and non-native hosts growing in close proximity to each other.

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Table 1. Source of *Lasiodiplodia theobromae* and *Lasiodiplodia pseudotheobromae* isolates in Cameroon.

Region	Location	Host	No of isolates	
			L. theobromae	*L. pseudotheobromae*
Centre	Mbalmayo	*Terminalia ivorensis*	16	22
		Theobroma cacao	16	8
	Nkong	*T. cacao*	9	4
South	Nkoemvone	*T. cacao*	28	18
		Terminalia superba	-	5

Table 2. Allele frequencies at 5 loci for *Lasiodiplodia theobromae* populations collected from *Terminalia* spp. and *Theobroma cacao* in Cameroon.

Locus	Allele length	Allele configuration	*Terminalia* spp.	*T. cacao*
las21-22	383	A	-	0.24
	388	B	1	0.76
las23-24	454	A	-	0.08
	458	B	0.333	0.48
	461	C	0.667	0.36
	463	D	-	0.08
las25-26	417	A	0.333	0.2
	420	B	0.5	0.48
	421	C	0.167	0.32
las35-36	376	A	0.333	0.4
	379	B	0.667	0.6
las37-38	117	A	1	0.8
	135	B	-	0.2
No isolates			**16**	**53**
No Alleles			**9**	**13**
No. Unique Alleles			**0**	**4**
Polymorphic loci			**3**	**5**
H			**0.3**	**0.484**

Table 3. Allele frequencies at 5 loci for *Lasiodiplodia pseudotheobromae* populations collected from *Terminalia* spp. and *Theobroma cacao* in Cameroon

Locus	Allele length	Allele configuration	*Terminalia* spp.	*T. cacao*
las15-16	351	A	0.833	0.625
	353	B	0.167	0.375
las21-22	383	A	0.833	0.625
	388	B	0.167	0.375
las25-26	415	A	0.333	0.25
	417	B	0.667	0.75
Las27-28	458	A	0.167	-
	463	B	0.167	0.375
	466	C	0.167	-
	471	D	0.167	0.125
	474	E	0.333	0.375
	477	F	-	0.125
Las29-30	180	A	0.833	0.875
	188	B	0.167	0.125
No isolates			**26**	**31**
No Alleles			**13**	**12**
No. Unique Alleles			**2**	**1**
Polymorphic loci			**5**	**5**
H			**0.41**	**0.44**

Table 4. Genotype estimation from multilocus profiles generated from 5 SSR loci for *Lasiodiplodia theobromae*.

GENOTYPES	*Terminalia* spp.	*T. cacao*
AAAAA	2	4
AABAA	5	5
ABABA	1	
ABBBA	3	3
ABBAA	4	1
ABCAA	1	3
BAAAA		1
BABBA		1
BABBB		1
BBBBA		1
BABAA		3
BBAAA		1
ACBAA		1
ACCAA		1
AAABA		1
AABBA		5
AABAB		1
AACBA		5
AACAA		4
AACAB		1
ABAAA		1
ABBAB		1
ABCBA		4
ABCAB		1
ADBBA		1
ADCBA		1
N	**16**	**53**
N(g)	**6**	**25**
$\hat{G}$	**4.751**	**17.024**
$\hat{G}$ (%)	**28.57**	**32.12**

N, number of isolates

N(g), number of genotypes
$\hat{G}$, Genotypic diversity (Stoddart & Taylor 1988)

$\hat{G}$(%), % max diversity

Table 5. Genotype estimation from multilocus profiles generated from 5 SSR loci for *Lasiodiplodia pseudotheobromae.*

GENOTYPES	*Terminalia* spp.	*T. cacao*
AAAAA	1	
AABBA	6	5
AABCA	5	8
AABCB	6	2
ABBDA	1	
BAAEA	7	9
AABFA		1
ABBCA		3
BBAEA		3
BBBEA		1
N	**26**	**31**
N(g)	**6**	**8**
Ĝ	**4.56**	**4.95**
Ĝ (%)	**17.57**	**15.97**

N, number of isolates

N(g), number of genotypes

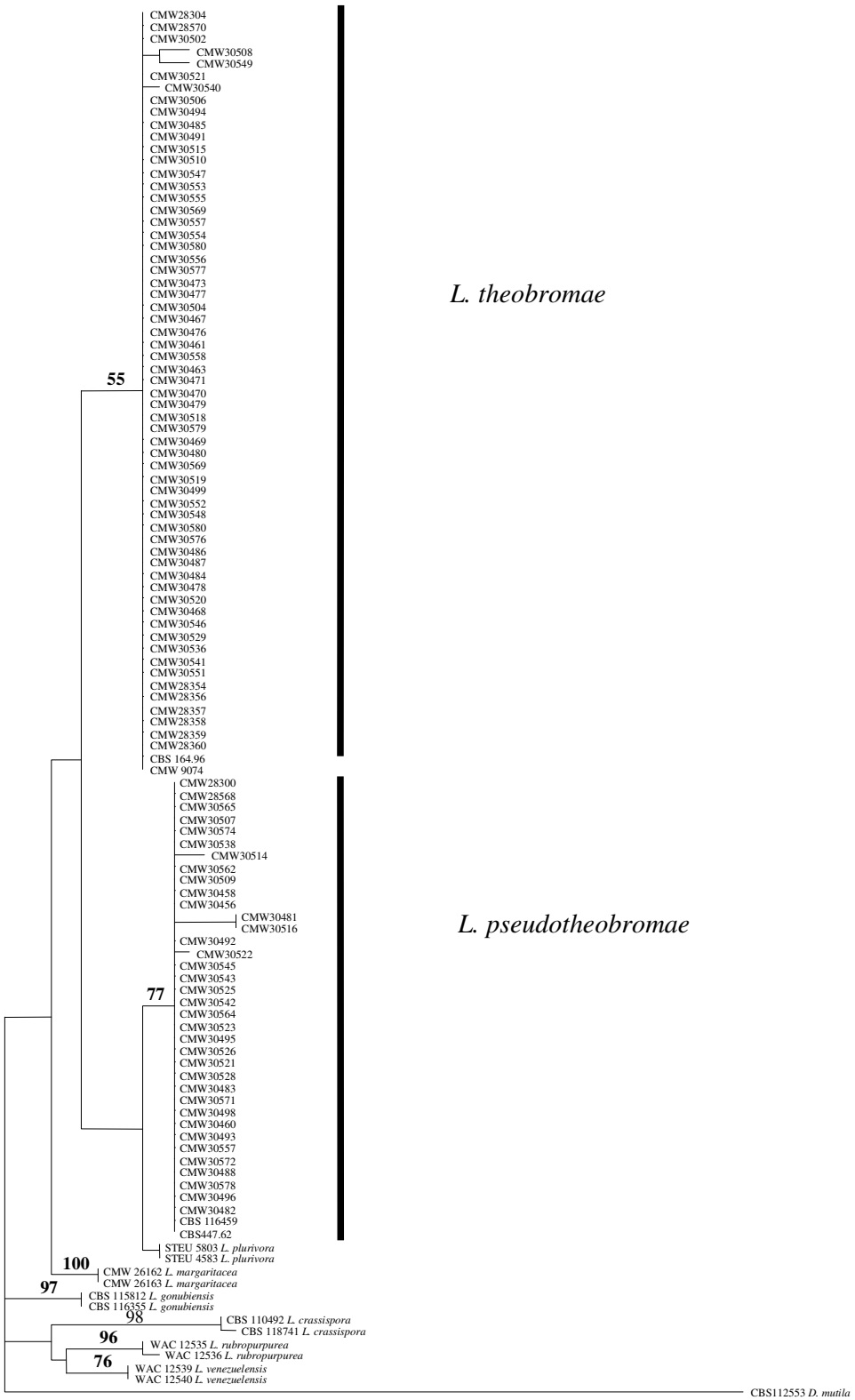
Ĝ, Genotypic diversity (Stoddart & Taylor 1988)

Ĝ(%), % max diversity

Table 6. Gene diversity (*H*) for the 5 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* and *L. pseudotheobromae* in Cameroon.

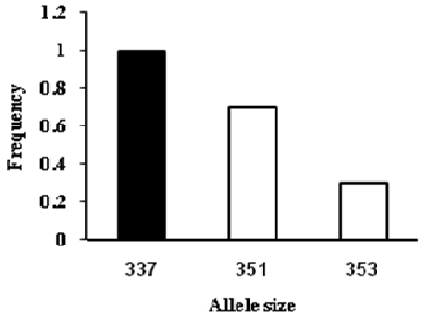
	Gene diversity (*H*)							
	L. theobromae		*L. pseudotheobromae*		*L. theobromae*		*L. pseudotheobromae*	
Loci	*Terminalia* spp.	*T. cacao*	*Terminalia* spp.	*T. cacao*	χ^2	df	χ^2	df
las15-15			0.28	0.47			0.7	1
las21-22	0.00	0.36	0.28	0.47	1.8	1	0.7	1
las23-24	0.44	0.63			2.3	3		
las25-26	0.61	0.63	0.44	0.37	0.8	2	0.1	1
las27-28			0.78	0.69			3.9	5
las29-30			0.28	0.22			0.04	1
las35-36	0.44	0.48			0.1	1		
las37-38	0.00	0.32			1.4	1		
N	6	25	6	8				
Mean	0.30	0.48	0.41	0.44				

Figure 1. Maximum parsimony phylogram of *Lasiodiplodia theobromae* and *L. pseudotheobromae* from this study obtained with sequences of ITS. Bootstrap support (%) from 1000 replications is given on the branches.

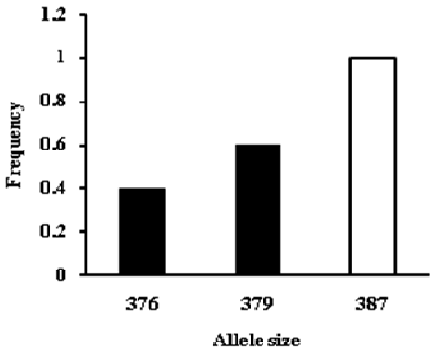


– 0.5 changes

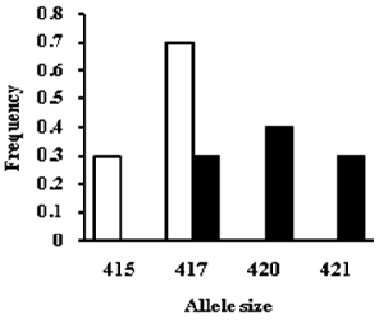
Figure 2. Distribution of alleles showing the size of PCR product at seven microsatellite loci for *Lasiodiplodia pseudotheobromae* (white) and *L. theobromae* (black).



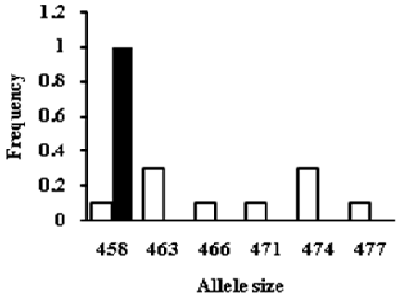
Las 15&16



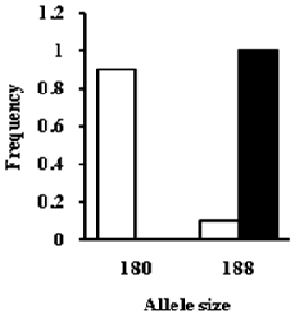
Las 35&36



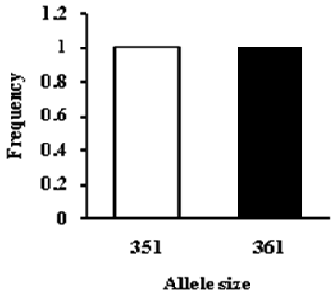
Las 25&26



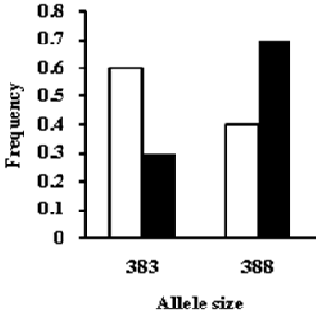
Las 27&28



Las 29&30

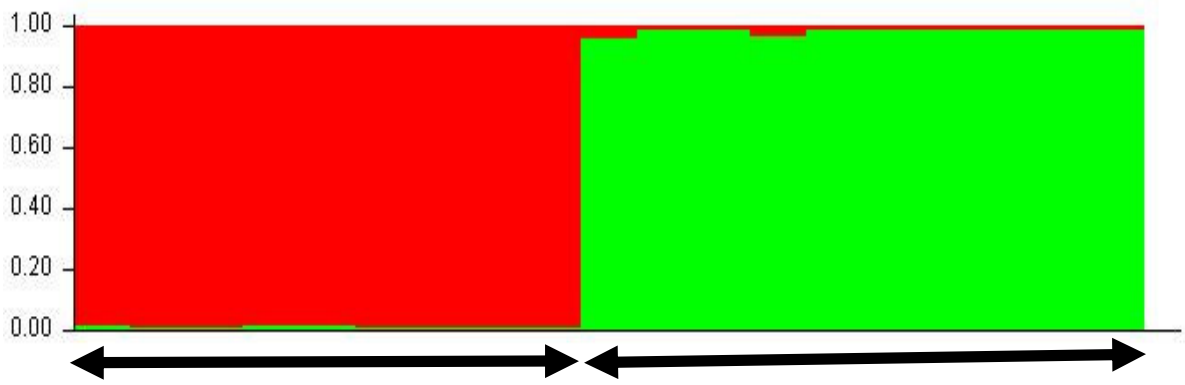


Las 3&4



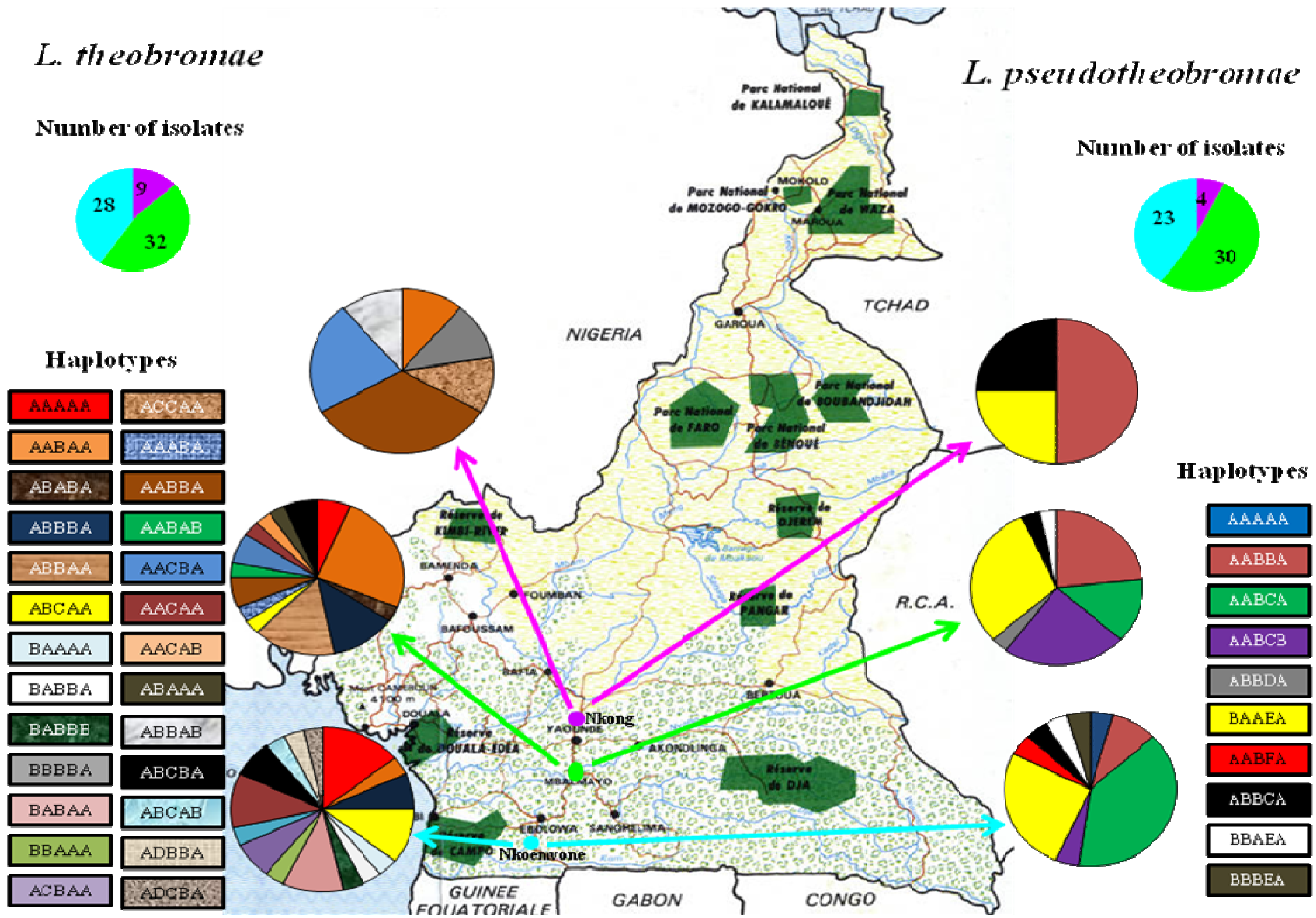
Las 21&22

Figure 3. Bayesian assignment of individuals into two clusters. Red : *Lasiodiplodia pseudotheobromae*; Green: *Lasiodilpodia theobromae*.



Lasiodiplodia pseudotheobromae *Lasiodiplodia theobromae*

Figure 4. Map of collecting sites and distribution of the 26 and 10 haplotypes of *Lasiodiplodia theobromae* and *L. pseudotheobromae,* respectively among the three locations. Each pie chart linked with arrows represents a collecting site and its haplotypes indicated by different color. The pie charts topping the left and right sides represent the number of isolates of *L. theobromae* and *L. pseudotheobromae,* respectively collected per locality.


L. theobromae
Number of isolates
9
28
32
Haplotypes
AAAAA
ACCAA
AABAA
AAABA
ABABA
AABBA
ABBBA
AABAB
ABBAA
AACBA
ABCAA
AACAA
BAAAA
AACAB
BABBA
ABAAA
BABBB
ABBAB
BBBBA
ABCBA
BABAA
ABCAB
BBAAA
ADBBA
ACBAA
ADCBA
L. pseudotheobromae
Number of isolates
4
23
30
Haplotypes
AAAAA
AABBA
AABCA
AABCB
ABBDA
BAAEA
AABFA
ABBCA
BBAEA
BBBEA
NIGERIA
TCHAD
R.C.A.
GUINEE
EQUATORIALE
GABON
CONGO
Nkong
Nkoemvone
GAROUA
YAOUNDE
AKONOLINGA
BERTOUA
DOUALA
BAFOUSSAM
FOUMBAN
BAMENDA
MBALMAYO
EBOLOWA
SANGMELIMA
MOKOLO
MAROUA

Chapter 6

Aurifilum, a new fungal genus in the Cryphonectriaceae from *Terminalia* species in Cameroon

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ABSTRACT

Native *Terminalia* spp. in West Africa provide a popular source of construction timber as well as medical, spiritual and social benefits to rural populations. Very little is, however, known regarding the diseases that affect these trees. During an investigation into possible diseases of *Terminalia* spp. in Cameroon, orange to yellow fungal fruiting structures, resembling those of fungi in the Cryphonectriaceae, were commonly observed on the bark of native *T. ivorensis*, and on dead branches of non-native *T. mantaly*. In this study the fungus was identified based on morphological features as well as DNA sequence data (ITS and β-tubulin) and its pathogenicity was tested on *T. mantaly* seedlings. Our results showed that isolates of this fungus represent a previously undescribed genus in the Cryphonectriaceae, which we describe as *Aurifilum marmelostoma* gen. et sp. nov. Pathogenicity tests revealed that *A. marmelostoma* is pathogenic on *T. mantaly*. These tests, and the association of *A. marmelostoma* with disease symptoms on *T. ivorensis,* suggest that the fungus is a pathogen of this important tree.

1. INTRODUCTION

The Cryphonectriaceae (Diaporthales) was described to include fungi belonging to the *Cryphonectria-Endothia* complex (Gryzenhout *et al.* 2006a). These fungi are characterized by a Diaporthe-type centrum and orange stromatic tissue in culture and on host tissue, as well as biochemical properties such as a pigment reaction with 3 % KOH and lactic acid. While morphological features, such as the degree of development, type and color of stromatic tissues, color and length of perithecial necks, color and shape of conidiomata and ascospore septation are used to distinguish genera in the Cryphonectriaceae, species differentiation is primarily based on spore shape and size (Micales and Stipes 1987; Gryzenhout *et al.* 2009).

Ten genera have been described in the Cryphonectriaceae (Gryzenhout *et al.* 2009; Lumbsch and Huhndorf, 2007), of which some, such as *Cryphonectria* and *Chrysoporthe,* accommodate virulent tree pathogens. For example, *Cryphonectria parasitica* is best known as the causal agent of chestnut blight that has devastated American chestnut trees (*Castanea dentata)* in North America (Anagnostakis 1987; 2001) and Europe (Heiniger and Rigling 1994). Examples of well-known pathogens in *Chrysoporthe* include *Chr. cubensis* and *Chr. austroafricana* (Gryzenhout *et al.* 2004) that have had a serious negative impact on *Eucalyptus* plantations in the tropics and subtropics, causing stem cankers and tree death (Hodges 1980; Wingfield 2003).

Species in the Cryphonectriaceae have a worldwide occurrence and their hosts include native and introduced tree species (Gryzenhout *et al.* 2009). Although the total inventory of trees susceptible to infection by the Cryphonectriaceae is incomplete, more than 100 tree species in over 14 families have been reported as hosts (Gryzenhout *et al.* 2009). In Africa, the Cryphonectriaceae are well known on trees in the Myrtales. Hosts recorded include *Eucalyptus* spp. and *Syzygium* spp. (Myrtaceae) (Gibson 1981; Heath *et al.* 2006; Nakabonge *et al.* 2006a), *Heteropyxis canescens* (Heteropyxidaceae) (Nakabonge *et al.* 2006a), *Tibouchina* spp. (Melastomataceae) (Myburg *et al.* 2002; Nakabonge *et al.* 2006a) and *Terminalia ivorensis* (Combretaceae) (Ofusu-Asiedu and Cannon 1976). These plants occur naturally (*Syzygium* spp., *H. canescens, T. ivorensis*), are grown as a source of pulp and timber (*Eucalyptus* spp. and *T. ivorensis*) or are non-native species grown as ornamentals (*Tibouchina* spp.).

In West Africa, native species of *Terminalia,* such as *T. ivorensis* and *T. superba* are important for forestry. Timber products from *T. ivorensis* and *T. superba* are commercially popular and ranked third in the national round wood export business in Cameroon (Laird 1999). Moreover, they are widely grown as a plantation crop where they are established by direct planting or in the "taungya" system where food crops are grown together with them (Lamb and Ntima 1971; Norgrove and Hauser 2002). Various species of *Terminalia* also provide medical, spiritual and social benefits to rural people (Batawila *et al.* 2005; Kamtchouing *et al.* 2006; Thiombiano *et al.* 2006).

Despite the economic and sociological importance of *Terminalia* spp., little research has been conducted on the fungal diseases affecting these trees (Ofusu-Asiedu and Cannon 1976; Hodges and Fereira 1981; Gryzenhout *et al.* 2005; Kamgan *et al.* 2008). As part of a larger project investigating diseases that affect *Terminalia* spp. in Africa, a survey was undertaken in Cameroon. Distinctive fungi with orange to yellow ascostromata resembling those of the Cryphonectriaceae were commonly observed in the bark of standing native *T. ivorensis*, and on dead branches of non-native *T. mantaly*. The objective of this study was to use DNA sequence and morphological comparisons to provide a taxonomic placement for this fungus. Furthermore, pathogenicity trials were performed to assess its potential ecological significance.

2. MATERIALS AND METHODS

2.1. Survey and specimen collection

Surveys were conducted in the central and southern parts of Cameroon in December 2007. These regions are located in the fifth agro-ecological zone of the country (http://www.irad-cameroon.org/carte_us.php) where the vegetation and climatic conditions are characterized by humid forests with bimodal rainfall and relatively high temperatures, averaging 26 ˚C. At the collection sites, native *T. ivorensi*s is grown in plantations while *T. mantaly* is planted as ornamentals alongside city roads and in villages. Bark segments bearing fungal fruiting bodies were collected from trees showing signs of disease and transported to the laboratory.

For isolation, the ascostromata on the bark were cut horizontally with a scalpel under a dissecting microscope and ascospore masses were extracted with a sterile needle and

transferred onto 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Merck, Midrand, Johannesburg, S.A.). Single germ tubes developing from the spores were transferred to fresh Petri dishes containing MEA and incubated at 25 °C. Pure cultures were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Duplicates of key isolates were also deposited with the Centaalbureau voor Schimmelcultures (CBS, Utrecht, Netherland), while bark specimens bearing fruiting bodies were deposited with the National Collection of Fungi, Pretoria, South Africa (PREM).

2.2. DNA extraction and sequence comparisons

Mycelium was scraped from the surfaces of 10-day-old cultures of five isolates with a sterile scalpel and transferred to 1.5 µl Eppendorf tubes for freeze-drying. The freeze-dried mycelium was ground to a fine powder by shaking for 2 min at 30.0 $1s^{-1}$ frequency in a Retsch cell disrupter (Retsch Gmbh, Germany) using 2 mm-diameter metal beads. Total genomic DNA was extracted following the method of Möller *et al.* (1992) and the concentration of the resulting DNA was determined on a NanoDrop (ND-1000 uv/Vis spectrometer, NanoDrop Technologies, Wilmington, DE USA) version 3.1.0.

The oligonucleotide primer pairs ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) (White *et al.* 1990), Bt1A (5’ TTCCCCCGTC TCCACTTCTTCATG 3’) and Bt1B (5’ GACGAGATCGTTCATGTTGAACTC 3’), Bt2A (5’ GGTAACCAAATCGGTGCTGCTTT C 3’) and Bt2B (5’ ACCCTCAGTGTAGTGACC CTTGGC 3’) (Glass and Donaldson 1995) were used to amplify and sequence the internal transcribed spacer (ITS) regions (including the complete 5.8S) and the β-tubulin 1 and 2 (*β-tub*) gene regions respectively. A “hot start” polymerase chain reaction (PCR) was carried out in an Icycler thermal cycler (BIO-RAD, Hercules, CA, USA) to amplify a 25 µl PCR reaction mixture containing 0.5 µl of each primer (10mM), 2.5 µl dNTPs (10mM), 4 µl of 10 mM $MgCl_2$, 2.5 µl of 10 mM reaction buffer (25 mM), 1 U of *Taq* polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 60-100 ng/µl of DNA and 13.5 µl of sterile SABAX water. The amplification conditions were an initial denaturation at 96 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, annealing for 1 min at 54 °C, extension for 90 s at 72 °C and a final elongation step of 10 min at 72 °C. The PCR amplification products were separated by

electrophoresis on 2 % agarose gels stained with ethidium bromide in a 1x TAE buffer and visualized under UV light.

The amplified PCR fragments were cleaned using 6 % Sephadex G-50 fine mini spin-columns (Sigma, Steinhein, Germany) following the manufacturer's instructions. Thereafter, 25 amplification cycles were carried out for each sample on an Icycler thermal cycler to generate sequences in both the forward and reverse directions using 10 µl mixes. Each mix contained 1µl reaction buffer, 2 µl ready reaction buffer (Big dye), 1 µl primer (10 mM), 3 µl of the PCR product and 3 µl Sabax water. The reaction cycles had the following parameters: one step at 96 °C for denaturation of the double stranded DNA (10 s), followed by an annealing step at 50 °C (5 s) and primer extension at 60 °C (4 min). The BigDye Terminator v 3.1 Cycle sequencing Kit (PE Applied Biosystems) was used for sequencing reactions, following the manufacturer's protocols, on an ABI PRISM 3130xl genetic analyzer using Pop 7 polymer (Applied Biosystems, Foster City, California, USA).

The sequences of the isolates from *Terminalia* spp. were edited using MEGA version 4 (Tamura *et al.* 2007). For the phylogenetic analyses, DNA sequences from this study were compiled into a matrix using the dataset produced by Gryzenhout *et al.* (2009) as a template (TreeBase number: S2003 Matrix M3737). The matrix was aligned using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh *et al.* 2005). The aligned sequences were transferred to PAUP version 4.0b10 (Swofford 2001) where a final manual alignment was made.

Phylogenetic analyses were run for each of the gene region datasets separately, as well as for a combined ITS and *β-tub* data set. In the analyses, gaps were treated as a fifth character (NEWSTATE) and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithm, and random taxon addition for the construction of MP trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. Sequences of two isolates of *Diaporthe ambigua* (Gryzenhout *et al.* 2009), which belong to the Diaporthaceae, another family in the Diaporthales (Castlebury *et al.* 2002; Rossman *et al.* 2007), were used as outgroups in all analyses, following examples of

previously published data sets (Gryzenhout *et al.* 2009). The outgroup was monophyletic in the phylogenetic analyses. The support of the branches for the most parsimonious trees was assessed with a 1000 bootstrap replications (Felsenstein 1985). Other measures noted were tree length, consistency index, rescaled consistency index, and retention index (Hillis and Huelsenbeck 1992). A partition homogeneity test of 500 replicates was conducted in PAUP to assess the possibility of combining the ITS and *β-tub* data sets.

Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with PAUP. Before launching the Bayesian analyses, the best nucleotide substitution models for each dataset were separately determined with MrModelTest version 2.2 (Nylander 2004) and included in each partition in MrBayes v3.1.2. (Huelsenbeck and Ronquist 2001). HKY+I+G were chosen as best-fitting model for both the ITS and *β-tub* datasets. The MCMC analyses, with four chains, started from random tree topology and lasted one million generations. Trees were saved every 100th generation. The burn-in number was graphically estimated from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after 50 % majority rule.

2.3. Morphology

A small piece of an original bark specimen bearing fruiting structures from which the fungal isolates were obtained was cut and boiled in water for 1 min to rehydrate the cells (Myburg *et al.* 2004; Gryzenhout *et al.* 2005). The structures were then broken from the bark and sections (12 µm thick) were made with a Leica CM1100 cryostat (Setpoint Technologies, Johannesburg, South Africa) at -20 °C. Sections were mounted on microscope slides in 85 % lactic acid. Stromata were also crushed on microscope slides in 3 % KOH in order to study the asci, ascospores, conidia, conidiophores and conidiogenous cells. Morphological features of fruiting bodies were photographed with a HRc Axiocam and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., München, Germany). For the holotype specimen, 50 measurements of each structure mentioned above were taken, whereas 20 measurements were made for the paratypes. These measurements were recorded as the extreme in brackets and the range calculated as the mean of the overall measurements plus and minus the standard deviation.

The physiognomy of fungal colonies was described from cultures grown on 2 % MEA at 25 °C under near UV-light for two weeks. Colony colours of the isolates were recorded using the color notations of Rayner (1970). Growth studies for isolates growing on 2 % MEA in the dark was performed by measuring the daily growth at 5 °C intervals ranging from 10 to 35 °C for five replicates of two isolates (ex-holotype isolate CMW28290, ex-paratype isolate CMW28285).

2.4. Pathogenicity

Seedlings of native species of *Terminalia* were not available for pathogenicity tests and these were consequently performed only on non-native *T. mantaly* trees. Pathogenicity experiments were carried out on one-year-old *T. mantaly* plants grown in the Yaoundé Urban Council nursery, Cameroon. The trees were maintained in 15 cm diameter plastic bags and watered daily. At the time of inoculation, the average stem diameter of the trees was approximately 10 mm. For inoculations, isolates were grown on 2 % MEA for 10 days prior to inoculation.

To inoculate trees, wounds were made on the stems, ~ 10 cm above soil level, by removing the outer bark with a 5 mm diameter cork-borer. A 5 mm-diameter plug bearing mycelium of each isolate was placed into each wound, with the mycelium facing the cambium, and wrapped with a strip of Parafilm (Pechiney Plastic Packaging, Chicago, USA) to prevent desiccation and cross contamination. The trees were divided into two separate blocks and within each block, six trees arranged in a completely randomized design, were used for each isolate. The entire trial was repeated once. For the control inoculations, a sterile MEA plug was used. After six weeks, the lengths of the lesions in the cambium were measured to obtain an indication of the virulence of the isolates tested. Single fruiting bodies were removed from the necrotic tissue and these were placed on MEA to produce cultures and to confirm the cause of the lesions. As no significant differences were noticed between the two pathogenicity tests ($P > 0.05$), the data for all isolates were pooled in a single dataset for analyses. Variation in lesion lengths was assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Survey and specimen collection

Terminalia trees at three sites, in two regions, in southern and central Cameroon were inspected. A total of seven trees were found with fruiting bodies of the unknown fungus. On standing *T. ivorensis*, disease symptoms included cankers on the tree trunks (Figure 1a), cracked bark containing yellow to orange fruiting structures (Figure 1b) and necrotic cambium including dead wood. On *T. mantaly*, cankers covered with abundant fruiting bodies were observed on the trunks of dead trees and on senescing branches (Figure 1c). Samples were obtained from one *T. mantaly* tree in Yaoundé, five *T. ivorensis* trees in Mbalmayo in the Central Region, and one *T. mantaly* tree in Kribi in the Southern Region of Cameroon (Figure 2).

3.2. DNA sequence comparisons

Five isolates resembling the Cryphonectriaceae, collected from *Terminalia* spp. in Cameroon, were selected for DNA sequencing (Table 1). Sequencing resulted in fragments of ~600 bps for the ITS and ~550 bps for each of the *β-tub* gene regions. BLAST searches against the NCBI (www.blast.ncbi.nlm.nih.gov) data base confirmed that the isolates collected represented species in the Cryphonectriaceae and indicated that isolates from Cameroon were most closely related to *Microthia havanensis*.

Data sets containing ITS and *β-tub* sequences were compiled using sequences obtained from isolates of Cryphonectriaceae in Cameroon and those obtained from Gryzenhout *et al.* (2009). These data sets comprised a total of 36 isolates each (Table 1), five isolates obtained from *Terminalia* spp. and 31 sequences that were used in the monograph of the Cryphonectriaceae (Gryzenhout *et al.* 2009) that represents the most complete database of sequences for this family.

Of the 592 characters present in the ITS data set, 250 were parsimony-informative. The MP analyses generated 60 identical trees (TL = 568, CI = 0.697, RI = 0.876, RC = 0.611). Isolates from *Terminalia* spp. grouped in a single, well supported clade, distinct from all

other recognized genera of the Cryphonectriaceae. A consensus tree generated through Bayesian analyses of the ITS data confirmed the uniqueness of the isolates from Cameroon.

In the analyses of the *β-tub* data set, sequences consisted of 991 characters with 486 parsimony informative characters of which 316 came from ambiguous portions representing introns (one for the *β-tub* 1 and two for the *β-tub* 2 gene region). The MP analyses yielded four most parsimonious trees (TL = 1382, CI = 0.614, RI = 0.834, RC = 0.512). The tree generated from the *β-tub* data (figure not shown) also separated the isolates from *Terminalia* spp. into a well supported clade, distinct from all the known genera in the Cryphonectriaceae. The tree obtained after Bayesian analyses confirmed results obtained from the MP analyses, suggesting that isolates from Cameroon represent a distinct genus and species. In a separate analyses of *β-tub* sequences without the ambiguous portions representing the introns (384 characters), a similar tree topology with high statistical support was obtained (tree not shown). However, the analysis of *β-tub* sequences containing the introns were preferred as it provided more informative characters.

Concordance among the ITS and *β-tub* datasets was confirmed by the results of the partition homogeneity test (P = 0.002) suggesting a lack of conflict between these gene genealogies, and they were thus combined. A total of 1583 bases were generated for the combined ITS and *β-tub* data sets. Of these, 735 characters were parsimony informative. After heuristic searches, one most parsimonious tree of 1980 steps (CI = 0.628, RI = 0.839, RC = 0.527; TreeBase Accession No: SN4451) was obtained (Figure 3). The consensus tree obtained from the combined analysis of ITS and *β-tub* sequences showed that isolates of the unknown fungus from *Terminalia* spp. formed a well supported clade (Bayesian Posterior Probability (BPP)/Bootstrap support (BS): 1/100). This clade is distinct from other phylogenetically related genera in the Cryphonectriaceae (Gryzenhout *et al.* 2009).

3.3. Morphology

Consistent with DNA sequence data, the fruiting bodies from *Terminalia* spp. showed typical microscopic characteristics of members of the Cryphonectriaceae (Table 2). These fruiting structures were characterized by distinct orange stromatic tissue, Diaporthe-type centra (Gryzenhout *et al.* 2006a) and a pigment that turns purple and yellow in culture and host

tissue when treated with 3 % KOH and lactic acid respectively (Castlebury *et al.* 2002; Rossman *et al.* 2007; Gryzenhout *et al.* 2009). The teleomorph fruiting structures were abundant on the bark, while conidiomatal structures were only occasionally seen on the bark specimens.

The fungus on *Terminalia* spp. in Cameroon resembled those species in the Cryphonectriaceae that have uniformly orange fruiting bodies as opposed to those with different colors between their anamorph and teleomorph states, such as species of *Chrysoporthe* (Gryzenhout *et al.* 2009). However, it had a number of features distinguishing it from all other Cryphonectriaceae. The most obvious of these characteristics were present in the anamorph. Conidiomata of the fungus from Cameroon were broadly convex, and thus wider than similar structures of *Amphilogia* and *Rostraureum* (Table 2). The presence of darkened ostiolar openings at the apex of the conidiomata was also unique to the fungus. Long paraphyses, or seemingly sterile cells (< 90 µm) (Walker *et al.* 1985; Venter *et al.* 2002), were observed between conidiophores, similar to those found for conidiomata of *Holocryphia* and *Microthia* (Gryzenhout *et al.* 2006b).

Gryzenhout *et al.* (2005), provided details of morphological characteristics of African specimens (IMI 187898 and IMI 288729) of Cryphonectriaceae obtained from *T. ivorensis* in Ghana and Kenya, respectively. The same specimens were also considered in this study. Conidiomata of the Ghanaian specimen were characterized by orange, pulvinate conidiomata without elongated necks, similar to the conidiomata of the Cameroonian specimens that are orange and broadly convex. The stromatic ascostromata of specimens from both Ghana and Kenya resemble the Cameroonian isolates. The similarity of specimens from Cameroon, Ghana and Kenya was, furthermore, supported by spore characteristics (one septate, fusoid to ellipsoid ascospores and minute, cylindrical conidia) and overlapping spore dimensions. The presence of conidiophores was not mentioned for specimens from Ghana and Kenya (Gryzenhout *et al.* 2005) and they were not present in specimens examined in this study.

3.4. Taxonomy

Comparisons of DNA sequences and morphology of the fungus from Cameroon with the genera in the Cryphonectriaceae revealed that the fungus from Cameroon represents a

previously undescribed genus in the family. A new genus and the linked species are described as follows:

Aurifilum Begoude, Gryzenh. & Jol. Roux, gen. nov.

Etymology

The name is derived from the Latin *Aureus* (golden) and *filum* (thread) referring to the orange, confluent stromata found in the cracks on the bark of infected trees.

Ascostromata magna, plerumque sub cortice vel erumpentia, pulvinata vel pyriformia, subimmersa. Ascosporae hyalinae, fusoideae vel ellipsoideae, septo singulo mediano.

Conidiomata ascomatorum partes pro loculis conidialibus vel structuris solitariis, aurantiaca cum apertura ostiolari, sine collo atrato, late convexa, subimmersa. Conidiophorae cylindricae hyalinae non septatae, cellulae conidiogenae phialidicae basibus inflatis apicibus attenuatis, cellulae nonnullae cylindricae steriles, paraphysibus similes. Conidia minuta hyalina cylindrica vel allantoidea, non septata. Species typical *A. marmelostoma Begoude et al.*

Ascostromata large, usually beneath or erumpent through bark, pulvinate to pyriform, semi-immersed, orange, upper region eustromatic, lower region pseudostromatic, pseudoparenchymatous to prosenchymatous tissue. *Perithecia* valsoid, embedded in stroma, fuscous black, bases globose to subglobose, necks emerge at stromatal surface with black ostioles, surround with orange stromatal tissue to form papillae. *Asci* fusoid to ellipsoidal, floating freely in perithecial cavity, unitunicate with non-amyloid, refractive apical ring. *Ascospores* hyaline, fusoid to ellipsoidal, one median septum.

Conidiomata part of ascomata as conidial locules or as solitary structures, orange, without a neck, tissue around ostiolar opening darkened, broadly convex, semi-immersed, uni- to multilocular, even to convoluted lining, tissue mostly prosenchymatous with pseudoparenchymatous tissue towards the margin depending on the developmental stage of the structures. *Conidiophores* cylindrical, aseptate, hyaline, conidiogenous cells phialidic with inflated bases and attenuated apices, some cylindrical cells sterile similar to paraphyses. *Conidia* minute, hyaline, cylindrical to allantoid, aseptate, exuded through ostioles as orange droplets or tendrils.

Aurifilum marmelostoma Begoude, Gryzenh. & Jol. Roux, sp. nov. MB 513488 Fig. 4

Etymology

The word "marmelo" is Greek for confectionary cooking practice using quinces with honey and from which the jam known as marmalade is derived. The name refers to the darkened stomatal (stoma = mouth) opening of the conidiomata giving the impression that they are covered with jam.

Ascostromata in cortice gregaria vel singula, saepe in rimis confluentia, mediocria vel magna, 300.0–830.0 µm supra corticem 760.0–1050.0 µm diametro crescentia, plerumque sub cortice vel erumpentia, subimmersa, pulvinata vel pyriformia, aurantiaca. Ascosporae hyalinae, fusoideae vel ellipticae, septo singulo mediano apice attenuata (9.0–) 10.0–12.0 (–13.5) x 3.0–4.0 (–4.5) µm.

Conidiomata ascomatarum partes pro loculis conidialibus vel structuris solitariis, aurantiaca, sine collis, apertura ostiolari atrata, late convexa, subimmersa, usque ad 660.0 µm supra superficiem corticis et 660.0 µm lata. Conidia minuta hyalina cylindrica vel allantoidea non septata (3.0–) 3.5–4.5 (–5.0) x 1.0–1.5 (–2.5) µm, pro guttulis vel cirrhis aurantiacis per aperturam in superficie stromatis exsudata.

Ascostromata on bark gregarious or single, often confluent in cracks, medium to large, ascostromata extending 300.0–830.0 µm high above the bark, 760.0–1050.0 µm diam, usually beneath or erumpent through bark, semi-immersed, pulvinate to pyriform, orange, upper region eustromatic, lower region pseudostromatic, pseudoparenchymatous to prosenchymatous tissue. *Perithecia* valsoid, up to nine per stroma, embedded in stroma at irregular levels with bases touching host tissue, fuscous black, bases globose to subglobose, 190.0–310.0 µm diam, perithecial walls 170.0–275.0 µm thick. Perithecial necks periphysate, black, 30.0–100.0 µm wide, emerging at stromatal surface as black ostioles, surrounded with orange stromatal tissue to form papillae, *textura porrecta*, extended necks up to 550.0 µm long. *Asci* fusoid to ellipsoidal, floating freely in the perithecial cavity, unitunicate with non-amyloid, refractive apical rings, non-stipitate, 8-spored, (44.5–) 47.0–53.5 (–61.0) × (7.0–) 7.5–9.0 (–10.5) µm. *Ascospores* hyaline, fusoid to ellipsoidal, one median septum with tapered apex, (9.0–) 10.0–12.0 (–13.5) × 3.0–4.0 (–4.5) µm.

Conidiomata part of ascomata as conidial locules or as solitary structures, orange, necks absent, tissue around ostiolar openings darkened, broadly convex, semi-immersed, up to 660.0 µm above the bark surface and up to 600.0 µm in diam, uni- to multilocular, even to convoluted lining, locule 80.0–300.0 µm diam, tissue mostly prosenchymatous with

pseudoparenchyma towards the margin depending on the developmental stage of the structure. *Conidiophores* cylindrical, aseptate, hyaline, (8.5–) 15.5–41.5 (–58.5) µm long, conidiogenous cells phialidic, sometimes with inflated bases, collarettes inconspicuous with attenuated apexes, (2.0–) 2.5–3.5 (–4.5) µm wide, long sterile cylindrical cells similar to paraphyses present, (22.5–) 33.5–66.0 (–89.0) x 2.5–3.5 (–4.0) µm. *Conidia* minute, hyaline, cylindrical to allantoid, aseptate, exuded through opening at stromatal surface as orange droplets or tendrils, (3.0–) 3.5–4.5 (–5.0) x 1.0–1.5 (–2.5) µm.

Cultural characteristics — mycelium fluffy, slightly aerial, creamy white to pale luteous. Conidiomata produced occasionally on old cultures. Optimum temperature of growth 25–30 °C, covering the 90 mm diameter Petri plate after one week in the dark. No growth at 10 and 35 °C.

Hosts — *Terminalia mantaly* H. Perrier, *Terminalia ivorensis* A. Chev.

Distribution — Cameroon: Kribi, Mbamalyo and Yaounde.

Specimens examined: CAMEROON, Mbamalyo, bark of *Terminalia ivorensis*, Dec 2007, *D. Begoude and J. Roux*, PREM 60256 – holotype, CMW28290/CBS124928 ex-type culture.

Additional specimens: CAMEROON, Yaoundé, bark of dead branches of *Terminalia mantaly*, Dec 2007, *D. Begoude and J. Roux*. PREM 60257 – Paratype, living cultures CMW28285/CBS124929; Mbalmayo: isolated from bark of *Terminalia ivorensis*, Dec 2007, *D. Begoude and J. Roux*. Paratype, living cultures CMW28288/CBS124930, CMW28592, CMW28289. The specimens, IMI 288729 and IMI 187898 obtained from *Terminalia* spp. in Ghana and Kenya, respectively, were also used, but could not be sequenced.

To facilitate identification of this genus, the dichotomous key presented by Gryzenhout *et al.* (2009) was updated to include this fungus. In this key, *A. marmelostoma* is compared with all members of the Cryphonectriaceae including those with dark fruiting bodies.

This key is based on characteristics of both the anamorph and teleomorph.

1a. Orange conidiomata ……..…….....…….. 2

1b. Black conidiomata ……..…….....……. 9

2a. Conidiomata pulvinate, ascospores septate or aseptate …… 3

2b. Conidiomata conical or rostrate or pyriform or convex, with or without a neck, ascospores septate …… 6

3a. Ascospores septate ……... 4

3b. Ascospores aseptate …….. 5

4a. Stromata strongly developed, erumpent, semi-immersed, usually no paraphyses..……..***Cryphonectria***

4b. Stromata small to medium, semi-immersed to superficial, paraphyses present ……..……...… ***Microthia***

5a. Stromata strongly developed, large, erumpent, mostly superficial, numerous conidial locules, no paraphyses in conidial locules .....……..…….. ***Endothia***

5b. Stromata small to medium, semi-immersed, few conidial locules or one convoluted locule, paraphyses in conidial locules ……... ***Holocryphia***

6a. Conidiomata with necks, ascospores single septate ……..……...…… 7

6b. Conidiomata without necks, ascospores septate…….…….....8

7a. Conidiomata rostrate, white sheath of tissue surrounding perithecial necks when sectioned longitudinally ……..... ***Rostraureum***

7b. Conidiomata rostrate to pyriform with large base, neck attenuated or not, teleomorph still unknown …….……...…… ***Ursicollum***

8a. Conidiomata conical without attenuated necks, uniformly orange, ascospores 1 to 3-septate…….....……. ***Amphilogia***

8b. Conidiomata convex, with blackened ostiolar openings, ascospores 1-septate ....***Aurifilum***

9a. Conidiomata uniformly black …….…… 9

9b. Conidiomata with orange neck, teleomorph still unknown …….... ***Aurapex***

10a. Conidiomata pulvinate to pyriform with attenuated neck, base tissue of *textura globulosa* when sectioned longitudinally, perithecial necks long and covered with dark tissue ……..……..... ***Chrysoporthe***

10b. Conidiomata pulvinate or conical, occasionally with short necks, base tissue prosenchymatous, perithecial necks short and of same color as stroma ……..……. ***Celoporthe***

3.5. Pathogenicity

Six weeks after inoculation, all isolates of *A. marmelostoma* yielded visible stem cankers on *T. mantaly* trees (Figure 5). Analysis of variance showed that lesion lengths on the cambium for all isolates were significantly different ($P < 0.0001$) to those associated with the negative control (Figure 5). Isolate CMW28290 from *T. ivorensis,* was the most virulent and produced significantly longer lesions in the cambium than the other isolates. Orange-colored fruiting bodies were observed on the lesions produced by all the isolates, and these provided the basis to confirm the association of *A. marmelostoma* with the lesions resulting from inoculation. In contrast, all control lesions were surrounded by callus, with no necrotic lesions, indicating a healed wound.

4. DISCUSSION

The Cryphonectriaceae was described as a family to accommodate fungal species previously treated in the *Cryphonectria-Endothia* complex (Gryzenhout *et al.* 2006a). This study records the discovery of a previously unknown genus in the Cryphonectriaceae with a single species. Comparisons of DNA sequences of isolates representing all the genera in the Cryphonectriaceae suggest that this fungus represents a new taxon, for which the name *Aurifilum marmelostoma* was provided.

Aurifilum marmelostoma shares characteristics with several taxa in the Cryphonectriaceae, especially those with uniformly orange fruiting bodies. While the teleomorph state was

especially similar to those of other Cryphonectriaceae, a suite of characters in the anamorph of *A. marmelostoma* differentiate this species. The broadly convex conidiomata without necks were similar but wider than conidiomata of *Amphilogia* and *Rostraureum*, and different in shape from the conidiomata of *Cryphonectria*, *Endothia*, *Holocryphia* and *Microthia* that have pulvinate structures. Furthermore, the ostiolar openings of *A. marmelostoma* were often darkened while this has not been observed for any other anamorph in the Cryphonectriaceae. The presence of long cylindrical cells, similar to paraphyses (Walker *et al.* 1985; Venter *et al.* 2002), provides additional characteristic to differentiate the anamorph of *A. marmelostoma* from the anamorphs of morphologically similar Cryphonectriaceae.

The morphological comparison of specimens previously obtained from *T. ivorensis* in Ghana and Kenya with those isolated from *Terminalia* spp. in Cameroon revealed some similarities in their teleomorph states. However, the presence of the long sterile cells, or paraphyses, present in anamorphs of the Cameroonian specimens were not observed in the other African specimens. Because molecular evidence to support morphological findings is not available, it is difficult to provide a conclusive taxonomic position to the specimens associated with *T. ivorensis* in the other African countries.

Aurifilum marmelostoma produced more ascostromata on the bark of *Terminalia* spp. than asexual fruiting structures. This inconsistency in the production of sexual and asexual fruiting structures is well-known in other species of the Cryphonectriaceae. For example, *Chr. austroafricana* in South Africa produces ascostromata on native *Syzygium* spp., but rarely produces these structures on non-native *Eucalyptus* spp. (Van Heerden and Wingfield 2001; Heath *et al.* 2006; Nakabonge *et al.* 2006b). However, in countries such as Malawi, Mozambique and Zambia, *Chr. austroafricana* produced both sexual and asexual structures on *Syzygium* spp. and *Eucalyptus* spp. (Nakabonge *et al.* 2006b). Surveys in other regions and on hosts other than *Terminalia* spp. will thus be necessary to determine whether the production of more sexual than asexual structures is consistent regardless of host or location. This is important since it is the anamorph structures that distinguish *A. marmelostoma* from related genera and species of Cryphonectriaceae with orange fruiting bodies.

Results of the pathogenicity trials showed that all isolates were pathogenic to young *T. mantaly* trees. This result and the consistent association of *A. marmelostoma* with disease symptoms on *T. ivorensis* suggest that the fungus is a pathogen of *Terminalia* trees. However,

pathogenicity tests on *T. ivorensis* will be needed to provide conclusive evidence of its impact and threat to these important native trees. Furthermore, *A. marmelostoma* caused lesions on *T. mantaly* during nursery inoculations, but on mature trees the fungus was present only on cut and dying branches lying on the ground.

Members of the Cryphonectriaceae are well known to occur on Myrtales in Africa. Prior to this study, three genera, including *Chrysoporthe*, *Celoporthe* and *Holocryphia,* were reported infecting trees in the Combretaceae, Heteropyxidaceae, Melastomataceae and the Myrtaceae (all Myrtales) in the sub-Saharan part of the continent (Ofusu-Asiedu and Cannon 1976; Gibson 1981; Myburg *et al.* 2002; Roux *et al.* 2003; Heath *et al.* 2006; Nakabonge *et al.* 2006b). Although members of the Cryphonectriaceae have been reported from Africa regularly (Gibson 1981; Conradie *et al.* 1990; Myburg *et al.* 2002; Gryzenhout *et al.* 2003; Roux *et al.* 2003; 2005; Nakabonge *et al.* 2006a,b), it is clear that their geographical and host distribution on the continent deserves further study.

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Table 1. Isolates of the Cryphonectriaceae used in this study.

Species	Isolates number [a]	Host	Origin	Collector	Genbank accession numbers [b]
Amphylogia gyrosa	CMW 10469	*Elaeocarpus dentalus*	New Zealand	G.J. Samuels	AF452111, AF525707, AF525714
	CMW 10740	*E. dentalus*	New Zealand	G.J. Samuels	AF452112, AF525708, AF525715
Aurapex penicillata	CMW 10030	*Miconia theazeans*	Colombia	C.A. Rodas	AY214311, AY214239, AY214275
	CMW 10035	*M. theazeans*	Colombia	C.A. Rodas	AY214313, AY214241, AY214277
Aurifilum marmelostoma	**CMW 28285**	*Terminalia mantaly*	Cameroon	D. Begoude and J. Roux	FJ882855, FJ900585, FJ900590
	CMW 28288	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ882856, FJ900586, FJ900591
	CMW 28289	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ890495, FJ900587, FJ900592
	CMW 28290	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ890496, FJ900588, FJ900593
	CMW 28592	*T. mantaly*	Cameroon	D. Begoude and J. Roux	FJ890497, FJ900589, FJ900594
Celoporthe dispersa	CMW 9976	*Syzygium cordatum*	South Africa	M. Gryzenhout	DQ267130, DQ267136, DQ267142
	CMW 9978	*S. cordatum*	South Africa	M. Gryzenhout	AY214316, DQ267135, DQ267141
Chrysoporthe	CMW 2113	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF046892, AF273067, AF273462

Species	Isolates number [a]	Host	Origin	Collector	Genbank accession numbers [b]
austroafricana	CMW 9327	*Tibouchina granulosa*	South africa	M.J. Wingfield	AF273473, AF273060, AF273455
Chrysoporthe cubensis	CMW 10639	*Eucalyptus grandis*	Colombia	C.A. Rodas	AY263419, AY263420, AY263421
	CMW 10669	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF535122, AF535124, AF535126
Chrysoporthella hodgesiana	CMW 10625	*Tibouchina semidecandra*	Colombia	R. Arbelaez	AY956970, AY956979, AY956980
	CMW 10641	*T. semidecandra*	Colombia	R. Arbelaez	AY692322, AY692326, AY692325
Cryphonectria parasitica	CMW 7048	*Quercus vaginiana*	USA	F.F. Lombard	AF368330, AF273076, AF273470
	CMW 13749	*Castanea mollisima*	Japan	unknown	AY697927, AY697943, AY697944
Cryphonectria japonica	CMW 13742	*Quercus grosseserrata*	Japan	T. Kobayashi	AY697936, AY697961, AY697962
	CMW 13747	*Q. serrata*	Japan	T. Kobayashi	AY697937, AY697963, AY697964
Cryhonectria radicalis	CMW 10455	*Castanea dendata*	Italy	A. Biraghi	AF452113, AF525705, AF525712
	CMW 10477	*Quercus suber*	Italy	M. Orsenigo	AF368328, AF368347, AF368346
Cryptodiaporthe corni	CMW 10526	*Cornus alternifolia*	USA	S. Redlin	DQ120762, DQ120769, DQ120770
Diaporthe ambigua	CMW 5288	*Malus domestica*	South Africa	W.A Smit	AF543817, AF543819, AF543821

Species	Isolates number [a]	Host	Origin	Collector	Genbank accession numbers [b]
	CMW 5587	*M. domestica*	South Africa	W.A. Smit	AF543818, AF543820, AF543822
Endothia gyrosa	CMW 2091	*Quercus palustris*	USA	R.J. Stipes	AF046905, AF368337, AF368336
	CMW 10442	*Q. palustris*	USA	R.J. Stipes	AF368326, AF368339, AF368338
Holocryphia eucalypti	CMW 7036	*Eucalyptus* sp.	South Africa	I. van der Westhuizen	AF232878, AF368341, AF368340
	CMW 7037	*Eucalyptus delegatensis*	Australia	K.M. Old	AF232880, AF368343, AF368342
Microthia havanensis	CMW 14550	*Eucalyptus saligna*	Mexico	C.S. Hodges	DQ368735, DQ368741, DQ368742
	CMW 11301	*Myrica faya*	Azores	C.S. Hodges and D.E. Gardner	AY214323, AY214251, AY214287
Rostraureum tropicale	CMW 9971	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	AY167425, AY167430, AY167435
	CMW10796	*T. ivorensis*	Ecuador	M.J. Wingfield	AY167428, AY167433, AY167438
Ursicullum fallax	CMW 18115	*Cocoloba uvifera*	USA	C.S. Hodges	DQ368756, DQ368760, DQ368761
	CMW 18119	*C. uvifera*	USA	C.S. Hodges	DQ368755, DQ368758, DQ368759

[a] **CMW**, Research collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[b] Accesssion numbers given as sequences from the ITS region, and two regions from the β-tubulin genes respectively.

Table 2. Morphological characteristics of genera in the Cryphonectriaceae with uniformly orange fruiting bodies, compared with those of *Aurifilum.*

	Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*
Teleomorph	**Structure of ascostroma**	Pulvinate, erumpent, slightly immersed to superficial	Large, pulvinate, erumpent, semi-immersed	Large, pulvinate to clavate, erumpent, superficial	Pulvinate, semi-immersed	Large, pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, immersed to semi-immersed	Not known	**Large, pulvinate to pyriform, semi-immersed**
	Ascospore shape	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Hyaline, cylindrical	Hyaline, cylindrical	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Not known	**Hyaline, ellipsoidal to fusoid**
	Ascospore septation	1-3 septate	One septate	Aseptate	Aseptate	One septate	One septate	Not known	**One septate**
Anamorph	**Structure of conidiomata**	Conical, superficial	Pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, superficial,	Pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, semi-immersed	Clavate to rostrate	Pyriform or rostrate, superficial	**Broadly convex**
	Conidiomatal neck	Absent	Absent	Absent	Absent	Absent	Present	Present	**Absent, ostiolar opening darkened**
	Conidiomatal stromatic tissue	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Of different textura type	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma

Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*
Paraphyses	Absent	Absent	Absent	Present	Present	Absent	Absent	**Present**

Figure 1. Symptoms of infection by *A. marmelostoma* on *Terminalia* spp. in Cameroon. a. canker on the basal parts of a *T. ivorensis* trunk; b. bark cracks containing yellow to orange fruiting structures; c. orange stromata on bark of *T. mantaly*.


a
b
c

Figure 2. Map of Cameroon showing sites where *Aurifilum marmelostoma* was collected from *Terminalia ivorensis* and *T. mantaly*.

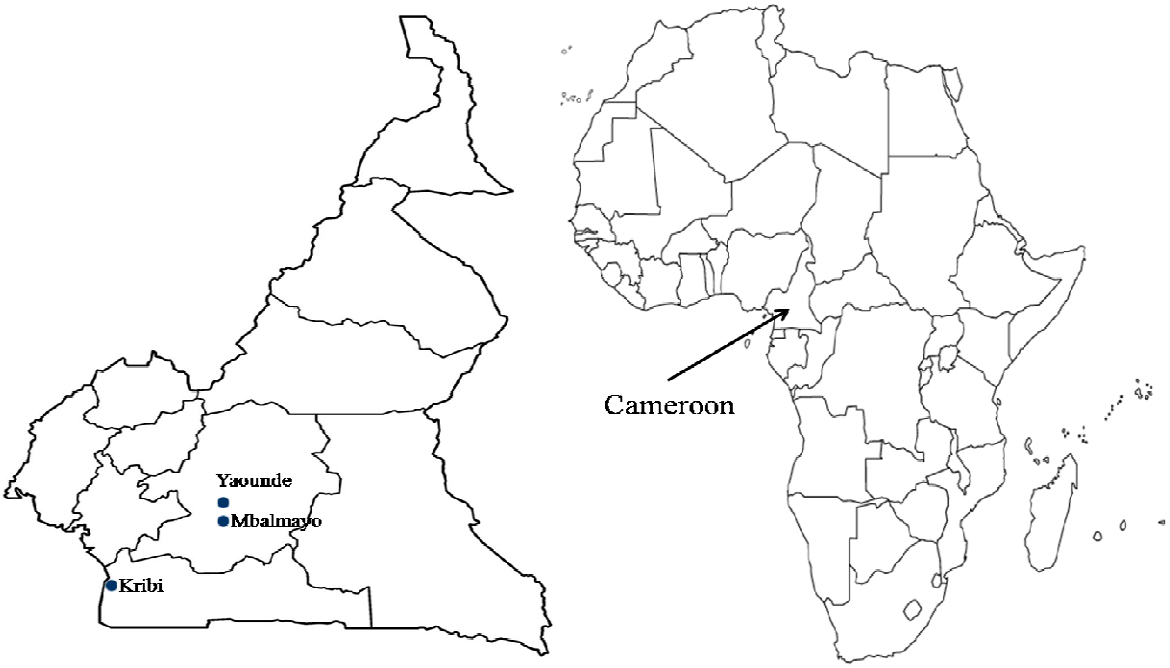

Yaounde
Mbalmayo
Kribi
Cameroon

Figure 3. Most parsimonious tree obtained from MP analyses of the combined ITS and BT sequence data of the Cryphonectriaceae. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (BPP/BS). Isolates marked in bold represent those obtained from *Terminalia* spp. — Scale "10 changes" reflects the graphical amount of nucleotide change between two sequences since their divergence from the common ancestor.

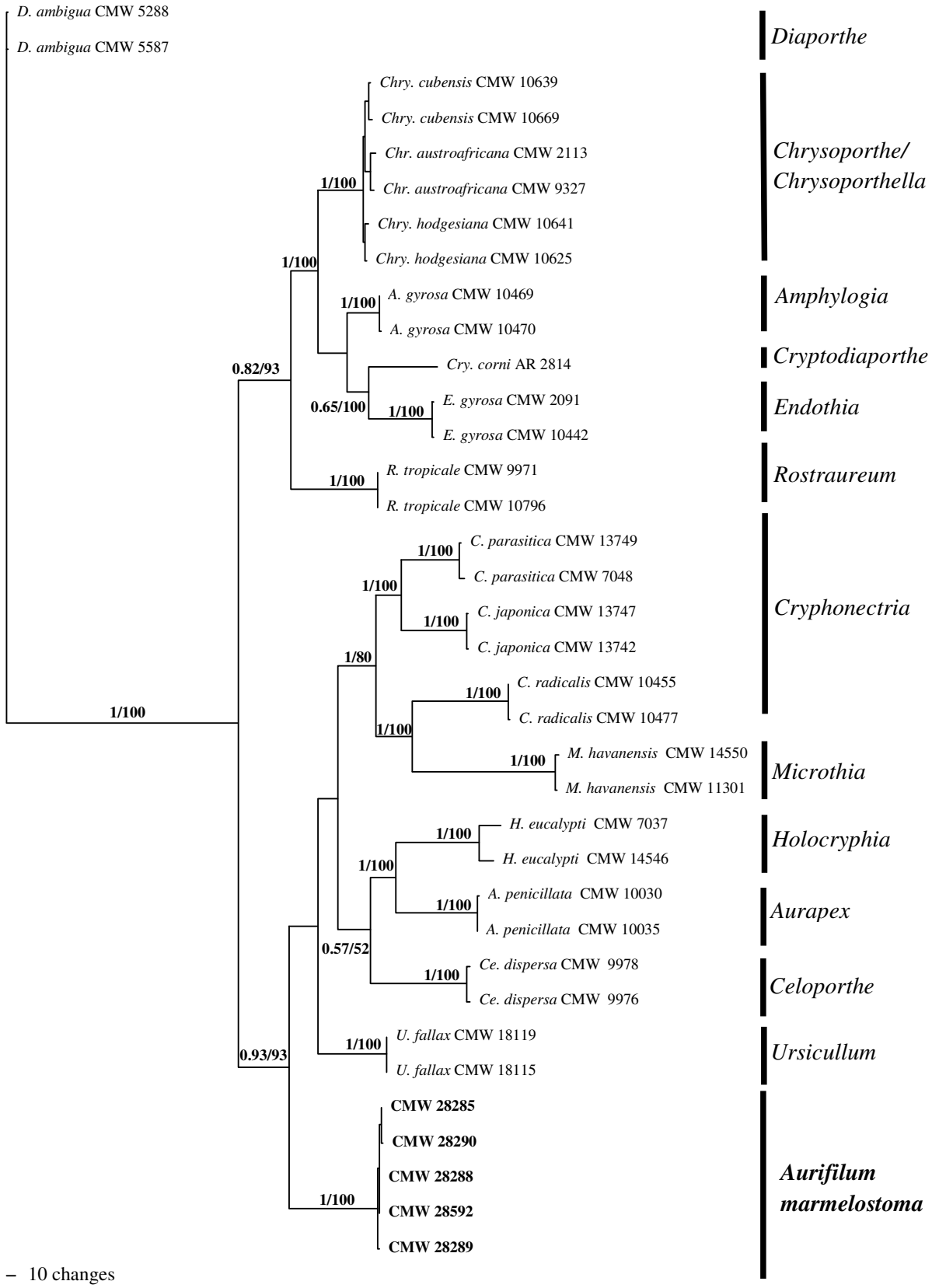

D. ambigua CMW 5288
D. ambigua CMW 5587
Chry. cubensis CMW 10639
Chry. cubensis CMW 10669
Chr. austroafricana CMW 2113
Chr. austroafricana CMW 9327
Chry. hodgesiana CMW 10641
Chry. hodgesiana CMW 10625
A. gyrosa CMW 10469
A. gyrosa CMW 10470
Cry. corni AR 2814
E. gyrosa CMW 2091
E. gyrosa CMW 10442
R. tropicale CMW 9971
R. tropicale CMW 10796
C. parasitica CMW 13749
C. parasitica CMW 7048
C. japonica CMW 13747
C. japonica CMW 13742
C. radicalis CMW 10455
C. radicalis CMW 10477
M. havanensis CMW 14550
M. havanensis CMW 11301
H. eucalypti CMW 7037
H. eucalypti CMW 14546
A. penicillata CMW 10030
A. penicillata CMW 10035
Ce. dispersa CMW 9978
Ce. dispersa CMW 9976
U. fallax CMW 18119
U. fallax CMW 18115
CMW 28285
CMW 28290
CMW 28288
CMW 28592
CMW 28289
1/100
1/100
1/100
1/100
1/100
0.82/93
0.65/100
1/100
1/100
1/100
1/100
1/100
1/80
1/100
1/100
1/100
1/100
1/100
0.57/52
1/100
1/100
0.93/93
1/100
Diaporthe
Chrysoporthe/
Chrysoporthella
Amphylogia
Cryptodiaporthe
Endothia
Rostraureum
Cryphonectria
Microthia
Holocryphia
Aurapex
Celoporthe
Ursicullum
Aurifilum
marmelostoma
– 10 changes

Figure 4. Fruiting structures of *Aurifilum marmelostoma*. a. orange ascostromata on bark; b. vertical section through ascostromata; c. ascus; d. ascospores; e. stromatic tissue of ascostromata; f. conidiomata showing a black ostiolar opening; g. vertical section though conidiomata;, h. stromatic tissue of conidiomata; i. conidia; j. conidiophores and sterile paraphyses (arrows); k. conidiophores. — Scale bars: a,d,f,i,j,k = 10 µm; c, e, g = 20 µm; b = 50 µm.

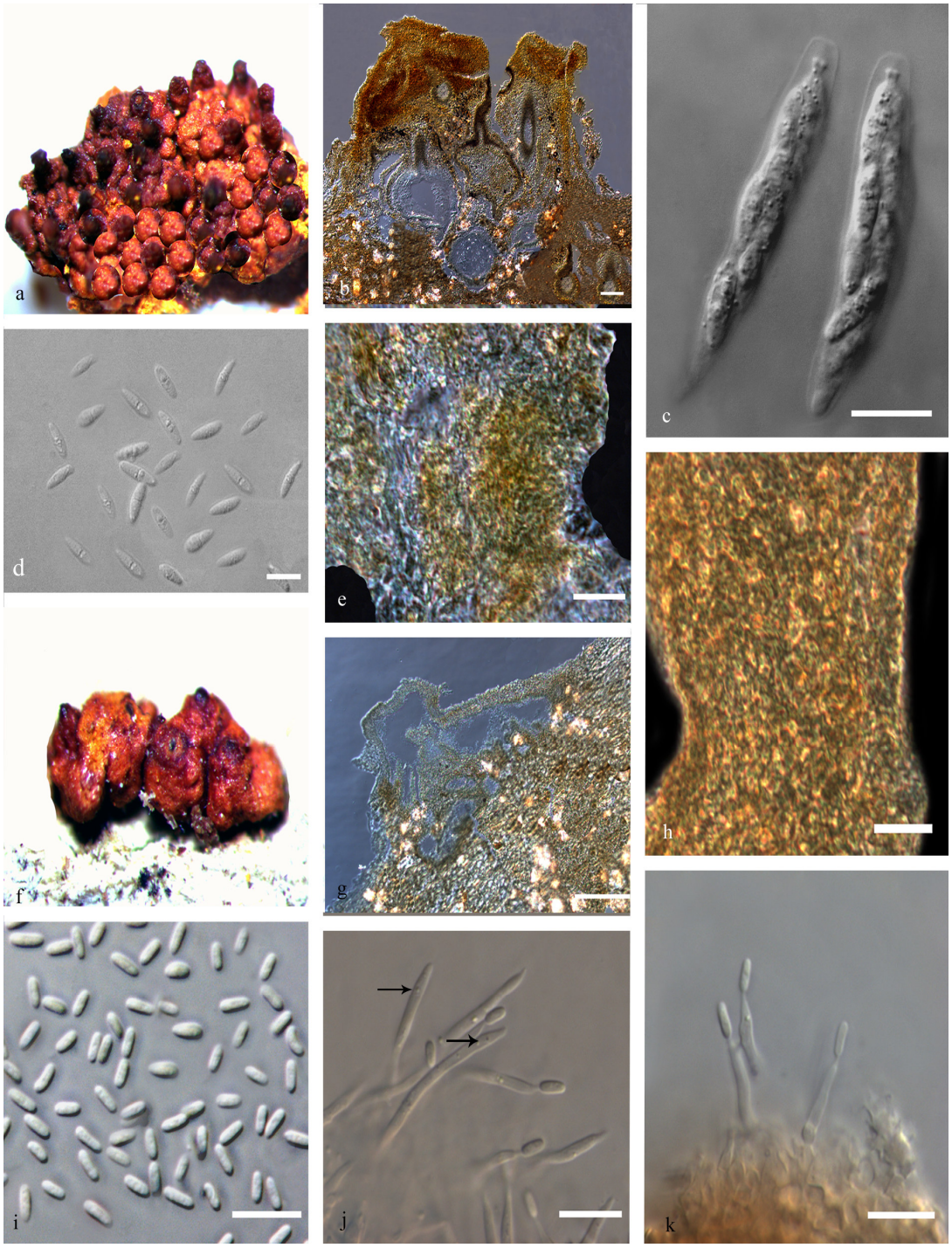
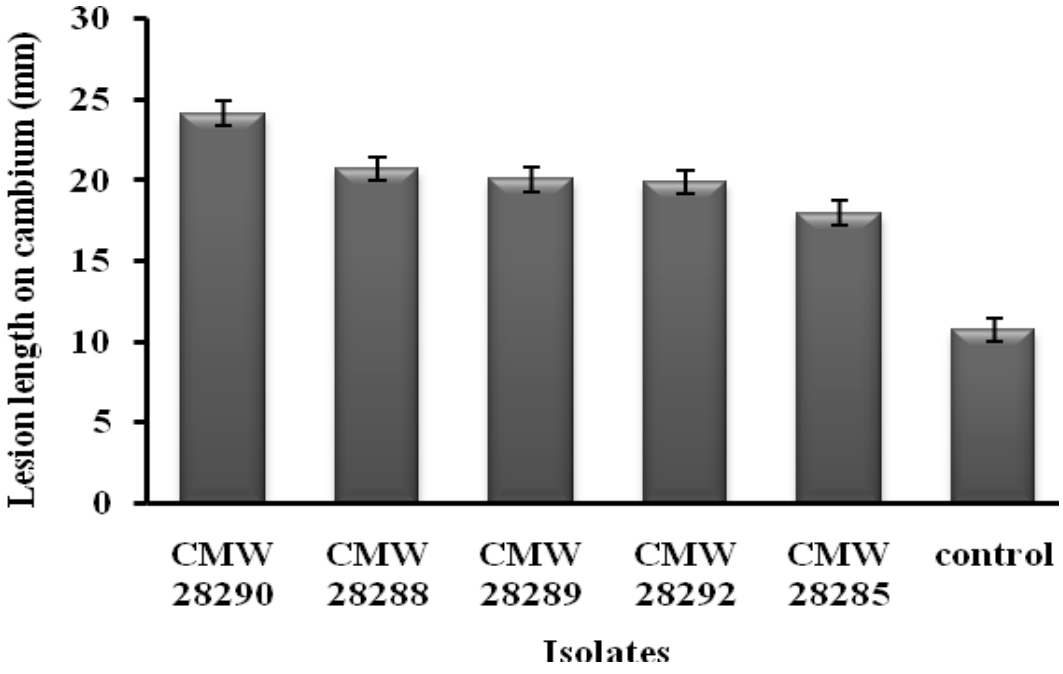

a
b
c
d
e
f
g
h
i
j
k

Figure 5. Mean lesion lengths (mm) on the cambium for each *A. marmelostoma* isolate six weeks after inoculation on *T. mantaly* ($P < 0.0001$).


Lesion length on cambium (mm)
30
25
20
15
10
5
0
CMW 28290
CMW 28288
CMW 28289
CMW 28292
CMW 28285
control
Isolates

Summary

Fungal diseases of trees are serious impediments to forest expansion and productivity. While the adverse effects of diseases of non-native plantation forest trees are increasingly recognized in Africa, the health of native trees has been virtually ignored. This is especially acute as many commercially propagated trees are related to native woody plants and recurrent movement of pathogens between native and non-native trees is increasingly being recorded.

The genus *Terminalia* accommodates important indigenous tree species in Africa that provide a variety of valuable wood and non-wood products. Despite the importance of *Terminalia* spp., very little information has been recorded on their health problems. Research for this dissertation aimed at studying some of the fungal diseases of native and non-native *Terminalia* species in Africa. Special reference was provided to fungi in the Botryosphaeriaceae, a group of pathogens that infect host plants without always producing clear symptoms.

A literature review, presented in Chapter One, highlighted the limited information available on the pathogens and pests associated with these trees. Most disease reports include minimal detail. Often the causal agents had been identified based only on morphology and were not classified to the species level.

Studies performed in chapter two compared the assemblages of endophytic species of the Botryosphaeriaceae from *T. catappa* in Cameroon, South Africa and Madagascar. Five species were identified and two of these are new to science and provided with the names *N. batangarum* and *L. mahajangana.* The assemblage of the Botryosphaeriaceae varied from one country to another, however, colonization patterns in the three areas were similar. Greenhouse inoculation trials performed on young *T. catappa* trees showed variation among isolates tested, with *L. pseudotheobromae* being the most pathogenic.

Chapter three represents the first investigation of the Botryosphaeriaceae on native *Terminalia* trees in Cameroon and Africa. Morphological and DNA sequence data were used to identify these species as *L. theobromae, L. pseudotheobromae, L. parva* and *E. endophytica.*

Pathogenicity trials performed on young *T. mantaly* and *T. catappa* trees revealed that *L. pseudotheobromae* was the most virulent species.

In chapter four, species of Botryosphaeriaceae on native *Terminalia* spp. in Southern Africa were studied. Nine species were identified from *T. sericea* and *T. sambesiaca*. Seven of these, *L. crassispora, L. pseudotheobromae, D. alatafructa, P. olivaceum, N. parvum, N. kwambonambiense* and *N. vitifusiforme* represented previously described species and two, *L. cryptotheobromae* and *N. terminaliae,* were described as new.

The genetic analyses of populations of *L. theobromae* and *L. pseudotheobromae* presented in chapter five showed clear genetic divergence between *L. theobromae* and *L. pseudotheobromae* supporting their distinction as separate species. Both *L. theobromae* and *L. pseudotheobromae* populations possessed high gene diversity, moderate degrees of genotypic diversity, and high levels of gene flow between isolates from *T. cacao* and *Terminalia* spp. Both sexual and asexual modes of reproduction were found.

The last chapter of this dissertation presents the description of a new genus in the Cryphonectriaceae, *Aurifilum marmelostoma,* using a combination of molecular and morphological tools. This fungus is the second genus in this family described from *Terminalia* spp. Pathogenicity tests conducted to assess its ability to cause disease revealed that the fungus is a possible pathogen of this tree.

An extensive number of potentially pathogenic fungi, including five new species were found associated with native and non-native *Terminalia* spp. in Africa. As some of these fungal species could serve as sources of inoculum onto economically important crops, research presented in this dissertation provides a foundation for understanding health issues affecting *Terminalia* and related genera in selected regions in Africa. Hopefully, results of this study will serve as valuable tools in forestry management in Africa.