Diseases associated with plantation forestry in Uganda

By

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B. Sc. Hons (Makerere University)

A Thesis submitted in fulfilment of the requirements for the degree

Magister Scientiae

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December 2002

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I dedicate this thesis to my dear friends and parents

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following people and institutions:

To the Norwegian Agency for International Development (NORAD), Strengthening of the Ugandan Forestry Department and the Faculty of Forestry and Nature Conservation, Makerere University for financial assistance provided.

My gratitude to Dr. J. R. S. Kaboggoza, your encouragement, the strength you gave me to persevere, your support and guidance, without which the completion of this study would not have been possible.

To Dr. Jolanda Roux for introducing me to the field of forest pathology, your guidance throughout this study, the knowledge you provided, being there during the times I needed your help and for the long hours you spent editing my scripts.

Prof. M. J. Wingfield who gave me an opportunity to join FABI, all the support and guidance you provided is highly appreciated.

Prof. T. A. Coutinho, for the guidance and training you offered me especially when you offered me work in the diagnostic clinic.

Uncle Kibira and Rev Fr Kennedy Lubega for all the support and encouragement, which have made me what I am today.

To my parents for being understanding during the time I haven't been there for you.

Mr. Hudson Andrua and Mr. Denis Mujuni who assisted me during fieldwork.

To the University of Pretoria and the Forestry and Agricultural Biotechnology Institute for the fascilities and equipment to undertake this study.

To the fabulous FABI family and collegues for sharing knowledge, and to my dear friends who have always given me courage especially during difficult times.

To Mr Bernard Slippers for translating the general summary to Afrikaans and help you provided with the *Botryosphaeria* chapter.

PREFACE

The growing demand for wood and wood products has increased worldwide dependency on plantation forestry. This has also resulted from the over exploitation of natural forests, especially in tropical countries. In Uganda, plantation forestry is of great importance. However, its success depends on appropriate management practices. During recent disease surveys, a number of serious plantation diseases have been reported in the southern part of the country. These diseases are known in other countries, where they have been shown to seriously reduce the productivity of exotic plantation forests. Of these diseases, Botryosphaeria canker, bacterial wilt and blight, as well as *Ceratocystis* wilt of *Eucalyptus grandis* and *Acacia mearnsii* have been identified as the most common diseases in Uganda. These diseases are responsible for the death of trees and they greatly reduce the growth, yield and quality of timber and poles.

In Chapter one of this thesis I present a general review of the development and status of plantation forestry in Uganda. This review gives a broad history of exotic plantation trees in the country, development of the forestry industry, importance and impact of the industry, its current status and it provides a perspective of the future of forestry in the country. Past and present plantation forestry problems are also considered. Special reference is made to the diseases that have been reported in *Eucalyptus*, *Pinus* and *A. mearnsii* plantations. The review emphasises the lack of general knowledge on the importance of diseases in plantation forestry. It, furthermore, attempts to highlight the impact these diseases may have on the developing industry and the measures that will need to be taken to avoid losses.

Botryosphaeria canker caused by *Botryosphaeria* spp. is the most widely spread disease of *Eucalyptus* spp. in Uganda. These pathogens are known to be endophytic and opportunistic on woody hosts, causing disease on stressed trees. Several *Botryosphaeria* spp. have been reported from *Eucalyptus* spp. worldwide, but the species causing disease in Uganda are not known. The results of a survey of *Botryosphaeria* stem canker in Southern Uganda are discussed, specifically focusing on the identification of the *Botryosphaeria* spp. in Uganda. Use is made of both morphological and molecular techniques, as morphology is known to provide variable results for this group of fungi.

Effective disease management relies fundamentally on understanding the population biology and phylogeny of a pathogen. Rapid adaptation to disease management practices by a pathogen depends on the diversity of the pathogen and also its ability to recombine. Knowledge of the population diversity, structure and reproductive mode of pathogens, thus provides clues to their origin and makes it possible to trace their movement, globally. Pathogen populations with low diversities are considered to be recently introduced and vice versa. Polymorphic Simple Sequence Repeats (SSR) are currently widely used to answer these questions, due to their high levels of polymorphism, and abundance throughout the genome. In chapter three, SSR DNA markers are used to determine the population diversity and structure of *Ceratocystis albofundus*, an important pathogen of *A. mearnsii*. Until recently, *C. albofundus* was known only from South Africa. With its discovery in Uganda in 2000, it was possible to determine the population structure and diversity of a Ugandan population. Results are further compared to those for a South African population in order to better understand the biology and origin of *C. albofundus*.

Bacterial blight of *Eucalyptus* spp. is a relatively newly recognised disease, caused by *Pantoea ananatis*. The disease was first discovered in South Africa on young *E. grandis* and *E. nitens* hybrids. During a survey conducted in Uganda, a disease causing similar symptoms was reported from *E. grandis*. Chapter four of this thesis deals with the characterization of the causal agent of bacterial blight in Uganda. Use was made of biochemical and sequence data comparisons of the 16S rRNA gene, to prove whether the pathogen is similar or different from that reported in South Africa.

Studies making up this thesis were aimed at expanding knowledge obtained from recent disease surveys in Uganda. It represents the first detailed study of plantation forestry diseases in this country. It also adds knowledge pertaining to the pathogens, to international forestry and the scientific community. Through this series of studies, a possible new species of *Botryosphaeria* has been discovered, several first reports of pathogens are made from Uganda and the geographic distribution of important pathogens is expanded. It has also provided information to the Ugandan Forestry Department on future strategies, which can be used to reduce the impact of plantation diseases in Uganda.

SUMMARY

Plantation forestry is important to Uganda as it represents a future resource of timber for fuel and structural purposes. Diseases, however, pose a serious threat to the productivity and sustainability of this emerging industry. A number of serious diseases have already been reported from surveys recently conducted in Uganda. The aim of the present study was to expand on the current knowledge of some of the diseases and to gain additional information regarding their causal agents.

The first chapter of this thesis reviewed the literature pertaining to diseases of plantation trees in Uganda. The observation is made that diseases were recognised as problematic, as early as the 1950's and trials were conducted at that time to select disease resistant planting stock. Due to political unrest, these initiatives ceased and a reliance on natural forests for the supply of wood and wood products persisted. Over exploitation of natural forests has now resulted in awareness that this resource is threatened and plantation forestry is widely recognised as the only alternative for the supply of wood and wood products. The Uganda Forestry Department is now actively promoting the establishment of plantations to reduce destruction of natural forests. This initiative has included surveys to identify the diseases affecting forest plantations in Uganda. These surveys have shown that diseases pose a serious threat to the establishment of plantation forests in the country.

Botryosphaeria canker is the most common disease of plantation *Eucalyptus* in Uganda. In chapter two of this thesis, I have shown that *B. parva*, *L. theobromae* and an undescribed species are associated with Botryosphaeria canker of *Eucalyptus* spp. in Uganda. Pathogenicity trials revealed statistically significant differences in virulence between different species, with *L. theobromae* being the least pathogenic and the undescribed species the most pathogenic. Further investigations are now needed to evaluate the pathogenicity of these fungi in the field and to consider their biology and relative importance.

Ceratocystis albofundus is an important pathogen of *Acacia mearnsii* and *A. decurrens* in South Africa. In 1999, *C. albofundus* was reported for the first time in south western Uganda. The results obtained in the current study indicate that the Ugandan population has high gene diversity similar to that of a South African

population. The results further show that the fungus reproduces clonally in both countries, with very little gene flow occurring between them. The high gene diversity values obtained in this study indicate that *C. albofundus* is native to Africa and not only South Africa, as previously hypothesized. The high gene diversity of *C. albofundus* revealed within the two populations gives a clear indication that management of Ceratocystis wilt should focus on breeding for resistance. Selection of resistant varieties is in progress in South Africa and in future, it will be useful in management of the disease.

Most *Eucalyptus* diseases are caused by fungi, but bacterial pathogens are increasing in importance. In the fourth chapter of this thesis, a bacterium known as *Pantoea ananatis* was identified as the causal agent of blight and dieback on *Eucalyptus* spp. in Uganda. This is the first report of the disease outside South Africa. The disease primarily damages young trees and it is thus of great concern in terms of plantation establishment. It will now be necessary to establish trials to select bacterial blight-tolerant planting stock. The bacterium has also been reported to infect agricultural crops which is especially important in the Ugandan situation, where trees are grown in close proximity to agricultural crops.

The results obtained in this thesis will help to manage diseases affecting plantation forest species in Uganda. The study will hopefully also provide farmers and foresters with an elevated understanding of the importance of diseases in plantation forestry. This should impact strongly on the capacity of Ugandans to deal with diseases, not only those caused by the pathogens identified in the study, but also other diseases, particularly of forest and fruit crops.

OPSOMMING

Plantasiebosbou is belangrik in Uganda, omdat dit 'n toekomstige bron van hout vir energie en strukturele behoeftes bied. Siektes is egter 'n ernstige bedreiging vir die produktiwiteit en volhoubaarheid van hierdie opkomende industrie. 'n Aantal ernstige siektes is alreeds aangemeld tydens vorige opnames wat in Uganda gedoen is. Die doelwit van die huidige studie is om die kennis oor sommige van hierdie siektes te verbreed en om verdere inligting oor siekteveroorsakende agente te verkry.

Die eerste hoofstuk van hierdie verhandeling gee 'n oorsig van die literatuur i.v.m. die siektes van plantasiespesies in Uganda. Dit word uitgewys dat siektes as problematies beskou is van so vroeg as die 1950's en dat proewe gedoen is om siekteweerstandbiedende plantmateriaal te selekteer. As gevolg van politieke oproer is hierdie inisiatiewe gestaak en het die land afhanklik gebly van natuurlike woude vir die verskaffing van hout en houtprodukte. Huidiglik is daar 'n bewuswording dat oorbenutting die voortbestaan van natuurlike woude bedrieg, en dat plantasiebosbou die enigste alternatief bied vir die verskaffing van hout en houtprodukte. Die Uganda Bosbou Departement promofeer nou aktief die vestiging van plantasies om die vernietiging van natuurlike woude te verminder. Hierdie inisiatiewe sluit in pogings om die siektes wat bosbouplantasies in Uganda beïnvloed, te identifiseer. Vroër het ondersoeke het getoon dat siektes 'n ernstige bedreiging inhou vir die vestiging van plantasiebosbou in die land.

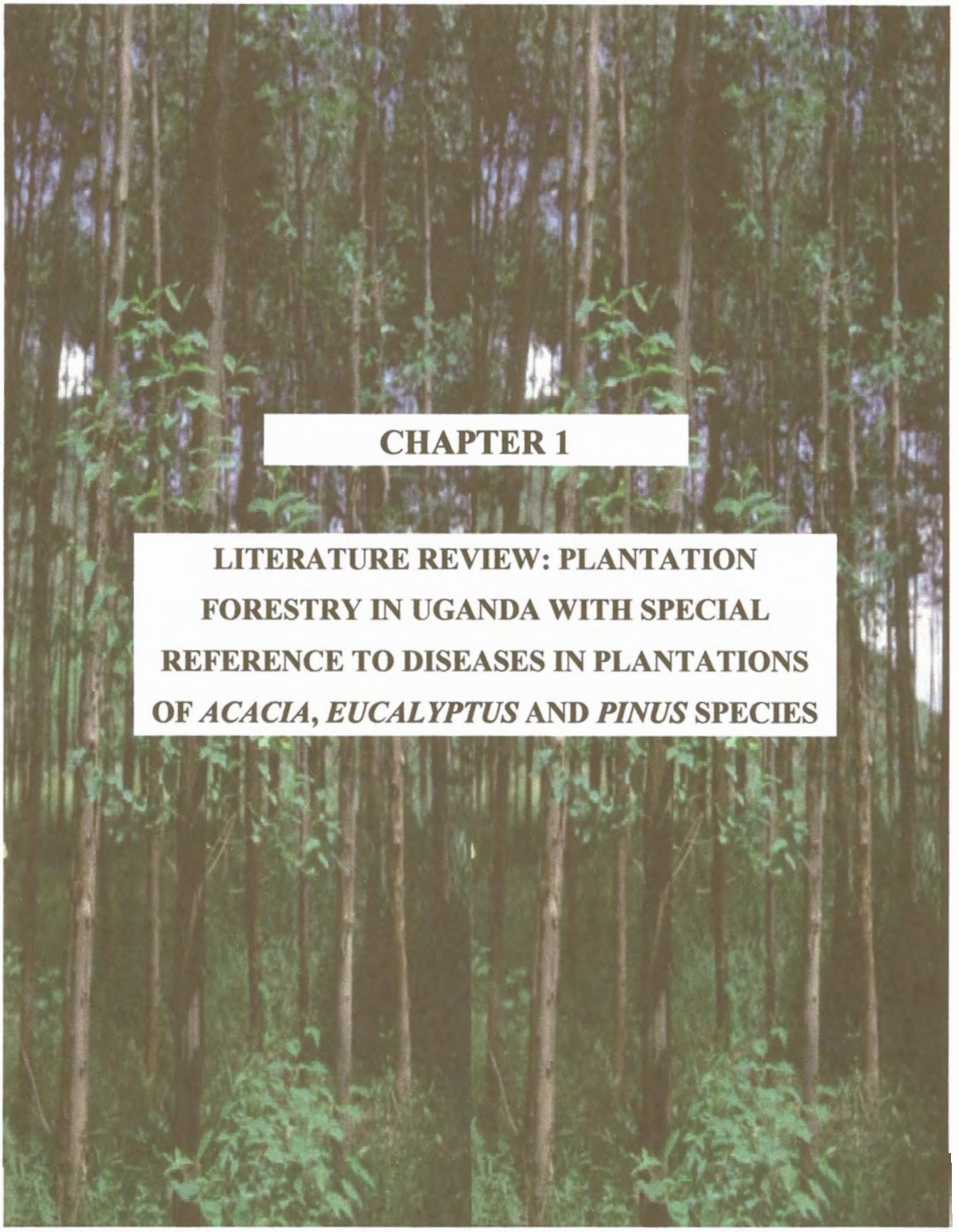
Botryosphaeria kanker is die mees algemene siekte van *Eucalyptus* spp. in Uganda. In hoofstuk twee van die verhandeling, wys ek dat *B. parva*, *L. theobromae* en 'n onbekende *Botryosphaeria* sp. geassosieer word met Botryosphaeria kanker van *Eucalyptus* in Uganda. Patogenisiteitstoetse het gewys dat daar statisties betekenisvolle verskille in die virulensie van die genoemde spesies is. *Lasiodiplodia theobromae* was die mins virulente spesie, terwyl die onbekende spesie die mees virulent was. Verdere ondersoek is nodig om die virulensie van hierdie swamme in die veld te toets en om hulle biologie en relatiewe belang te bepaal.

Ceratocystis albofundus is 'n belangrike patogeen van *Acacia mearnsii* en *A. decurrens* in Suid-Afrika. In 1999, is *C. albofundus* die eerste keer geidentifiseer in die suid weste van Uganda. Die resultate in die huidige studie wys daarop dat die

Ugandese populasie hoë geendiversiteit het, wat vergelykbaar is met die Suid Afrikaanse populasie van die swam. Die resultate wys verder dat die swam klonaal voortplant in beide bg. lande, met baie min genetiese vloei tussen hulle. Die hoë geendiversiteits-waardes wat in hierdie studie verkry is, wys dat *C. albofundus* inheems is aan Afrika en nie Suid-Afrika soos 'n vroeëre hipotese voorstel nie. Die hoë geendiversiteit van *C. albofundus* in die twee populasies, wys daarop dat daar gefokus moet word op die kweek van weerstandbiedende variëteite as 'n beheerstrategie. Die seleksie van weerstandbiedende variëteite is reeds aan die gang in Suid-Afrika en sal baie bruikbaar wees vir beheer van die siekte in die toekoms.

Meeste siektes op *Eucalyptys* word veroorsaak deur swamme, maar bakteriese patogene word al meer belangrik. In die vierde hoofstuk van die verhandeling word die bakterium, *Pantoea ananatis,* geïdentifiseer as die oorsaak van skroeisiekte en terugsterwing van *Eucalyptus* spesies in Uganda. Dit is die eerste aanmelding van die siekte buite Suid-Afrika. Die siekte affekteer hoofsaaklik jong boompies en is daarom 'n bron van ernstige kommer vir die vestiging van plantasies. Dit is nou nodig om proewe op te stel om plantmateriaal te selekteer wat weerstanbiedend is teen bakteriese skroeisiekte. Die bakterium infekteer ook ander landbou-gewasse. Laasgenoemde is belangrik in Uganda, aangesien bome dikwels naby aan ander landbou gewasse geplant word.

Die resultate van hierdie verhandeling sal help met die beheer van siektes van bosbouplantasies in Uganda. Die studie sal hopelik ook dien as hulpmiddel om land- en bosbouers 'n beter begrip te gee van die belang van siektes in bosbouplantasies. Dit sal ook 'n wesenlike impak hê op die kapasiteit van die mense van Uganda om plantasiesiektes te hanteer, nie net die wat veroorsaak word deur die patogene wat in hierdie verhandeling geïdentifiseer is nie, maar ook ander siektes in die bosbou- en vrugtebedrywe.



CHAPTER 1

LITERATURE REVIEW: PLANTATION FORESTRY IN UGANDA WITH SPECIAL REFERENCE TO DISEASES IN PLANTATIONS OF *ACACIA*, *EUCALYPTUS* AND *PINUS* SPECIES

INTRODUCTION

The demand for wood and wood products has increased tremendously throughout the world (Evans 1982, Kanowski 1997, Sutton 1999). Many countries that had large areas under natural forests are now embarking on plantation forestry. This is because natural forests have been exploited by agriculture, industrial expansion, charcoal burning, firewood collection, overgrazing, wars and fires (Evans 1982, Sutton 1995, 1999). In addition, many tropical countries are promoting nature conservation, with the result that many natural forests have been converted to sites for ecotourism (Evans 1982).

Plantation forests have several advantages over natural forests. First of all they can be planted in areas where they are needed, thus, solving the problem of transportation, especially for rural people (Sutton 1999). They can be grown for a specific purpose, for instance the production of pulp and paper, timber, fuel, essential oils, tannins, and poles. They also play other important roles, such as in soil conservation by providing canopy cover, acting as wind breaks, as water catchment areas and for carbon dioxide sequestration. Very importantly, they provide an alternative source of wood, reducing the negative impact on natural and non-renewable forests (Sutton 1995, 1999).

Globally, plantation forestry is estimated to cover 135 million ha, 75 % of which are found in temperate regions and about 25% in the tropics and subtropics. Of these, 5% are in Africa (Anonymous 1993). The most commonly/widely planted plantation trees include species and interspecific hybrids of *Acacia*, *Eucalyptus*, *Picea* and *Pinus* (Evans 1982, Kanowski 1997). Plantation forest ownership includes government bodies, non-governmental organisations, private companies as well as individual farmers (Kanowski 1997). Management also varies tremendously from simple and low input to highly sophisticated and intensive (Anonymous 1993).

Serious disease problems affect both exotic plantations and native forests. Poor understanding of the threat of diseases has resulted in serious epidemics such as Chestnut blight caused by *Cryphonectria parastica* (Murrill) M. E. Barr, which has led to the near elimination of chestnut as a forest species in North America (Boyce 1961, Anagnostakis 1987, Sinclair, Lyon & Johnson 1987). In exotic plantation forestry, there are many examples of diseases that have caused devastating loss. For

example, Mycosphaerella leaf blotch led to the termination of the planting of *Eucalyptus globulus* Labill. in South Africa (Purnell & Lundquist 1986).

For successful plantation forestry, much research is needed in the areas of pathology, entomology, silviculture and genetic resources (Kanowski 1997). In countries with a strong research base in plantation forestry the cloning of hybrids has contributed to the success of plantation forestry (Denison & Kietzka 1993). It has made possible, the selection of clones resistant to a range of pathogens, as well as with high growth rates and tolerance to harsh environmental conditions. Most developing forestry countries, however, still rely entirely on seedling-based forestry (Kanowski 1997).

The aim of this review is to discuss the development and status of plantation forestry in Uganda. Special reference is made to the diseases that have been reported in *Eucalyptus*, *Pinus* and *Acacia mearnsii* plantations.

History of exotic plantation forestry in Uganda

In 1907, the colonial government (Uganda under British protectorate) realized that the supply of firewood and building poles from natural forests was rapidly declining (Karani 1972). The natural forests around Entebbe (25 km to the south of Kampala on the shores of lake Victoria), for instance, had been cleared and firewood supplies for Kampala were collected from as far away as 10–13 kms from the city. This was true for all major towns in the country. As a reaction to the rapidly increasing deforestation problem, the government promoted the planting of trees in these areas (Karani 1972).

Plantation forestry in Uganda began with the growing of indigenous trees. These included *Markhamia platycalyx (*Bark.) Sprague, *Milicia excelsa* (Syn. *Chlorophora excelsa*) (Wewl.) Benth. & Hoof. F. and *Maesopsis eminii* Engl. (Anonymous 1951). Exotic tree species were later introduced for their faster growth rates as compared to those of indigenous tree species (Karani 1972). The first exotic trees to be grown included, among others, *Cupressus lusitanica* Mill (Karani 1972). In 1910, other species such as *Pinus patula* Schl. & Cham. and *P. radiata* D. Don. were introduced (Anonymous 1951).

Eucalyptus spp. were introduced into Uganda in 1912 (Ruyooka 1999). The first species to be grown were *E. creba* F. Muell, *E. polyanthemos* Schaner, *E. hemiphloia* F. Muell and *E. tereticornis* Domin (Karani 1972). A total of 23 species of *Eucalyptus* have been introduced into Uganda, but the most widely grown species is *E. grandis* W. Hill (Ruyooka 1999).

Development of the forestry industry in Uganda

In 1918, fuel plantations were established around Kampala and Entebbe (Anonymous 1951). These consisted of a mixture of *Eucalyptus* spp. and *Cassia* spp. *Cassia* spp. are resistant to termites and are thus commonly grown in areas where *Eucalyptus* spp. cannot survive (Anonymous 1951). By 1926, the Buganda (Central) region had 230 acres under plantations and these were dominated by *Eucalyptus* spp. Native species such as *M. excelsa* and *M. platycalyx* were, however, preferred due to their higher quality timber. However, their survival in plantations was poor. For example, in 1915 the Forestry Department established 10 acres of *M. excelsa* and *M. platycalyx* plantations in Busoga (Central region), but their survival was very low and the project was abandoned (Anonymous 1951).

By 1939, 50 acres of *M. excelsa* had been established nationwide (Anonymous 1951). These plantations grew well and by 1941 a programme of establishing 50 acres per year was implemented for timber production (Anonymous 1951). In 1942, softwood plantations were established, dominated by *C. lusitanica* and by 1949 a total of 869 acres had been planted (Anonymous 1951). Furthermore, every farmer was encouraged to own a tree plot and by 1950 approximately 44684 ha of fuel and pole plantations were under the jurisdiction of the Forest Department and 2845 ha under the local government (Webstar & Osmaston 1999).

The growth and development of forest plantations was good until the 1970s. At this time management ceased due to a lack of facilities as a result of political instability. During this period no new plantings were undertaken and maintenance of existing plantations was neglected (Webstar & Osmaston 1999).

Since 1986, the Forest Department of Uganda has undertaken a rehabilitation programme for all plantations. The Department is currently encouraging private and foreign investments in commercial tree growing and permits to grow commercial

timber plantations have been issued for over 25000 ha (Webstar & Osmaston 1999). There has also been an increase in demand for poles since 1995. This has been as a result of a boom in the construction industry and the accelerated economic growth, which has averaged 6% per year since 1986. These factors have once again increased the demand for timber and are contributing to a stronger forestry industry (Webstar & Osmaston 1999) (Figure 1). The planting of forest trees has also been boosted by an increase in prices being paid for poles (Table 1). It is expected, therefore, that within the next few years the plantation forestry industry in Uganda will grow exponentially.

Importance and impact of exotic plantation forestry

The development of exotic plantation forestry is of great social, environmental and economic importance to Uganda. Plantation forestry comprises mostly of *Eucalyptus* and *Pinus* spp., which are important as sources of building poles, transmission poles, firewood and sawn timber (Karani 1972, Ruyooka 1999, Anonymous 2000a). Building and transmission poles are the major products from *Eucalyptus* plantations (Ruyooka 1999). These trees are being harvested at approximately 4 years for building poles and between 8-12 years of age for transmission poles (Ruyooka 1999).

It is estimated that 96% of Uganda's population depends on fuelwood as a source of energy. This is equivalent to 20 million m^3 of wood per annum. In 1999, the total wood production from both natural and plantation forests was estimated at about 24 million tonnes, with a gross output, including charcoal making of over 173 billion Uganda shillings (Ush.) (Anonymous 2001). The major consumers include tea and tobacco factories, bakeries, brick burning and sugar jaggeries (Ruyooka 1999). Other consumers include among others, schools, colleges and restaurants. The prices for fuelwood range between Ush. 2000-6000 per cubic meter for 60-80 pieces of wood on average (Ruyooka 1999). Since fuelwood is cheaper than electricity or other sources of fuel/power, it is estimated that for many years to come fuelwood will continue to be the preferred source of energy (Karani 1972, Ruyooka 1999, Anonymous 2000b). Since natural forests have been severely depleted, plantation species should and will be the most important alternative source of fuelwood (Ruyooka 1999).

In the new National Forest Policy, the government of Uganda emphasizes the conservation of the remaining natural forests. This implies that the importance of commercial forestry will increase in future (Anonymous 2000b). The production of

fuelwood, construction poles and timber will now depend solely on plantations, including both exotic and endangered indigenous tree species (Anonymous 2000b).

FOREST PLANTATION DISEASES IN UGANDA

Reports of diseases and pests affecting exotic trees are increasing throughout the world, where exotic plantation forestry is practiced (Gibson 1975, Sinclair *et al.* 1987, Keane *et al.* 2000). Although exotic trees have been removed from their natural enemies, their concentration in monocultures increases the risk of being seriously affected by diseases (Leakey 1987). These diseases may be caused by native pathogens attacking exotic hosts or pathogens from the native range of the exotic trees gradually appearing in their new country. These diseases can cause large-scale damage, due to the narrow genetic base linked to monoculture (Wingfield 1999, Wingfield *et al.* 2001).

Uganda is similar to other countries where exotic plantations have been established, in that it faces the threat of increasing disease problems. Diseases were reported on forest trees in Uganda as early as the 1950's, although research pertaining to the aetiology of the pathogens was never undertaken. In general, there is a lack of knowledge on diseases and general plantation forestry in Uganda. Recently disease surveys have were undertaken in 1999 and 2000 in both nurseries and plantations. The surveys have revealed the presence of a number of diseases, which if no concerted control efforts are undertaken, are capable of causing devastating losses to the emerging forestry industry in the country. The most important of these diseases are discussed briefly in the following sections.

Root diseases

Armillaria root rot of Pinus spp.

Armillaria mellea (Vahl. Ex Fr.) Kummer was reported as a serious problem on *P. radiata* during the 1960's in the southern and western parts of Uganda. Field trials were thus established to select species tolerant to this pathogen (Webstar & Osmaston 1999). The fungus was most common where plantations were growing on areas previously occupied by natural forests. This is typical of *Armillaria* spp., which occur naturally on native trees and is commonly known to cause serious loss in newly established plantations (Ivory 1968, Gibson 1975).

All age classes of *P. radiata* were susceptible to Armillaria root rot in Uganda (Gibson 1975, Webstar & Osmaston 1999). The appearance of white/creamy sheets of mycelium under the bark, the presence of basidiocarps at the base of the infected trees and the wilting and death of trees, were the most common symptoms, as is characteristic of Armillaria root rot in other countries (Gibson 1975).

Armillaria spp. have a cosmopolitan distribution and have been reported from many other African countries, including neighbouring countries such as Ethiopia and Kenya (Mwangi, Lin & Hubbes 1989, Mengistu 1992). They mostly spread through root contact or through growth of rhizomorphs between susceptible trees. They can also spread via basidiospores, although this is rare (Gibson 1975, Webstar & Osmaston 1999).

Wilt diseases

Bacterial wilt of Eucalyptus spp.

Ralstonia solanacearum (synonyms *Pseudomonas solanacearum* and *Burkholderia solanacearum*) Yabuuchi *et al.* is a well-known cause of bacterial wilt (Hayward 1964, Yabuuchi *et al.* 1995). In Uganda it is a very important and destructive pathogen of *E. grandis* in areas around Entebbe and Kampala (major cities on the northern shores of L. Victoria) (Roux *et al.* 2001).

The first report of *R. solanacearum* on *Eucalyptus* spp. was in Brazil in the 1980's (Dianese 1986). Since then, other reports of its occurrence have been made from Australia (Akiew & Trevorrow 1994), China (Wu & Liang 1988), South Africa (Coutinho *et al.* 2000) and the Republic of Congo (Roux *et al.* 2000a). Given the relatively rapid increase in new reports of this disease, it appears to have a relatively wide distribution on *Eucalyptus* and is growing in importance in plantation forestry.

Ralstonia solanacearum is a soil borne pathogen, with a wide host range (Hayward 1964, Seal *et al.* 1993, Hayward 1994, Brown & Ogle 1997). Infection normally starts from the roots and spreads up the stem disrupting the vascular system (Hayward 1991, Brown & Ogle 1997). The xylem shows a brown discoloration and bacterial exudates ooze out when the stem is cut through longitudinally (Hayward 1991, Coutinho *et al.* 2000, Roux *et al.* 2001). Weeding the fields before planting and

removing and burning the infected trees is one of the strategies, which can reduce the rate of spread of the bacterium (Hayward 1991, Hartman & Elphinstone 1994, Akiew & Trevorrow 1994).

Ceratocystis wilt of Eucalyptus spp.

Ceratocystis fimbriata Ellis & Halst., is an important fungal pathogen of many woody plants (Kile 1993) and causes *Ceratocystis* wilt of *Eucalyptus* spp. in Uganda (Roux *et al.* 2001). Symptoms of this disease include, discoloration of the xylem, formation of epicormic shoots, wilting and death of the trees (Roux *et al.* 2000b, Roux *et al.* 2001). The disease was reported for the first time on *E. grandis* trees in the Tororo district (Eastern Uganda). It was estimated that 50% of the trees in the affected plantation were diseased and dying (Roux *et al.* 2001). Uganda was only the second country in Africa and the third in the world where the disease had been reported from *Eucalyptus* spp. (Roux *et al.* 2001).

Ceratocystis spp. typically infect through wounds, which may result from unfavourable environmental factors, silvicultural practices and insects. Insects are attracted by the sweet aroma produced by the fungus and thus also serve as vectors (De Vay *et al.* 1963, Christen, Meza & Revah 1997). No insect vectors have, however, as yet been identified as vectors of *C. fimbriata* in plantation forestry.

Ceratocystis wilt of Acacia mearnsii

Ceratocystis wilt of *Acacia mearnsii* is caused by *C. albofundus* Wingfield, De Beer & Morris (Morris, Wingfield & De Beer 1993, Wingfield *et al.* 1996). The disease was reported from wounded *A. mearnsii* in the Kabale District, South Western Uganda, where stems had been harvested for fuelwood (Roux & Wingfield 2001). Symptoms include streaking in the xylem, formation of lesions and cankers on the bark of the affected trees, wilting and death. The disease was first reported from South Africa in 1989, causing rapid wilt and death of *A. mearnsii* (Wingfield *et al.* 1996). Uganda is the second country in the world where the disease has been reported (Roux & Wingfield 2001). The only other host for *C. albofundus* is *Protea* spp. and it has been speculated that it might be native to South Africa (Roux, Dunlop & Wingfield 1999, Roux *et al.* 2001). With the report of the fungus from Uganda, this is currently under re-evaluation.

Canker diseases

Cytospora canker of Eucalyptus spp.

Cytospora eucalypticola Van der Westhuizen causes Cytospora canker of *Eucalyptus* species. In Uganda the disease was first reported during the early 1970's (Gibson 1975). Recently, in 1999, *C. eucalypticola* was isolated from *E. grandis* growing in wetland areas (Roux *et al.* 2001). The pathogen causes cankers on branches and stems, thus, interfering with the quality and strength of the wood (Van der Westhiuzen 1965, Gibson 1975). *C. eucalypticola* is commonly associated with stressed trees, for instance wounded trees and those weakened by bacterial wilt and termite damage (Gibson 1975, Roux *et al.* 2001). Results from a recent phylogenetic study indicate that Ugandan isolates are related to Australian isolates of *C. eucalypticola* (Gerard Adams, personal communication), which gives an impression that the fungus may have entered the country with seeds from Australia.

Sphaeropsis sapinea Canker and Die-back of Pinus spp.

Sphaeropsis sapinea (Fr.:Fr.) Dyko and Sutton (syn *Diplodia pinea* (Desm.) Kickx) causes canker, die-back and root rot, on *Pinus* spp. (Gibson 1975, Swart & Wingfield 1991). In Uganda, it was also reported to be responsible for the cause of blue stain of timber at various sawmills (Roux *et al.* 2001).

Sphaeropsis sapinea is an opportunistic stress-related pathogen and endophyte (Swart & Wingfield 1990, 1995, Smith *et al.* 1996a). It may infect trees where wounding has occurred, either due to pruning, hail or other wounding agents (Zwolinski, Swart & Wingfield 1990, Stanosz *et al.* 1997). Selection for resistance and appropriate silvicultural practices can reduce the spread and impact of the pathogen (Wingfield & Roux 2000).

Botryosphaeria canker of Eucalyptus spp.

Botryosphaeria spp., are the causative agents of Botryosphaeria canker of *Eucalyptus* spp. This is the most widespread disease of *Eucalyptus* spp. in Uganda, having been reported from all the plantation areas surveyed in 1999 by Roux *et al.* (2001). Symptoms include kino exudation, branch die-back, stem cankers and cracking of the bark (Roux *et al.* 2001). These symptoms are similar to those described from South Africa where Botryosphaeria canker is the most common disease of *Eucalyptus*

(Smith, Kemp & Wingfield 1994, Smith, Wingfield & Petrini 1996b, Wingfield & Roux 2000).

Botryosphaeria spp. are capable of surviving as endophytes in healthy plants, and as saprophytes on dead plant material (Smith *et al.* 1996b, Smith *et al.* 1996). They are commonly known as stress-related pathogens affecting trees of all ages (Smith *et al.* 1994). This is a serious problem in Uganda, where *E. grandis* is the most common species grown in plantations and is often subject to stress due to poor site matching (Roux *et al.* 2001). Breeding for resistance, site/species matching, cultural management practices and reduction of wounding are the major management strategies, which will reduce the impact of Botryosphaeria canker of *Eucalyptus* in Uganda (Wingfield & Roux 2000, Roux *et al.* 2001).

Leaf Diseases

Dothistroma needle blight of Pinus spp.

Dothistroma needle blight is caused by *Dothistroma pini* Hulbury (synonym *Dothistroma septospora* Dorog. Morelet). It was reported in Uganda between 1961 and 1963, on *P. radiata*, causing severe defoliation (Gibson 1975). This was a serious disease in Uganda and East Africa in general where, in some areas it led to the abandonment of *P. radiata* (Ivory 1968, Paterson & Ivory 1968). Symptoms include abnormal chlorosis and necrosis of the needles. This starts from the lower branches and spreads up the tree. With severe and repeated defoliation, trees may die (Gibson 1975).

Eucalyptus leaf diseases

During a survey conducted by Roux *et al.* (2001), spots were observed on leaves of young *Eucalytpus* spp. Isolations revealed *Mycosphaerella* spp., *Harkenesia* spp., and *Cryptosporiopsis eucalypti* Sankaran & Sutton (Roux *et al.* 2001). Powdery mildew was also identified resulting in leaf distortion and necrosis (Roux *et al.* 2001). Of these fungi, *Mycosphaerella* spp. are probably the most important in plantation forestry but, at the present time, these pathogens are of minor importance in Uganda.

Nursery diseases

During a survey of nursery seedlings conducted by Maiteki *et al.* (1999) and Roux *et al.* (2001), a number of important diseases were reported on both *Pinus* and

Eucalyptus seedlings. On *Eucalyptus* spp., amongst others were *Pestalotiopsis* and *Cercospora* spp., which appeared to be responsible for brown leaf spots resulting in defoliation and severe plant stress (Maiteki *et al.* 1999). *Fusarium*, *Pythium* and *Rhizoctonia* spp. were reported to be responsible for damping off of seedlings in most of the nurseries surveyed (Maiteki *et al.* 1999).

Powdery mildew was a common disease in nurseries. Infected plants had the typical whitish, powdery growth on the surface of the leaves, which interferes with photosynthetic capacity. Infection may also result in malformation of the leaves, stunting of the trees as well as leaf drop (Roux *et al.* 2001).

On *Pinus* spp., *Fusarium solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans. and *Fusarium oxysporum* (Schlecht. Emend. Synd. & Hans.) have been reported as serious problems causing necrotic lesions on the stems and roots as well as death of seedlings (Maiteki *et al.* 1999). *Sphaeropsis sapinea* was reported at Magamaga nursery to be responsible for tip die-back and death of *P. radiata* (Roux *et al.* 2001).

MANAGEMENT OF PLANTATION FOREST DISEASES

With the increasing exploitation and degradation of natural forests in Uganda it is now clear that the future supply of wood and wood products, as well as the protection of native forests, will depend largely on exotic plantations. Appropriate management of these plantations is a key factor in the development of the industry. In plantation forestry, the most common management strategies include selection, breeding and chemical control. Of these, selection and breeding will provide the most sustainable option.

Successful plantation health management requires a thorough understanding of the biology of the pathogen in question (Gadgil *et al.* 2000). It is also necessary to obtain detailed knowledge pertaining to the growth and development of trees under various environmental conditions (Brown 2000, Gadgil *et al.* 2000, Simpson & Podger 2000). Thus, in Uganda, it will be necessary to access the population biology, taxonomy and aetiology of the most serious pathogens. This will bring our results into context with international disease reports. At the same time knowledge will be gained on the

origin and spread of the pathogens, while also answering questions on their taxonomy and management.

Selection of species to match site and local environmental factors, combined with breeding for disease tolerance are so far the most sustainable management strategies in forest plantations (Gadgil *et al.* 2000, Roux *et al.* 2001, Wingfield *et al.* 2001). This approach has been effective in many countries where plantation forestry is practiced on a large scale. In South Africa, for instance, breeding for resistance has reduced the impact of diseases such as Cryphonectria stem canker of *Eucalyptus* spp. to such a degree that it is no longer considered to be amongst the most threatening diseases in the country (Wingfield *et al.* 2001).

Quarantine measures, which prevent the introduction of new pathogens, and silvicultural practices, which provide favourable conditions for tree growth and disease avoidance are crucial in disease management (Gibson 1975, Colquhoun & Elliott 2000, Gadgil *et al.* 2000). Use of chemicals has also proved to be very effective in control of a variety of diseases especially in forest nurseries, although in most cases the costs are very high (Wingfield *et al.* 2001).

The future sustainability of Ugandan forestry will undoubtedly rely on the implementation of a stable forest protection programme and strategies to ensure disease avoidance. Only through active integrated forestry management systems will forestry be practiced optimally. The challenge to Ugandan forestry now is to include pests and diseases, silviculture, site selection and other forestry practices into a single, combined operation to ensure maximum yield.

CONCLUSIONS

Plantation forestry is of crucial importance to Uganda. Diseases, however, pose a serious threat to the productivity and sustainability of plantation forestry in the country. A number of serious diseases have already been reported from surveys conducted in the Southern part of the country. These surveys should be expanded to the central and western areas of Uganda to ensure a clear understanding of plantation forestry diseases.

A major component of disease management includes awareness of diseases amongst all farmers and foresters. In Uganda, this is currently lacking. A major thrust of the future plantation health programme of Uganda should thus include field days and training of foresters at university and college level, focused on disease diagnoses and their management. Breeding for resistance, selection of species to match sites, silvicultural and cultural practices that reduce disease incidence should all become an integral part of forestry operations and training to ensure healthy plantations in Uganda.

Diseases such as Botryosphaeria canker and bacterial wilt are capable of causing considerable loss. Already these, and other diseases, are having a negative impact on the Ugandan forestry industry. Very little is, however, known of diseases and their causal agents in Uganda. Their aetiology needs to be properly understood in order to design appropriate management strategies to avoid losses.

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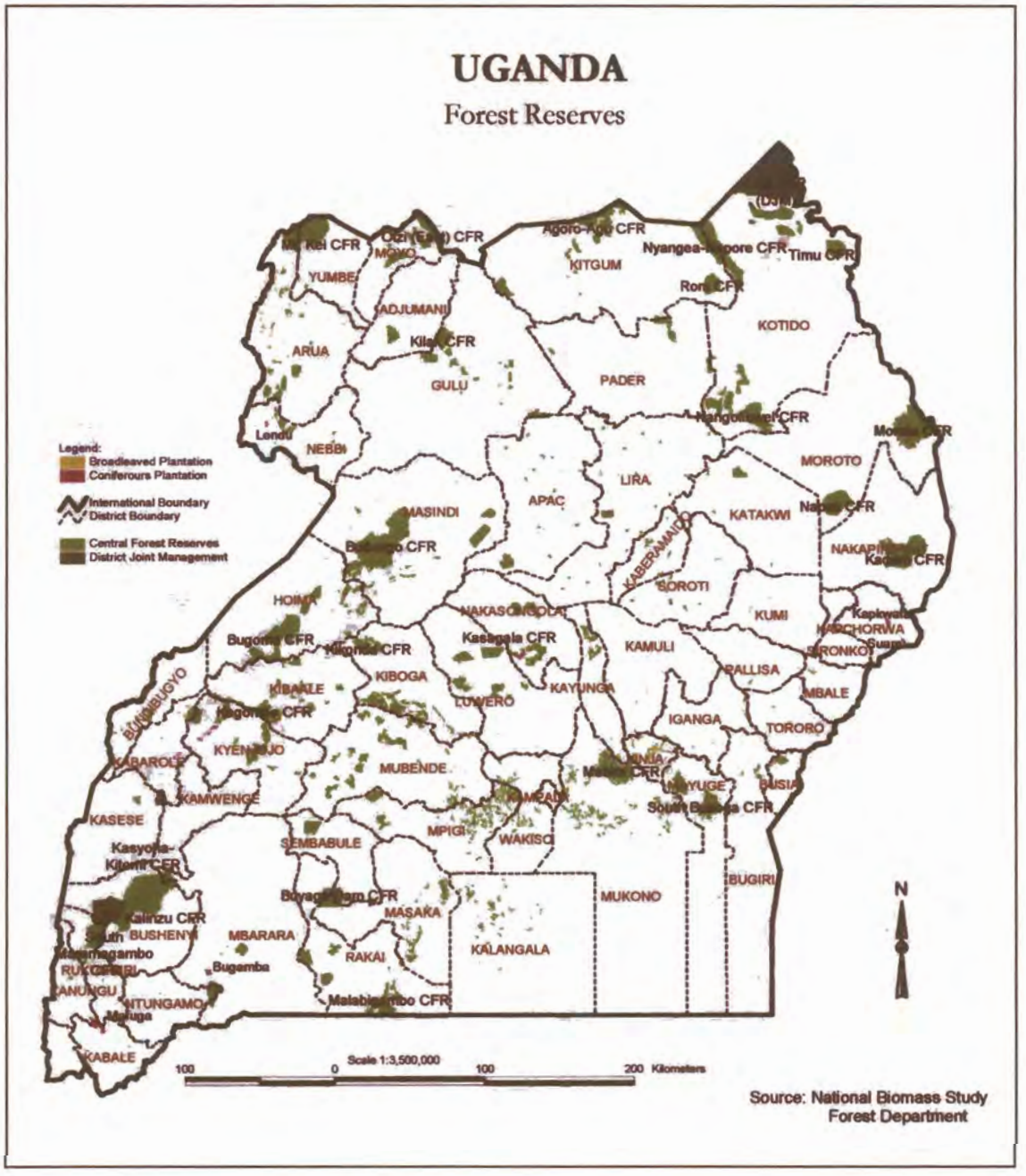
Table 1. Old and newly proposed Uganda government prices for tree poles of four different size classes (Ruyooka 1999).

Class	Size in cm (thick end)	Old price (Ushs) 1999	Proposed new prices (Ushs)
I	5-9	112.50 per pole	350 per pole
II	10-14	67.50 per running metre	600 per pole
III	15-19	75.00 per running metre	600 per running metre
IV	20-24	80.00 per running metre	800 per running metre

Figure 1. Location of plantation forestry areas in Uganda (National Biomass study, Forestry Department 2000).



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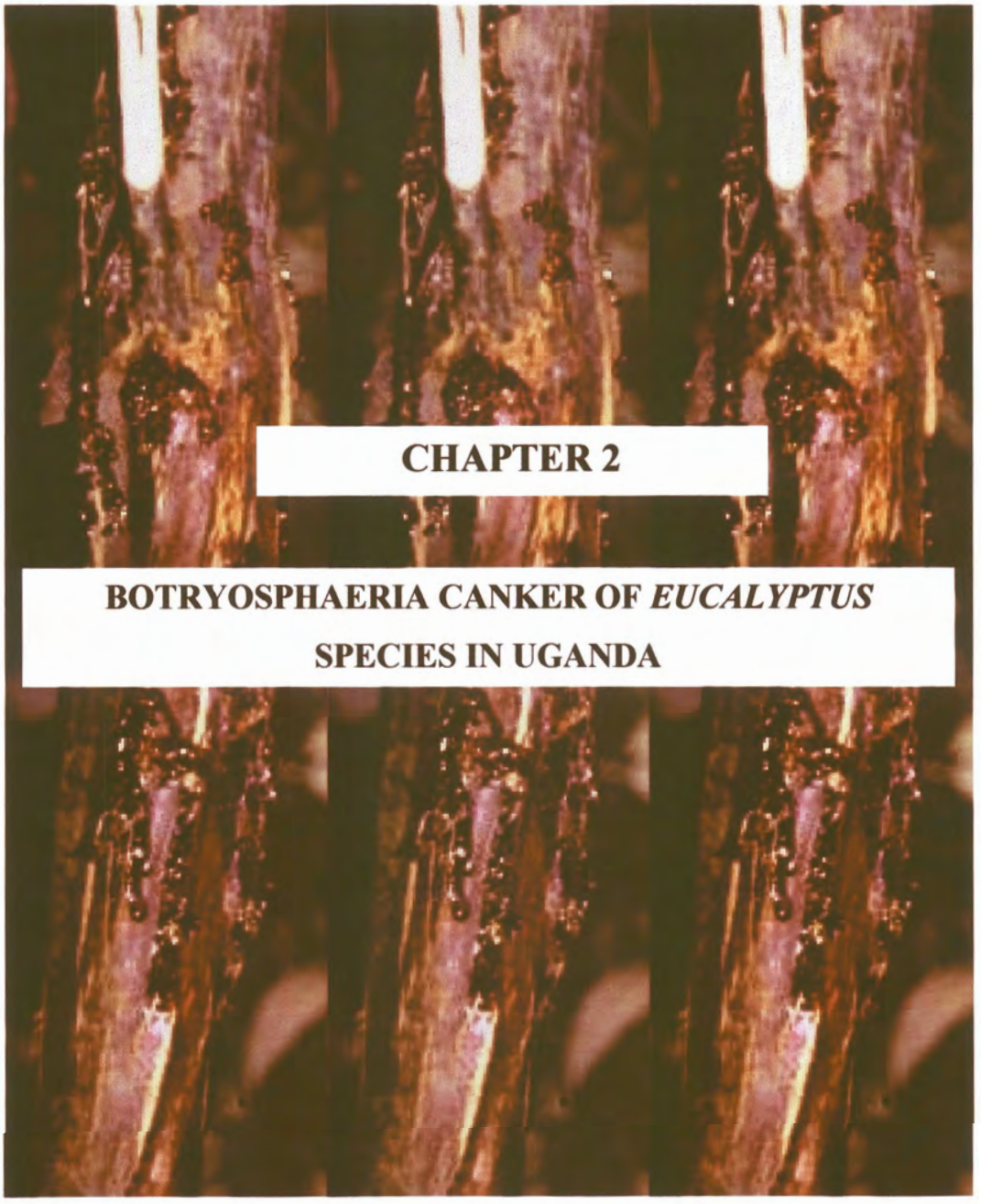

CHAPTER 2

BOTRYOSPHAERIA CANKER OF *EUCALYPTUS* SPECIES IN UGANDA

ABSTRACT

In Uganda, more than 90% of energy production comes from wood. Previously most of this wood was from natural forests, however, with increasing over exploitation of natural forests, plantation forestry has become an alternative resource. *Eucalyptus* spp. are amongst the most widely planted exotic trees in Uganda. Disease surveys conducted in 1999 and 2001 revealed that Botryosphaeria canker is the most common disease of plantation grown *Eucalyptus* in the country. The aim of this study was to determine the identity of the *Botryosphaeria* species associated with cankers on *Eucalyptus* in Uganda. Isolations were made from twigs collected from symptomatic trees in eastern, western and central parts of the country. Identifications were based on morphological characteristics as well as DNA based techniques, including RFLP's and sequence data of ITS rDNA and EF1-α gene regions. Molecular data and pathogenicity trials showed that *B. parva, B. rhodina* and an unknown species of *Botryosphaeria* are responsible for Botryosphaeria canker of *Eucalyptus* spp. in Uganda. These trials further showed that *B. rhodina* was the least pathogenic and the unknown species the most pathogenic. This study represents the first report of *B. parva* from *Eucalyptus* in Uganda.

INTRODUCTION

The development and improvement of plantation forestry is of importance for the continued supply of wood and wood products worldwide (Kanowski 1997). This will prevent environmental degradation and contribute to increased economic activity, especially for poor communities in tropical Africa and Asia (Evans 1982). For example, in Uganda more than 90% of energy is produced from wood and this represents 20 million metric tones per annum (Ruyooka 1999, Anonymous 2001). Since most of this wood is from natural forests, it is expected that most indigenous sources will be completely depleted within the next few years. To avoid this situation, there is a strong drive by the Ugandan Forestry Department to develop plantation forestry (Anonymous 2001).

In a recent survey, diseases caused by fungi were reported to significantly reduce productivity in Ugandan *Eucalyptus* plantations (Roux *et al.* 2001). Stem canker caused by *Botryosphaeria* sp. was found to be the most widely distributed disease in the areas surveyed (Roux *et al.* 2001). *Botryosphaeria* spp. are opportunistic pathogens taking advantage of stress caused by drought, hail, frost, water logging, nutritional imbalances and wounding (Swart, Wingfield & Knox-Davies 1987, Arauz & Sutton 1989, Pusey 1989, Zhonghua, Morgan & Michailides 2001). Symptoms of disease include tip die-back, stem cankers, cracking, kino exudation, death of the xylem and eventually, in extreme infections, death of the tree (Smith, Kemp & Wingfield 1994, Shearer, Tippett & Bartle 1987). The deposition of kino in the tree reduces the strength of the wood, thus making it unsuitable for construction (Smith *et al.* 1994, Smith, Wingfield & Petrini 1996, Shearer *et al.* 1987).

Relatively recent research has shown that *Botryosphaeria* spp. commonly exist as endophytes in healthy plant tissue (Fisher, Petrini & Sutton 1993, Smith *et al.* 1996). They are thus present in most woody plants and are able to invade tissues when stress ensues. They can also be semiparasitic and saprophytic on dead wood and other plant material (Sivanesan 1984).

Species in the genus *Botryosphaeria* Ces. & De Not (Pleosporales, Loculoascomycetes), have anamorph states residing in the genera *Fusicoccum* Corda in Sturm., *Dothiorella* Sacc., *Diplodia* Fr. In Mont., *Lasiodiplodia* Ellis & Everh., *Sphaeropsis* Sacc and *Phyllosticta* Pers. (Von Arx 1987, Jacobs & Rehner 1998, Denman *et al.* 2000). The identification of *Botryosphaeria* spp. is based mainly on the anamorph characters, since teleomorphic characters are very similar among species. However, characteristics of the anamorphs in some *Botryosphaeria* spp. are also similar and can be influenced by the media on which they are produced (Zhou & Stanosz 2001, Zhonghua & Michailides 2002), complicating identification of these fungi. Recently, DNA sequence data obtained from the variable regions of the genome, such as the internally transcribed regions (ITS1, 5.8S and ITS 4), β-tubulin and the elongation factor (EF-1α) have been used to successfully distinguish the species in the genus (Smith *et al.* 2001, Zhou & Stanosz 2001, Slippers *et al.* 2002). These studies have added considerable understanding to the taxonomy of the group and now facilitate further work on *Botryosphaeria* spp. on various hosts.

Botryosphaeria spp. are widely distributed in the sub-tropical and tropical regions of the world (Von Arx & Müller 1954, Punithalingam & Holiday 1973, Denman *et al.* 2000). Members of this genus have been reported to cause disease mainly on woody species including *Eucalyptus* (Smith *et al.* 2001). In South Africa, *B. parva* Pennycook & Samuels., *B. dothidea* (Moug.) Ces. & De Not. and *B. eucalyptorum* Crous, H. Smith et M. J. Wingf. have been reported to cause dieback and canker symptoms on *Eucalyptus* spp. (Smith, Kemp & Wingfield 1994, Smith *et al.* 2001, Slippers *et al.* 2002). In Uganda, *B. rhodina* (Cooke) Von Arx (anamorph *L. theobromae* (Pat.) Griffson & Maubl. has been reported on *Eucalyptus* spp. causing canker symptoms (Roux *et al.* 2001). *B. ribis* Grossenb. & Dugg. causes death of *Eucalyptus radiata* D. Don. in Australia (Shearer *et al.* 1987) and it is also the cause of basal cankers and coppice failure of *E. grandis* Hill ex Maid. in Florida (Barnard *et al.* 1987). However, the name *B. ribis* was used in these studies prior to recent taxonomic revisions based on DNA sequence data and this may be in error.

Preliminary surveys showed *B. rhodina* to be one of the pathogens responsible for the disease (Roux *et al.* 2001), detailed studies were necessary to determine whether other species might also be present. The aim of this study was, therefore, to identify *Botryosphaeria* spp. responsible for the stem canker on *Eucalyptus* spp. in Uganda.

This was done using identifications based on morphological and molecular characteristics of isolates. In addition, we considered the relative pathogenicity of *Botryosphaeria* species collected from *Eucalyptus* spp.

MATERIALS AND METHODS

Collection and isolation

Isolates were collected from three geographically distinct regions (Western, Eastern and Central) in Southern Uganda. Collection sites were selected to represent the agro-ecological regions within the commercial forestry production areas (Table 1, Figure 1). Sites and plantations from which collections were made were based on previous surveys by Roux *et al.* (2001). Dry twigs with fungal fruiting bodies were obtained from plantations with Botryosphaeria canker (Figure 2), packed in paper bags and transferred to the laboratory where they were stored at 4°C until isolations could be made.

For isolations, perithecia or pycnidia were picked from the twigs using a dissection microscope (Nikon Model: SMZ645 - Japan), and plated directly onto 2% malt extract agar (MEA) (20 g/l malt extract, 15 g/l agar, Biolab, Midrand, Johannesburg). The MEA cultures were incubated under near UV light for 10 days. Isolates resembling *Botryosphaeria* spp. were sub cultured until pure cultures were obtained. Fruiting structures from field-collected tissue were mounted on slides in a drop of lacto-phenol or water at this stage, for later morphological comparisons. Cultures were stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table 1).

To induce sporulation, *Botryosphaeria* cultures were transferred to water agar (2% Biolab Agar, Midrand, Johannesburg) plates containing sterile pine needles and incubated for 14 days at 25°C under near UV light. Isolates that did not sporulate on pine needles were transferred to sterile *Eucalyptus* leaves on water agar. Fruiting bodies formed on the pine needles (Figure 3A) and on *Eucalyptus* leaves (Figure 3B) were dissected and mounted in a drop of lacto-phenol for morphological characterisation of conidia. To obtain single spore cultures, conidia from the pine needles were dispersed on water agar and incubated for 8-24 hours at 25°C. Single germinating conidia for each sample were then transferred onto MEA plates.

incubated for 2 days at 25°C and then sub-cultured separately onto MEA plates. Cultures were stored at 4°C until required for DNA isolation.

Morphology

Length and width measurements were made for seven randomly selected conidia and/or ascospores for each isolate, using a light microscope fitted with a calibrated micrometer eyepiece (Carl Zeiss). Measurements for conidial length, width and length/width ratios were analysed using the general linear model of analysis of variance (ANOVA) and means were separated using Tukey's Honest Significant Difference (HSD) method available in STATISTICA for Windows (StatSoft 1995).

DNA isolation

For each single conidial isolate, actively growing mycelium on a MEA plate, was scraped off the surface of the culture using a sterile scalpel and transferred to a 1.5 µl eppendorf tube. The tubes were centrifuged at 12,000 rpm for 1 minute and all excess liquid was removed. The pellets were used for DNA extraction using a modified version of the method described by Raeder and Broda (1985). Mycelium was crushed using sterilized toothpicks and homogenised in 800 µl extraction buffer (200 mM Tris-HCL, pH 8.0; 150 mM NaCl; 25 mM EDTA pH 8.0; 0.5% SDS). A phenol-chloroform (1:1) mixture (400 µl) was added to each sample, mixed using a vortex mixer and centrifuged. This was repeated until the interface between the aqueous phase was clear of proteins and cell debris. Nucleic acids were precipitated by addition of 10% 3 M sodium acetate (pH 4.6) and 2 volumes of ice cold 100% ethanol, followed by centrifugation at 10,000 rpm for 30 minutes. A wash step involving the addition of 500 µl of 70% ethanol to the resulting pellet followed by centrifugation was included. The pelleted DNA was vacuum dried using a Speed vac Sc 100 vacuum drier (Savant Instruments Inc., Farmingdale, New York) and re-suspended in 30 µl sterile distilled water. RNA was degraded by addition of 5 µl RNase (1 mg/ml) to the sample and incubated for 3 hours in a 37°C water bath. DNA concentrations were estimated visually on a 1% agarose gel using known concentrations of lambda (λ) DNA under UV illumination.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify the rDNA (ITS 1, 5.8S and ITS 2) region in all isolates using the flanking primers ITS1 (5'-TTT CCG TAG GTG AAC CTG C-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (MWG Biotech, Germany) (White *et al.* 1990). For the amplification of the elongation factor (EF1-α), forward primer EF1 – 728F (5' CAT CGA GAA GTT CGA GAA GG – 3') and reverse primer EFI – 986R (5' TAC TTG AAG GAA CCC TTA CC-3') was used (MWG Biotech, Germany) (Slippers *et al.* 2002). The PCR reaction mixture contained 2 ng DNA template, 0.2 mM dNTPs (Promega, Madison, Wisconsin, USA), 0.15 µM of each primer, 5 U/µl Expand™ High Fidelity Taq polymerase (Roche Molecular Biochemicals, Almeda, CA), 10 x PCR reaction buffer containing 1.5 mM $MgCl_2$ (Roche diagnostic, Mannheim, Germany) and 17.4 µl water to a total reaction volume of 25 µl. The PCR reaction was carried out on a thermal cycler (Model: Mastercycle$^{(R)}$ Eppendorf) using the following amplification programme: Initial denaturation at 96°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 1 min at 56°C, followed by extension at 72°C for 1.5 min. A five sec elongation step was added to each cycle after the first 25 cycles. Finally, an extension at 72°C for 10 min completed the reaction. Five µl of the PCR reaction mixture was loaded onto a 2% agarose gel, also containing 1% ethidium bromide. This was exposed to UV light to visualize the PCR products.

Restriction Fragment Length Polymorphism (RFLP) analysis

Jacobs (2002) developed a PCR-RFLP method for reliable identification of South African isolates of *Botryosphaeria* that had been collected from mango trees and *Eucalyptus* spp. The method involves restriction digestion of PCR products of amplified ITS regions (ITS1, 5.8S, ITS2) with either *Cfo*I, *Alu*I or *Bst*I restriction enzymes. *Cfo*I restriction enzyme was found to produce the highest number of polymorphisms using computer aided restriction site analysis. This enzyme was thus selected for preliminary identification of the Ugandan isolates in this study.

A restriction digest was performed on all Ugandan isolates in 23 µl volumes containing 100 ng PCR product, 10 U/µl *Cfo*1 restriction enzyme and 1 ml 10X conc Buffer L (Promega, Madison, Wisconsin, USA). The reaction was incubated at 37°C for 3 hours. Polymorphic bands of the fragmented DNA were visualised and photographed after separation on a 2% agarose gel containing 1% ethidium bromide,

run at 60V for 3 hours. Banding patterns obtained were compared to known patterns of *Botryosphaeria* species obtained from Jacobs (2002).

DNA sequencing and phylogenetic analysis

Based on preliminary analysis of morphological characteristics, the *Botryosphaeria* isolates from Uganda were placed in three groups. ITS rDNA and elongation factor sequences (EF1-α) were determined for representative samples from each of the groups. The PCR products were purified using the High Pure PCR Product Purification kit following the manufacturer's published protocol (Instruction Manual Version 2.0, Roche Molecular Biochemicals, Mannheim, Germany). After purification, a sequencing PCR was performed on a thermal cycler (Model: Mastercycle® Perkin Elmer Corporation) in a 10 µl volume containing 10X ready reaction mix BD (ABI Prism BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit; Applied Biosystems), ~2.0 pmol/µl forward or reverse primer for each area sequenced (using the same primers used for PCR amplification), 5X dilution buffer, DNA (PCR product ~50 ng DNA) and 4.5 µl sterile distilled water. PCR was subsequently performed with the following parameters: initial denaturation at 96°C for 10 sec, annealing at 56°C for 30 sec and elongation at 60°C for 4 min for a total of 25 cycles. The PCR reaction was diluted to 20 µl with water and 3 M sodium acetate (pH 4.6) and 50 µl ice cold 100% ethanol added. The mixture was incubated for 10 min on ice and then centrifuged at 12000 rpm for 30 min. The supernatant was discarded and the DNA pellet washed with 80 µl ethanol (70%). The pellet was vacuum dried for 2 min using a vacuum drier (SpeedVac Sc 100 – Savant Instruments Inc. Farmingdale, New York). Automated sequencing was performed on an ABI Prism 3100 auto sequencer (Perkin-Elmer Applied Bio Systems, Foster City, CA, USA).

Sequence analysis involved manual editing using Sequence Navigator Version 1.0.1™ (Perkin-Elmer Applied BioSystems, Foster City, CA, USA). Homology searches were done from the GenBank/EMBL databases using the BLAST program (National Centre for Biotechnology Information, U. S. National Institute of Health, Bethesda. http://www.ncbi.nlm.nih.gov/BLAST). Four sequences with homologies >80%, known to be of *Botryosphaeria* spp. (Table 3), were selected and co-aligned with sequences obtained from Slippers *et al.* (2002) (Table 3) and Ugandan sequences

obtained in this study (Table 3) using the program ClustalX (Thompson, Higgins & Gibson 1994).

Phylogenetic analysis was first done for each gene region separately and then for a combined data set of the ITS rDNA and EF1-α sequences. This was preceded with performance of a partition homogeneity test to determine the congruence and combinability of the two sequence data sets (Huesenbeck, Bull & Cunnigham 1996). The software package, Phylogenetic Analysis Using Parsimony (PAUP) Version 4.01b (Swofford 1998) was used for the phylogenetic analysis.

The most parsimonious trees were obtained with heuristic searching using stepwise addition, tree bisection and reconstruction (TBR) as the branch swapping algorithms. All equally parsimonious trees were saved and all branches equal to zero were collapsed. Gaps were treated as fifth characters. Bootstrap confidence levels (1000 reps.) were done on the consensus parsimonious trees (Felsenstein 1985). The fungus *Guignardia philoprina* (Ellis) Viala & Ravaz, known to be related to *Botryosphaeria* spp. was used as the outgroup to root the trees (Slippers *et al.* 2002). It was treated as a monophyletic sister group to the other taxa.

Pathogenicity tests

Based on morphology, RFLP and sequence data, three distinct groups were identified from the Ugandan isolates. One representative isolate was selected from each of the two groups (CMW7236 and CMW7233) and two isolates from the third group (CMW7231 & CMW8052) for pathogenicity tests. An *Eucalyptus* clone (ZG14) that grows well in tropical and subtropical climates, and is also known to be highly susceptible to fungal pathogens (Van Heerden & Wingfield 2001) was selected for the experiment. Ten trees (~1-2 cm diameter) were selected for inoculation with each isolate and the control and these were acclimatised in a phytotron with regular day/night intervals (~20-25°C). A randomised experimental design was used with 5 treatments replicated 10 times. The whole experiment was repeated once in a second phytotron.

Agar disks were made from MEA plate cultures (10-day-old) completely covered with actively growing mycelium of each isolate using a 9 mm cork borer. The same size wounds were made in the bark of the trees to expose the cambium. Wounds were

made on the stems ~ 40 cm from the soil level. Trees were immediately inoculated by placing an agar disk, with the mycelium side facing the cambium into each wound. The site of inoculation was rapidly sealed with Parafilm (Pechiney plastic packing, Chicago, USA) to prevent desiccation and contamination. Control trees were inoculated with sterile MEA disks. After 3 weeks, the lengths and widths of the resulting lesions were recorded. The fungus was re-isolated from the lesions by cutting small pieces of wood from the leading edges of lesion margins and plating them directly onto MEA. Data were analysed using the general linear model of analysis of variance (ANOVA) and means were separated using the Least Significant Difference (LSD) method available in STATISTICA for Windows (StatSoft 1995).

RESULTS

Collection and isolation

A total of 40 *Botryosphaeria* isolates from *Eucalyptus* spp. in Uganda, were obtained from surveys. Twenty three isolates were from Bweyogerere (Kampala district, Central Uganda), ten from Kagwale, (Tororo district, Eastern Uganda), four from Baita Ababiri (Wakiso district, Entebbe, Central Uganda) and three from Mafuga (Kabale district, Western Uganda) (Table 1, Figure 1). Anamorph states of the fungi were obtained for most isolates by inoculation onto pine needles (Figure 3A). For some isolates, structures were only successfully obtained using *Eucalyptus* leaves in culture (Figure 3B). Pseudothecia containing asci and ascospores were obtained on naturally infected tissue, for 26 of the 40 collections.

Morphology

All ascospores on naturally infected tissue resembled those of *Botryosphaeria* spp. Conidia were characteristic of the *Fusicoccum* state of *Botryosphaeria* spp. A single isolate (CMW7233), with conidial characteristics of *L. theobromae* (Figure 4D) was obtained. This isolate was excluded from other comparisons, as there was no doubt as to its identity. There appeared to be no significant differences in cultural morphology for isolates growing on MEA, which generally displayed greyish fluffy mycelium (Figure 3D). The fluffiness, however, reduced with culture maturity. The underside of cultures appeared black. Teleomorph structures from naturally infected tissue (Figure 4A), appeared similar with ascospore lengths ranging from 17.7 to 22.3 µm and widths ranging form 5.4 to 7.4 µm (Table 2). Most conidia appeared hyaline

(Figure 4B), while conidia for three isolates, (CMW8036, CMW8286 and CMW7231) appeared granular (Figure 4C). Conidial lengths obtained ranged from 12.4 to 23.2 µm, while the widths ranged from 4.7 to 10.2 µm.

Analysis of variance for conidial length and length/width ratios among isolates was found to be highly significant ($p < 0.0001$). Graphs of length of conidia and length/width ratio were constructed based on 95% confidence limits (Figure 5 A & B). From the analysis, three groups could be distinguished. Group A had large (21-23 µm) conidia, group B had conidia of intermediate (19.5-21 µm) size and group C had small (17.6-19 µm) conidia. Analysis of the length/width ratio of conidia did not provide additional data to those for length measurements.

Polymerase chain reaction and Restriction Fragment Length polymorphisms (RFLP)

DNA was successfully isolated from all the samples and polymerase chain reaction amplifications of the ITS rDNA produced fragments of ~550bp in size. The EF1-α regions produced fragments of ~ 309bp. After restriction digests of the PCR products with *Cfo*I, all but one isolate, produced a banding pattern similar to that of *Fusiccocum* spp., based on previous reports of Jacobs (2002) (Figure 6). Isolate CMW7233 produced a banding pattern characteristic of *L. theobromae* (Jacobs 2002) (Figure 6), confirming its identity as determined based on morphology.

DNA sequencing and phylogenetic analysis

Complete sequences were obtained for both the ITS rDNA and EF1-α regions. All isolates used for sequencing could be aligned for both regions. The total aligned length for the ITS rDNA was 558 bp, elongation factor EF1- α was 309 bp and 867 bp for the combined regions (Figure 12).

Phylogenetic analysis of the ITS rDNA resulted in 558 characters of equal weight. Of these, 430 were constant, 65 variables were parsimony-uninformative and 63 were parsimony informative. A total of 11 most parsimonious trees were retained with a length of 164, a consistency index (CI) of 0.878 and retention index (RI) of 0.894. A bootstrap analysis of 1000 replicates resulted in a tree with the same topology as the most parsimonious trees (Figure 7). The ITS rDNA tree consisted of seven clades (Figure 7). Clade I contained *B. obtusa* (Schwein.) Shoem., and a *Diplodia* sp., clade

II was comprised of *B. stevensii* Shoem., while clade III contained two *B. dothidea* isolates (Slippers *et al.* 2002). Clade IV contained one Ugandan isolate together with *B. ribis* isolates (Slippers *et al.* 2002). Clade V contained *B. parva* and Ugandan isolates. Clade VI contained Ugandan isolates grouping separately and clade VII contained *Fusicoccum luteum* Pennycook & Samuels isolates.

Phylogenetic analysis of the EF1-α region resulted in 309 characters of equal weight where, 120 of the characters were constant, 79 variable characters were parsimony uninformative and 110 were parsimony informative. Eight trees were obtained. The most parsimonious tree was obtained with a length of 285, a consistence index (CI) of 0.874 and a retention index (RI) of 0.864. A bootstrap analysis of 1000 replicates resulted in a tree with the same topology as the most parsimonious trees (Figure 8). Seven clades were obtained (Figure 8). Clade I contained Ugandan isolates (CMW8286, CMW7231, CMW8041 & CMW7230) forming a separate group, but most closely to *B. ribis*. Clade II contained *B. ribis* isolates. Clade III contained Ugandan isolates (CMW8045, CMW7500, CMW7238, CMW7236, CMW7237 & CMW8052) together with *B. parva* isolates (Slippers *et al.* 2002). Clade IV contained *B. eucalyptorum* isolates (Smith *et al.* 2001), while clades V to VII consisted of known species used in the analysis only for comparative purposes (Figure 8).

A combined phylogenetic analysis of both ITS and EF1-α sequence data generated 847 characters of equal weight, with 482 constant characters of which, 229 were parsimony uninformative and 136 were parsimony informative. Two most parsimonious trees were retained, with a length of 428, a consistence index (CI) of 0.986 and retention index (RI) of 0.972. A bootstrap analysis of 1000 replicates resulted in a tree with the same topology as the most parsimonious trees (Figure 9). The most parsimonious tree consisted of four clades. Clade I contained Ugandan isolates together with *B. parva,* clade II contained *B. ribis* isolates, while clade III contained some Ugandan isolates grouping separately, but more closely to *B. ribis*. However, Ugandan isolate CMW8052 grouped slightly separate from the rest and could not be designated to a different clade due to a low bootstrap value (56). It was, however, most similar to *B. ribis*. Clade IV contained *B. dothidea* isolates (Slippers *et al.* 2002) (Figure 9).

Pathogenicity tests

Three weeks after inoculation, dark to light brown lesions, stretching from the site of inoculation, up and down the stems and extending into the xylem (observed by peeling off the bark and sectioning) were observed. In many cases lesions appeared sunken, indicating cell necrosis characteristic of *Botryosphaeria* canker (Figure 10).

Mean inner lesion lengths in the first experiment, ranged from 110 mm for CMW7233 to 200 mm for CMW8052. Bark lesion lengths ranged from 61 mm for isolate CMW7233 to 129 mm in isolate CMW7231. The differences observed between isolates were significant ($p<0.001$). These differences were generally similar in both trials (Figure 11). Isolate CMW8052 showed greatest pathogenicity, which was significantly different from isolates CMW7236 and CMW7233, in both trials (Figure 11A, B). The Pearson product moment correlation analysis between the two trials produced high and significant correlations for bark lesion lengths ($r= 0.95$) and inner lesion lengths ($r=0.98$). All lesions associated with inoculations differed significantly from the controls (Figure 11).

DISCUSSION

Results of this study have shown that at least three *Botryosphaeria* spp. are associated with Botryosphaeria canker of *Eucalyptus* spp. in Uganda. Of these, *B. parva* and an unidentified species are most abundant, *L. theobromae* represented by a single isolate appears to be rare. Cankers associated with *Botryosphaeria* spp. represent the most common disease of *Eucalyptus* trees in Uganda, resulting in loss of growth and greatly reducing product quality.

Initial identification of isolates based on conidial morphology showed that three distinct groups exist amongst the Ugandan isolates. These were characterised by conidia with septa, conidia containing granular structures and hyaline conidia without granules. From these observations it was clear that one of the isolates represented *L. theobromae*, which has very characteristically shaped two-celled conidia with striations (Punithalingam 1976). Based on conidial measurements the remaining isolates appeared to represent three different species of *Botryosphaeria* with *Fusicoccum* anamorphs (Jacobs & Rehner 1998, Denman *et al.* 2000).

PCR-RFLP characterisation distinguished only two groups among the Ugandan isolates. One group was represented by the single isolate (CMW7233) that had been identified as *L. theobromae* based on morphology. All other isolates showed the same RFLP banding pattern as that of *B. parva*. It was thus not possible to distinguish the isolates, which had *Fusicoccum* conidia, even though they differed in appearance and size. The PCR-RFLP method did not seem to offer enough resolution to be able to concur with the observed morphological differences. Sequence analysis of the ITS rDNA and EF1-α were therefore attempted as they have been shown to reflect a proper phylogeny (Taylor *et al.* 2000, Slippers *et al.* 2002).

DNA sequence data showed that, apart from *L. theobromae*, two other *Botryosphaeria* spp. are associated with Botryosphaeria canker in Uganda. One group clearly represents *B. parva* as recently defined by Slippers *et al.* (2002). The second group of isolates, although grouping close to *B. ribis* (Slippers *et al.* 2002), formed a separate cluster with relatively high bootstrap support. The fact that morphologically different isolates group together based on their ITS rDNA sequence information has been observed and reported previously (Denman *et al.* 2000, Ogata, Sano & Harada 2000, Zhonghua & Michailides 2002, Slippers *et al.* 2002). Jacobs and Rehner (1998) for instance, despite having grouped *B. dothidea* isolates into two groups based on ITS rDNA information, showed that these groups contained more than five morphologically distinct groups.

Lasiodiplodia theobromae is known as an important pathogen on a variety of fruit and forest trees, worldwide (Cilliers, Swart & Wingfield 1993, Punithalingam & Holliday 1973). It has been reported on *Hevea brasiliensis* Mull. Arg. and *Pinus* spp. causing dieback and blue stain of timber (Fu, Shi & Li 1988), on *Pyrus* spp. resulting in canker and dieback (Avtar, Aulakh & Chahal 1990), on *Eucalyptus* spp. as the cause of root collar canker and wilting (Sharma, Mohanan & Florence 1985), on *Carica papaya*. L. causing fruit rot (Hunter, Buddenhagen & Kojima 1969) and on *Mangifera indica* L. causing pre- and postharvest diseases (Punithalingam 1976). The occurrence of *L. theobromae* on *Eucalyptus* spp. in Uganda was noted by Roux *et al.* (2001), although detailed studies to quantify the extent of damage have not been made. During the current study only a single isolate of *L. theobromae* was obtained. This might suggest that it does not play a major role in Botryosphaeria canker of

Eucalyptus spp. in Uganda. This is also confirmed by its relatively low level of pathogenicity in the greenhouse inoculations.

Botryosphaeria parva was first described in 1985 causing ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand (Pennycook & Samuels 1985). It is known worldwide to be a pathogen of woody plants (Von Arx 1987). *B. parva* has for example, also been reported on mangoes causing pre- and post harvest diseases (Ramos *et al.* 1991, Johnson 1992) and has been described as an endophyte in healthy Mango tissue (Jacobs 2002). Considerable controversy exists regarding the identity of *B. parva*. Many morphological features overlap with those of *B. ribis*, a well-known pathogen of *Eucalyptus* spp. (Shearer *et al.* 1987). Suggestions have been made that these two species are synonyms, however, recent research using both morphological and molecular data has confirmed that they are distinct (Slippers *et al.* 2002). Results of the present study show that *B. parva* from Uganda is highly pathogenic on *Eucalyptus* and we believe that it is one of the most common causes of Botryosphaeria canker in that country.

The third *Botyrosphaeria* sp. isolated in this study cannot be named at present. Although most closely related to *B.ribis*, it forms a distinct clade, with high Bootstrap support. The genus *Botryosphaeria* especially species associated with plantation diseases are currently undergoing major revision (Slippers *et al.* unpublished, Slippers *et al.* 2002). Once this process is completed, the known species from Uganda might acquire an identity otherwise it will be described as a new species in the near future.

Greenhouse inoculations revealed that all three *Botryosphaeria* spp. obtained from *Eucalyptus* in Uganda are pathogenic to *E. grandis*. Although significantly different from the control, *L. theobromae* produced the smallest lesions. Two of the isolates representing the unidentified *Botryosphaeria* sp. were the most pathogenic of the fungi tested, with their pathogenicity significantly different to that of isolates representing *B. parva* and *L. theobromae*. Bark lesions were closely correlated with cambium lesions, thus giving similar results. Although based on a very limited number of isolates, data suggest that *L. theobromae* is not a major cause of disease in Uganda. The unknown *Botryosphaeria* sp. and *B. parva* are considerably more virulent and probably the major causes of Botryosphaeria canker in the country. What

is now required is inoculations on mature trees in the field to confirm these observations.

Based on these preliminary results, the Ugandan Forestry Department should include *Botryosphaeria* spp. as a potential constraint to *Eucalyptus* propagation in that country. Certainly further research on these pathogens is justified. The situation in Uganda appears to be similar to that in South Africa (Smith *et al.* 1994, 1996, 2001) where *Botryosphaeria* canker is one of the most common diseases of *E. grandis*. Thus, steps should be taken to improve the quality of planting stock and to ensure stringent site/genotype matching. Because *Botryosphaeria* spp. are known to be stress related, opportunistic pathogens of *Eucalyptus* spp. (Smith *et al.* 1994, 1996), failure to avoid stressful situations could result in substantial loss.

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Table 1. *Botryosphaeria* isolates from Uganda used in this study and their areas of collection.

CMW No.	District	SubCounty	Village	Agro ecological zone	Average temperature (°C)	Average rainfall ($mmyr^{-1}$)	Altitude (m)
7229	Tororo	Tororo Municipality	Kagwale	Lake Victoria Crescent	22.5	1427	1170
7230	"	"	"	"	"	"	"
7231	"	"	"	"	"	"	"
7232	Kabale	"	Mafuga	Kabale-Rukungiri Highlands	15..3	1181	2241
7233	"	"	"	"	"	"	"
7234	"	"	"	"	"	"	"
7235	"	"	"	"	"	"	"
7236	"	"	"	"	"	"	"
7237	"	"	"	"	"	"	"
7238	"	"	"	"	"	"	"
7494	"	"	"	"	"	"	"
7496	"	"	"	"	"	"	"
7497	"	"	"	"	"	"	"
7499	"	"	"	"	"	"	"
7500	"	"	"	"	"	"	"
7561	"	"	"	"	"	"	"
7562	Wakiso (South)	Entebbe	Abayita Ababiri	Lake Victoria Crescent	21.5	1538	1115
7959	"	"	"	"	"	"	"
8036	"	"	"	"	"	"	"
8037	"	"	"	"	"	"	"
8038	"	"	"	"	"	"	"
8039	"	"	"	"	"	"	"
8040	"	"	"	"	"	"	"
8041	"	"	"	"	"	"	"
8042	Kampala (East)	Nakawa	Bweyogerere	"	21.5	1538	1115
8044	"	"	"	"	"	"	"
8045				"	"	"	"
8046	"	"	"	"	"	"	"
8047	"	"	"	"	"	"	"
8048	"	"	"	"	"	"	"
8049	"	"	"	"	"	"	"
8050	"	"	"	"	"	"	"
8051	"	"	"	"	"	"	"
8052	"	"	"	"	"	"	"
8053	"	"	"	"	"	"	"
8286	"	"	"	"	"	"	"
8615	"	"	"	"	"	"	"
10171	"	"	"	"	"	"	"
10177	"	"	"	"	"	"	"

Table 2. Morphological characteristics of conidia and ascospores of *Botryosphaeria* isolates from Uganda.

	Anamorph[a]		Morphology (Anamorph)	Teleomorph[a]	
CMW No.	Length	Width		Length	Width
7229	18.5	6.2	hyaline, non-septate, non-granular	-	-
7230	19.5	5.3	"	-	-
7231	18.4	4.6	hyaline, non-septate, granular	-	-
7232	19.9	5.3	hyaline, non-septate, non-granular	19.8	5.4
7233	21.0	10.2	dark, septate, non-granular	-	-
7234	17.9	4.7	hyaline, non-septate, non-granular	-	-
7235	18.6	4.7	"	-	-
7236	18.7	5.2	"	-	-
7237	19.0	4.7	"	19.9	5.8
7238	18.6	4.7	"	-	-
7494	20.0	6.2	"	-	-
7496	19.3	5.3	"	-	-
7497	18.6	4.7	"	20.6	5.8
7499	19.0	4.7	"	19.4	5.8
7500	20.5	5.5	"	20.3	7
7561	18.8	6.2	"	20.3	7.4
7562	20.2	4.7	"	19.9	5.8
7959	20.2	4.7	"	19.7	5.8
8036	23.2	6.2	hyaline, non-septate, granular	-	-
8037	18.3	5.1	hyaline, non-septate, non-granular	20.9	6.2
8038	18.6	4.7	"	22.3	7.2
8039	21.8	6.2	"	-	-
8040	18.7	4.7	"	20.8	5.8
8041	22.4	5.5	"	19.9	6.2
8042	19.9	4.7	"	19.9	6.6
8044	19.1	4.9	"	19.8	7.4
8045	19.3	4.7	"	19.4	6.2
8046	19.5	5.5	"	20.5	6.6
8047	19.7	5.5	"	17.7	5
8048	18.6	5.1	"	20.3	5.4
8049	18.6	6.2	"	20.6	7.4
8050	18.6	5.5	"	20.2	5.8
8051	17.4	4.7	"	19.4	7.4
8052	21.4	6.2	"	19.8	5.4
8053	18.6	5.5	"	20.2	5.8
8286	21.9	6.2	hyaline, non-septate, granular	-	-
8615	18.8	5.5	hyaline, non-septate, non-granular	21.0	7.4
10171	19.2	4.9	"	19.8	5.4
10177	18.7	5.1	"	21.1	6.6

[a] measurements in µm. Values represent an average of length and width measurements.

Table 3. *Botryosphaeria* isolates used for phylogenetic analysis.

Culture No.	Identity	Host	Origin	Collector	Accession No.
CMW7780	*B. dothidea*	*Fraxinus excelsior*	Switzerland	B. Slippers	
CMW8000	*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	
CMW10125	*B. eucalyptorum*	*Eucalyptus grandis*	S. Africa	H. Smith	AF283686
CMW10126	*B. eucalyptorum*	"	S. Africa	H. Smith	AF283687
CMW992/3	*B. lutea*	*Actinidia deliciosa*	New Zealand	G.J. Samuels	AF027743
CMW9076	*B. lutea*	*Malus X domestica*	New Zealand	S.R .Pennycook	
CMW7772	*B. ribis*	*Ribis* sp.	New York	B. Slippers/G. Hudler	
CMW7773	*B. ribis*	"	New York	B. Slippers/G. Hudler	
CMW9077	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	
CMW9078	*B. parva*	"	New Zealand	S.R. Pennycook	
CMW7774	*B. obtusa*	Apple	USA	T. Sano	AB034822
CMW7060	*B. stevensii*	-	-	S. S. Zhou/G.R. Stanosz	AF243407
-	*Diplodia* sp.	*Pinus sylvestris*		S. Schroeder/Sterflinger	AJ292761
[a] CMW8052	Unknown	*Eucalyptus grandis*	Uganda	G. Nakabonge/J. Roux	AY226856, AY228104
[a] CMW7238	*B. parva*	"	"	"	AY226851, AY228097
[a] CMW7500	"	"	"	"	AY226849, AY228095
[a] CMW8045	"	"	"	"	AY226848, AY228096
[a] CMW7236	"	"	"	"	AY226850, AY228100
[a] CMW7237	"	"	"	"	AY228103
[a] CMW7231	Unknown	"	"	"	AY226853, AY228105
[a] CMW8036	"	"	"	"	AY228099
[a] CMW7230	"	"	"	"	AY226855, AY228089
[a] CMW8041	"	"	"	"	AY226854, AY228101
[a] CMW8286	"	"	"	"	AY226852, AY228102
[a] CMW7063	*Guignardia philoprina*	*Taxus baccata*	Netherlands	H.A. van der Aa	

[a] Isolates sequenced in this study.

Figure 1. Map of Uganda showing sites from where *Botryosphaeria* isolates were collected for this study.

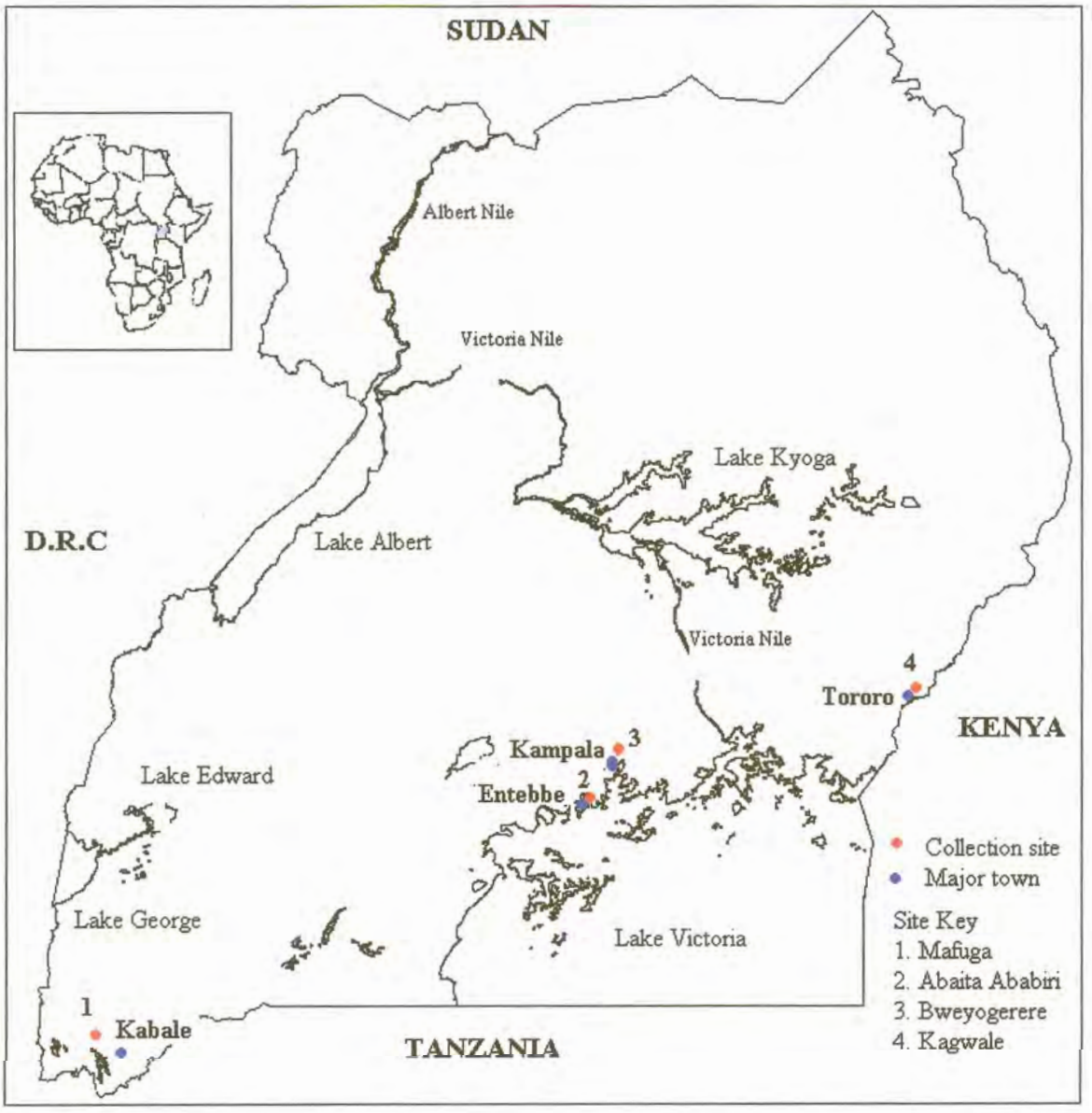
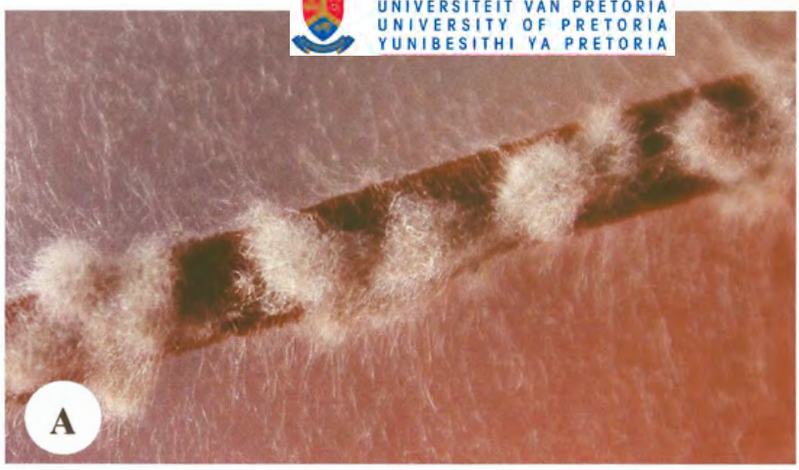
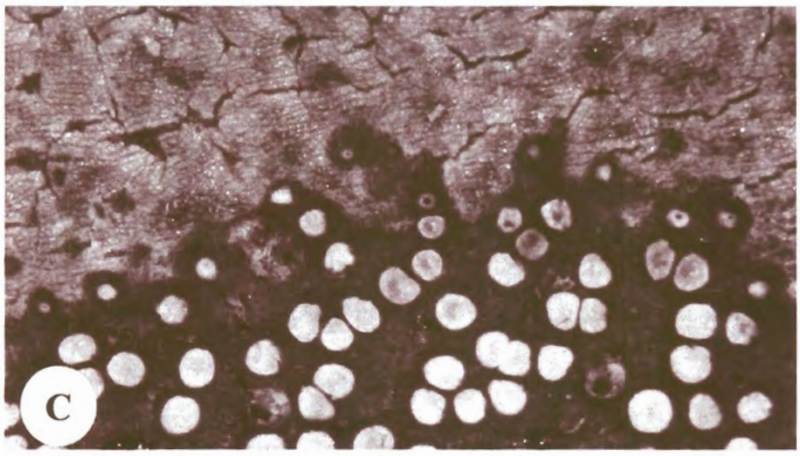

SUDAN
Albert Nile
Victoria Nile
Lake Kyoga
D.R.C
Lake Albert
Victoria Nile
4
Tororo
KENYA
Kampala
3
Lake Edward
Entebbe
2
Collection site
Major town
Site Key
1. Mafuga
2. Abaita Ababiri
3. Bweyogerere
4. Kagwale
Lake George
Lake Victoria
1
Kabale
TANZANIA

Figure 2. Symptoms associated with infection of *E. grandis* with *Botryosphaeria* spp. **A)** Death of stem and formation of double-leaders. **B)** Stem cankers, characterised by cracks and kino exudation. **C)** Kino pockets/rings in the xylem of infected trees.

Figure 3. Cultural characterists of *Botryosphaeria* isolates associated with canker on *Eucalyptus* spp. in Uganda. **A)** Typical *Botryosphaeria* isolate growing on a sterilised pine needle on which they were inoculated to induce sporulation. **B)** Typical *Botryosphaeria* isolate which did not grow on pine needles, growing on an *Eucalyptus* leaf. **C)** Cross section of pycnidia growing on dry *Eucalyptus* twigs. **D)** Cultural characteristics of *Botryosphaeria* isolates growing on malt extract agar (MEA). Note that they all looked similar.





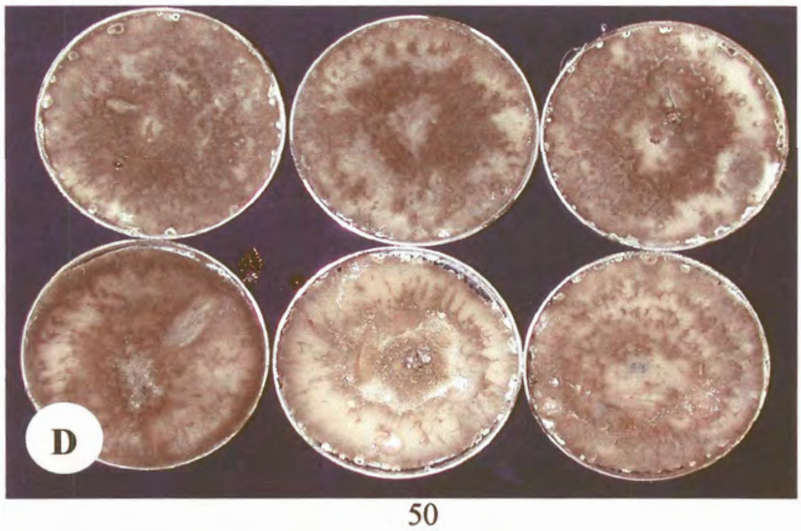


Figure 4. Morphological structures of *Botryosphaeria* spp. from *Eucalyptus* in Uganda. **A)** Teleomorph showing asci and ascospores. **B)** *Fusicoccum* state of *Botryosphaeria* sp. **C)** *Fusicoccum* state with granular appearance of conidia. **D)** *Lasiodiplodia theobromae* with typical septa. (All scale bars = 10 μm).

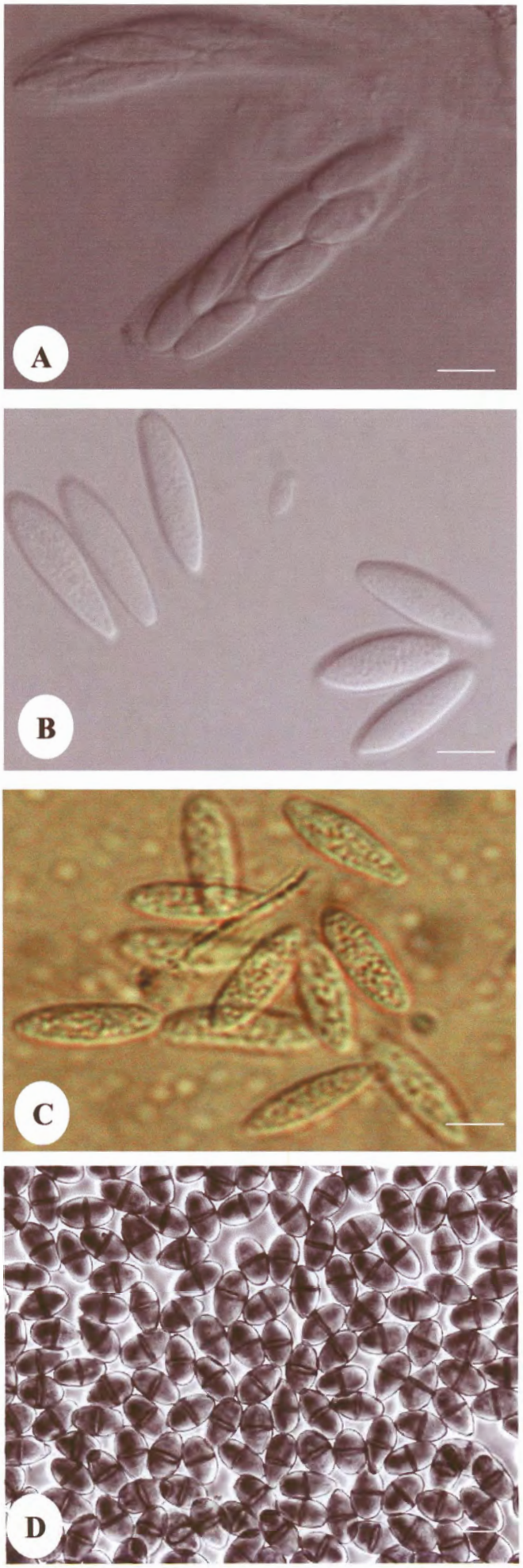
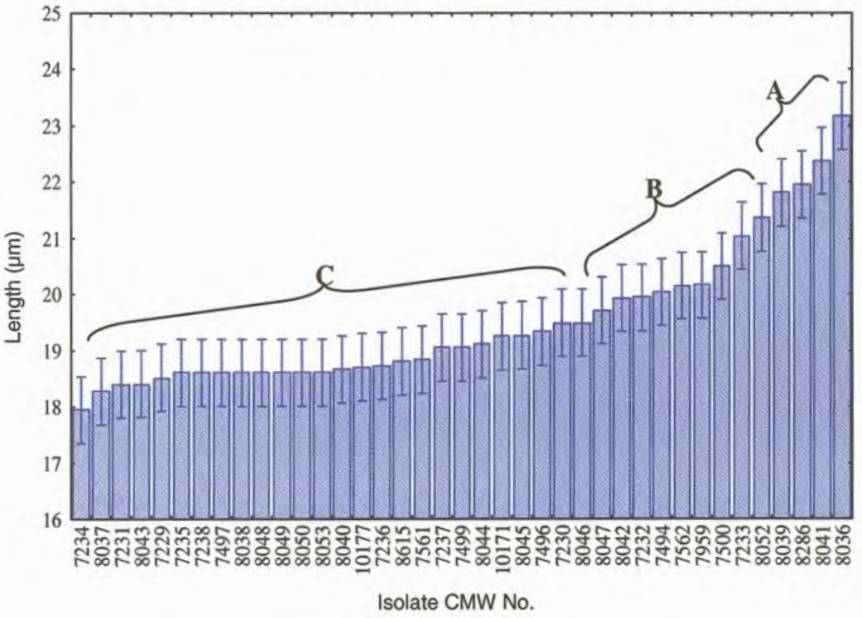

A
B
C
D

Figure 5. Conidial measurements of *Botryosphaeria* isolates from Uganda. **A)** Comparisons of length. **B)** Comparisons of length/width ratio. Horizontal bars indicate groups in which the means are significantly different ($p < 0.0001$).

A


Length (µm)
25
24
23
22
21
20
19
18
17
16
A
B
C
7234 8037 7231 8043 7229 7235 7238 7497 8038 8048 8049 8050 8053 8040 10177 7236 8615 7561 7237 7499 8044 10171 8045 7496 7230 8046 8047 8042 7232 7494 7562 7959 7500 7233 8052 8039 8286 8041 8036
Isolate CMW No.

B

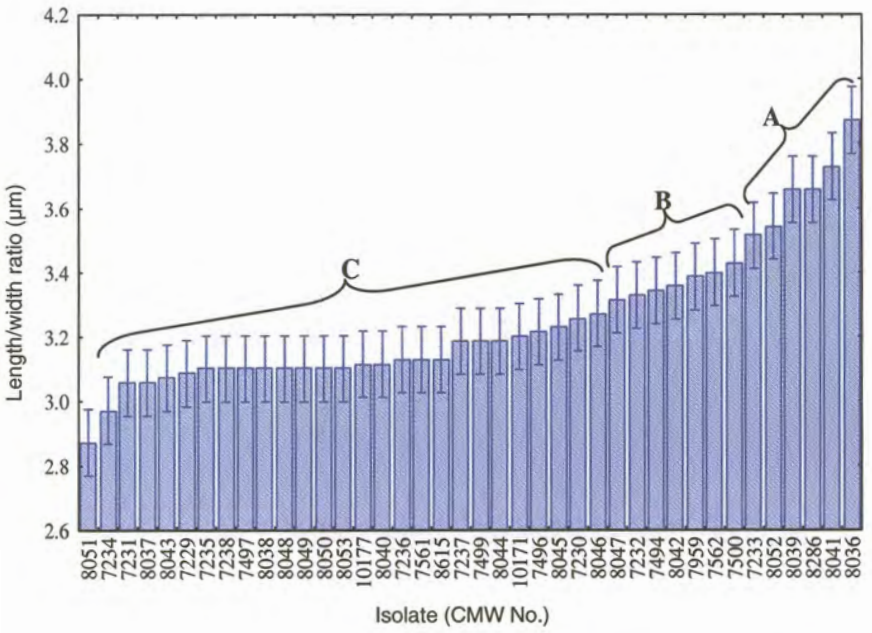

Length/width ratio (µm)
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
A
B
C
8051 7234 7231 8037 8043 7229 7235 7238 7497 8038 8048 8049 8050 8053 10177 8040 7236 7561 8615 7237 7499 8044 10171 7496 8045 7230 8046 8047 7232 7494 8042 7959 7562 7500 7233 8052 8039 8286 8041 8036
Isolate (CMW No.)

Figure 6. An agarose gel (2%) profile showing polymorphic banding patterns obtained after restriction digestion of a PCR amplicon of *Botryosphaeria* isolates with *Cfo*I restriction enzyme. Lane 1 represents a 100bp marker, lanes 1-19 and 21 show *Fusicoccum parva* like banding patterns while lane 20 shows a *Lasiodiplodia theobromae* banding pattern (Jacobs 2000).

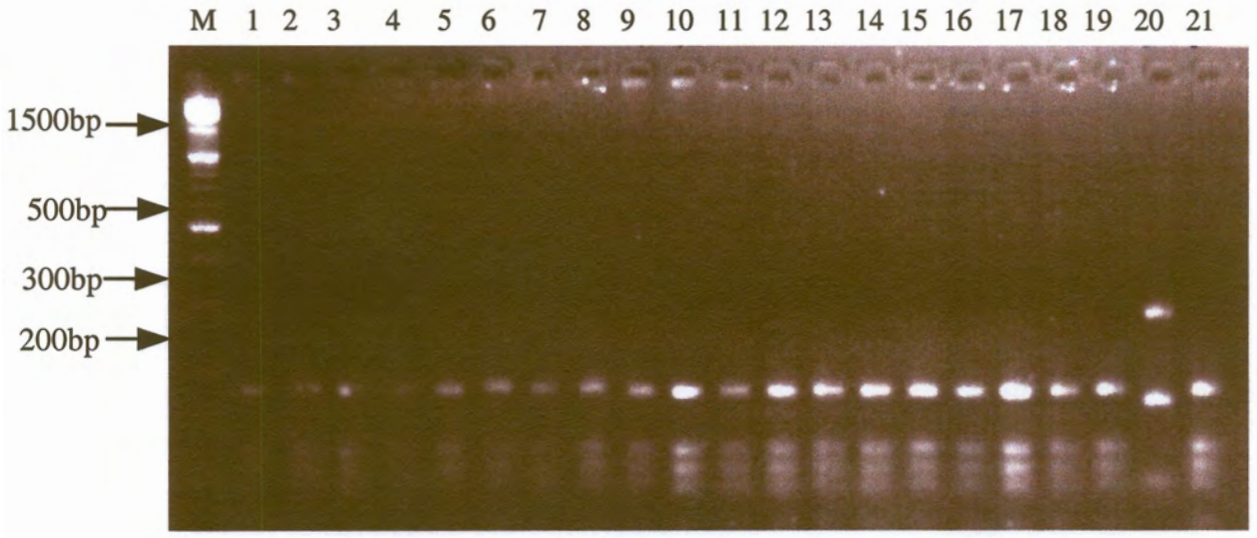

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
1500bp
500bp
300bp
200bp

Figure 7. Most parsimonious phylogenetic tree obtained from a heuristic search of the ITS rDNA data of *Botryosphaeria* isolates from Uganda (red font) compared to other known isolates (black font). Numbers above and below the branches are distances and bootstrap values respectively.

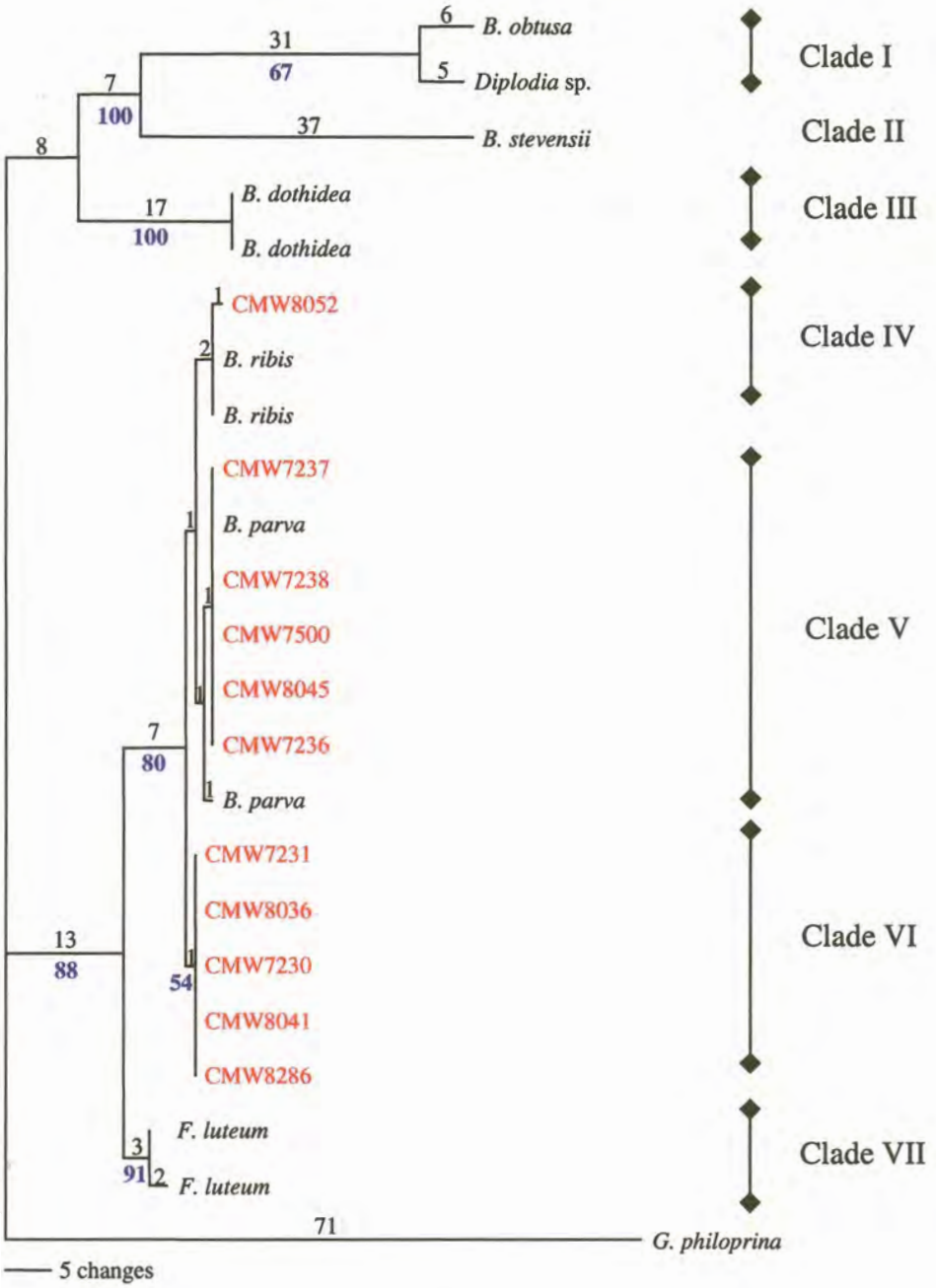


Figure 8. Most parsimonious phylogenetic tree obtained from a heuristic search of the EF1-α sequence data of *Botryosphaeria* isolates from Uganda (red font) compared to other known isolates (black font). Numbers above and below the branches are distances and bootstrap values respectively.

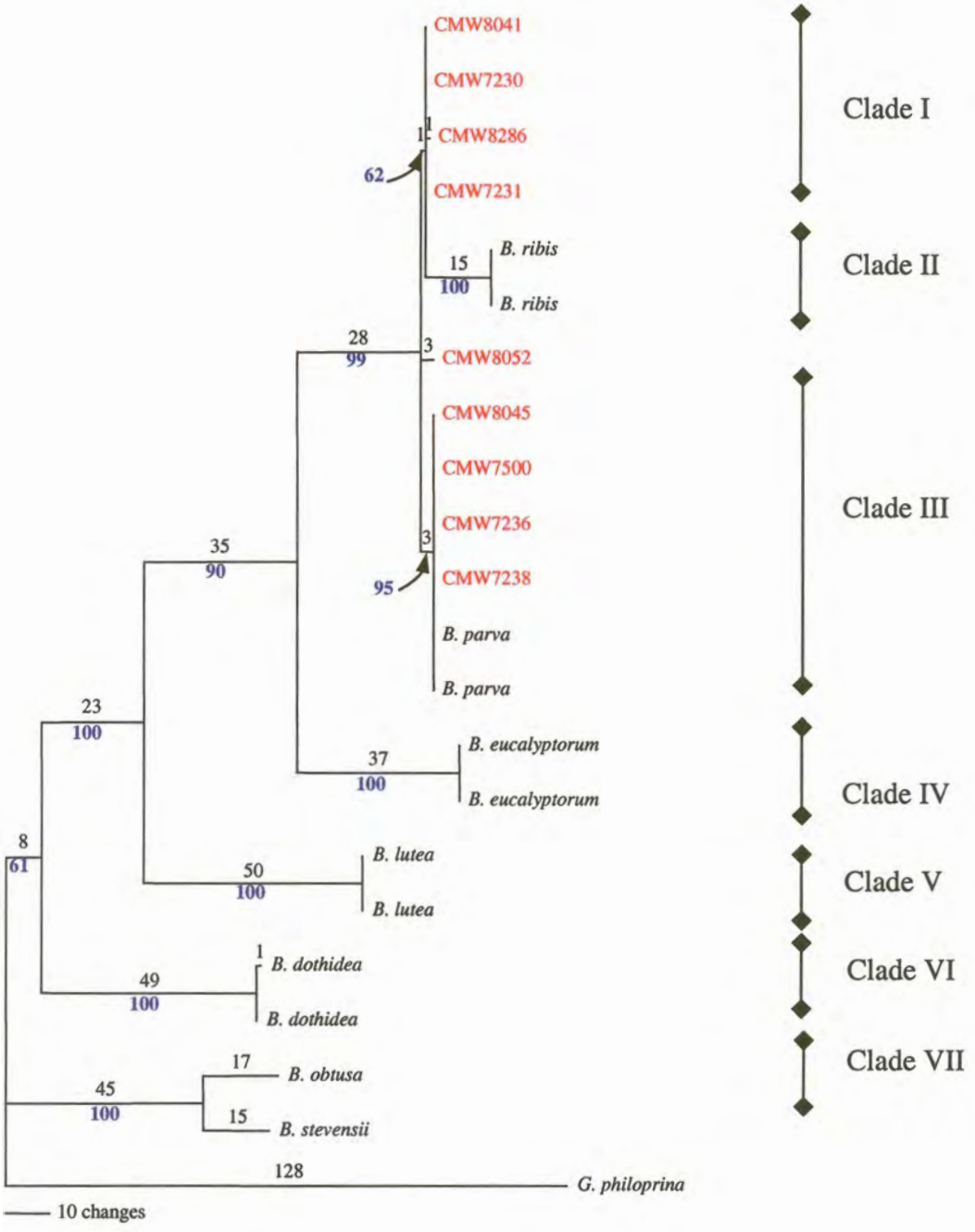

CMW8041
CMW7230
1
1
CMW8286
62
CMW7231
B. ribis
15
100
B. ribis
28
99
3
CMW8052
CMW8045
CMW7500
CMW7236
3
35
90
95
CMW7238
B. parva
B. parva
23
100
B. eucalyptorum
37
100
B. eucalyptorum
8
61
B. lutea
50
100
B. lutea
1
B. dothidea
49
100
B. dothidea
17
B. obtusa
45
100
15
B. stevensii
128
G. philoprina
10 changes
Clade I
Clade II
Clade III
Clade IV
Clade V
Clade VI
Clade VII

Figure 9. Most parsimonious phylogenetic tree obtained from a heuristic search of combined ITS rDNA and EF1-α sequence data of *Botryosphaeria* isolates from Uganda (red font) compared to other known isolates (black font). Numbers above and below the branches are distances and bootstrap values respectively.

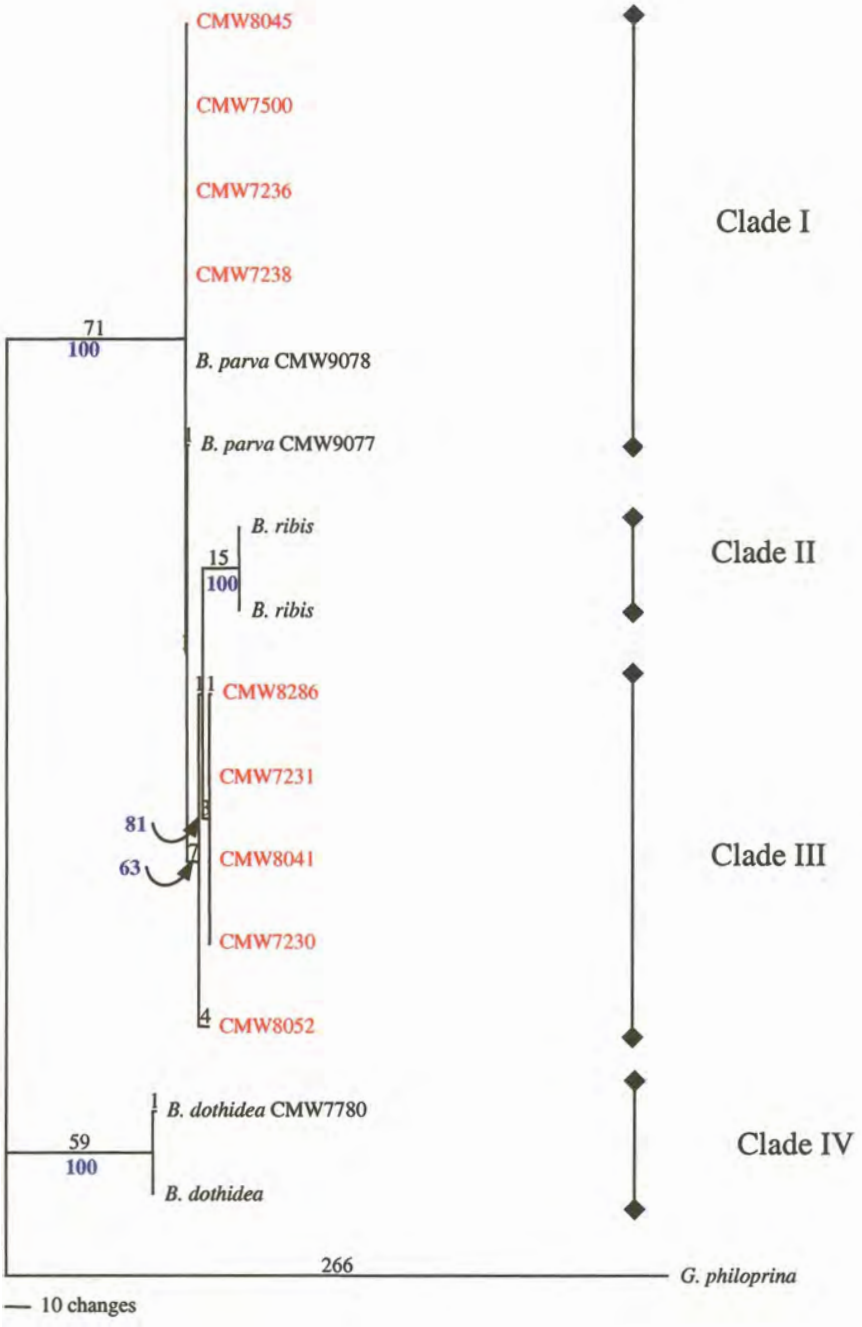

CMW8045
CMW7500
CMW7236
CMW7238
71
100
B. parva CMW9078
B. parva CMW9077
B. ribis
15
100
B. ribis
11
CMW8286
CMW7231
81
63
CMW8041
CMW7230
4
CMW8052
1
B. dothidea CMW7780
59
100
B. dothidea
266
G. philoprina
10 changes
Clade I
Clade II
Clade III
Clade IV

Figure 10. Development of symptomatic lesions on an *E. grandis* clone (ZG14) after inoculation with *Botryosphaeria* isolates from Uganda. **A)** and **B)** Symptom development on inoculated trees. **C)** Formation of callus tissue (indicated by arrow) around the wound of a control treatment (water agar).

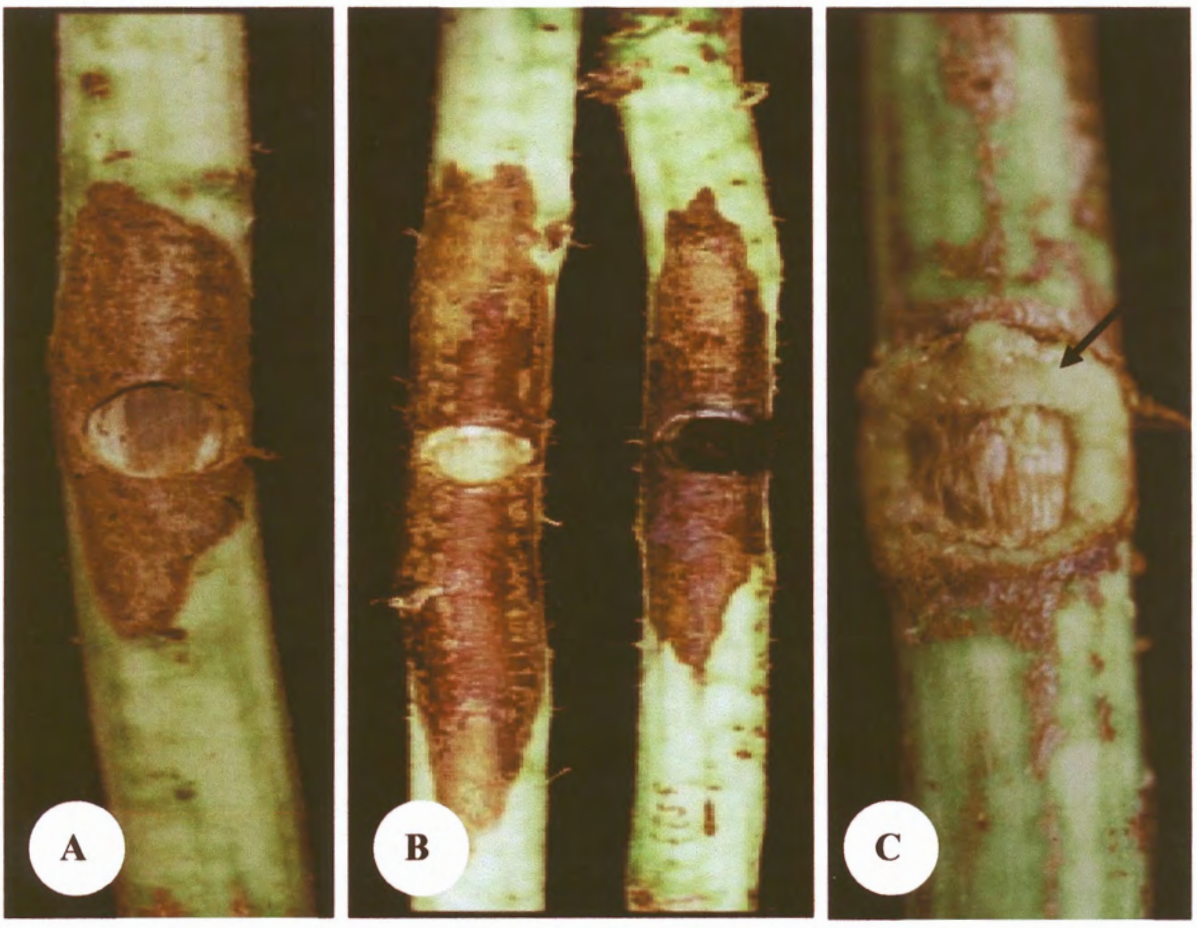
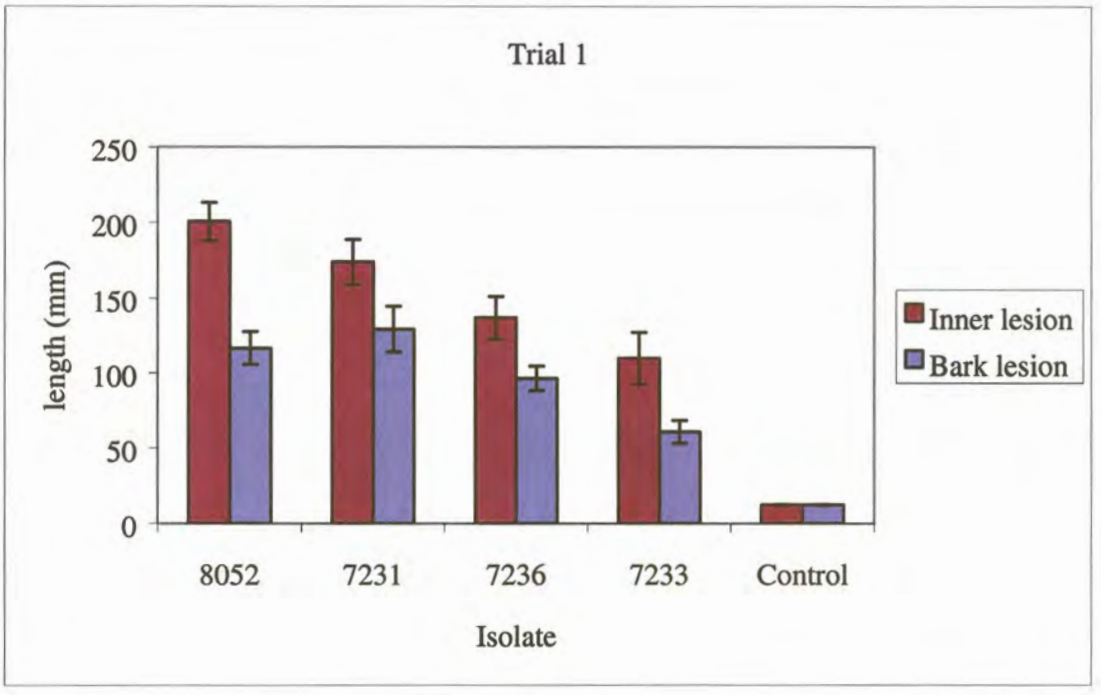

A
B
C

Figure 11. Comparison of pathogenicity of *Botryosphaeria* isolates on an *E. grandis* hybrid (ZG14) tested in two greenhouse trials using inner lesion length and bark lesion length (mm).
Unknown spp. (CMW8052 & CMW7231), *B. parva* (CMW7236) and *L. theobromae* (CMW7233).

NB. Error bars derived from standard error of means.

A


Trial 1
250
200
150
100
50
0
length (mm)
8052
7231
7236
7233
Control
Isolate
Inner lesion
Bark lesion

B

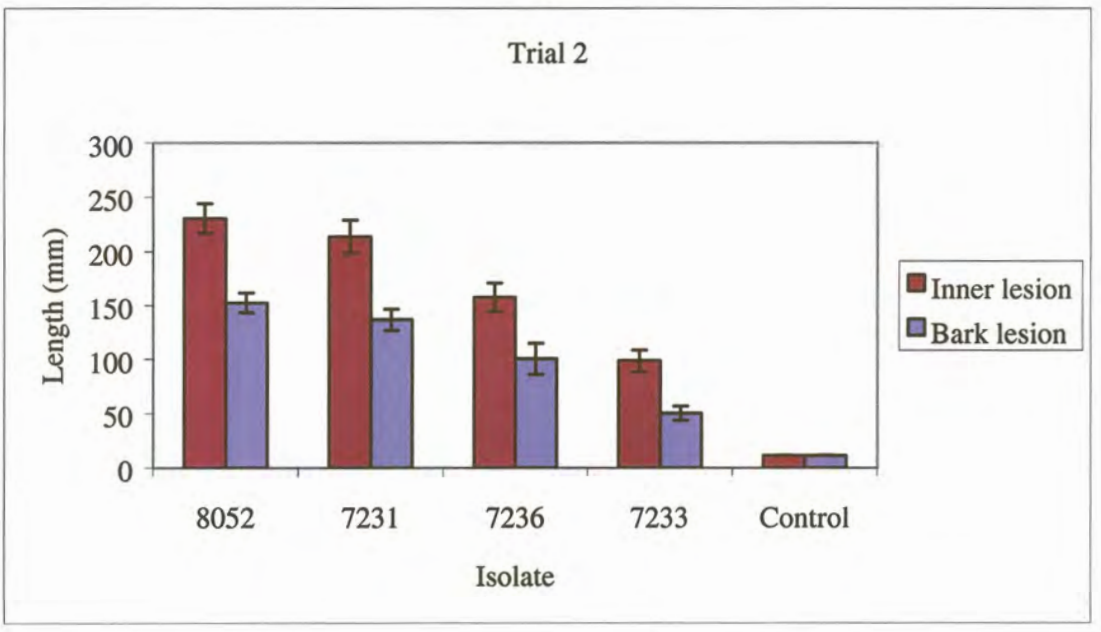

Trial 2
300
250
200
150
100
50
0
Length (mm)
8052
7231
7236
7233
Control
Isolate
Inner lesion
Bark lesion

Figure 12. Combined DNA sequence data for ITS rDNA and EF1-α of Ugandan isolates, aligned against sequences of *B. parva, B. ribis,* and *B. dothidea* species obtained from Slippers *et al.* (2002).
(- represents gaps, ? represents missing data, . represents identical bases)

```
                                  10         20         30         40         50         60
B.parva          (CMW8042)  AGAAGGTAAG AAAGTTTTTC CTTCCGCTGC ACGCGCTGGG TGCCAGG--- ----------
B.parva          (CMW7500)  .......... .......... .......... .......... .......--- ----------
B.parva          (CMW7236)  .......... .......... .......... .......... .......--- ----------
B.parva          (CMW7238)  ?......... .......... .......... .......... .......--- ----------
B.parva          (CMW9078)  .......... .......... .......... .......... .......--- ----------
B.parva          (CMW9077)  .......... .......... .......... .......... ..-------- ------CCAG
Unknown          (CMW8052)  .......... .......... .......... .......... ..-------- ------CTGG
Unknown          (CMW8041)  .......... .......... .......... .......... ..-------- ------CTGG
Unknown          (CMW7230)  .......... .......... .......... .......... ..-------- ------CTGG
Unknown          (CMW7231)  ---------- ----?????? ?????????? ?????????? ?????????? ???????TGG
Unknown          (CMW8286)  .......... .......... .......... .......... ..-------- ------CTGG
B.ribis          (CMW7772)  .......... .......... .......... .......... ...TG..TGC TGGGTGCTGG
B.ribis          (CMW7773)  .......... .......... .......... .......... ...TG..TGC TGGGTGCTGG
B.dothidea       (CMW8000)  .......... C.CACA...T ..GTGC.... ...T.----- ---------- ----------
B.dothidea       (CMW7780)  .......... C.CACA...T ..GTGC.... ...T.----- ---------- ----------
G.philoprina     (CMW7063)  ---------- ---------- ---------- ----AGAA.. .AAG.ACAGC CACTCCTTTG

                                  70         80         90        100        110        120
B.parva          (CMW8042)  -TGCTGGGTT CCCGCACTCA ATTTGCCTTA TCGCTTCGGT GAGGGGCATT TTGGTGGTGG
B.parva          (CMW7500)  -......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  -......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  -......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  -......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  G......... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  G......... .......... .......... .......... .......... ..........
Unknown          (CMW8041)  G......... .......... .......... .......... .......... ..........
Unknown          (CMW7230)  G......... .......... .......... .......... .......... ..........
Unknown          (CMW7231)  G......... .......... .......... .......... .......... ..........
Unknown          (CMW8286)  G......... .......... .......... .......... .......... ..........
B.ribis          (CMW7772)  G......... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  G......... .......... .......... .......... .......... ..........
B.dothidea       (CMW8000)  -......... ..T..G.CG. .......... ..A..CT... ........A. ..CT......
B.dothidea       (CMW7780)  -......... ..T..G.CGG .......... ..A..CT... ........A. ..CT......
G.philoprina     (CMW7063)  A.A.CCA.A. .GT..GGC-- G.C.CG.A.C ..A.AC.T.- ----------. C..-------
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```
                                130        140        150        160        170        180
B.parva          (CMW8042)  GGTTGGCCCG CGCTAAGCCT CGTTTGGGCT -CGGCAAAAT GTCCGCATCT GGTTTTTTTG
B.parva          (CMW7500)  .......... .......... .......... -......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... -......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... -......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... -......... .......... ..........
B.parva          (CMW9077)  .......... .......... .......... -......... .......... ..........
Unknown          (CMW8052)  .......... .......... .......... -......... .......... A.........
Unknown          (CMW8041)  .......... .......... ....C..... -......... .......... ..........
Unknown          (CMW7230)  .......... .......... ....C..... -......... .......... ..........
Unknown          (CMW7231)  .......... .......... ....C..... -......... .......... ..........
Unknown          (CMW8286)  .......... .......... ....C..... -......... .......... ..........
B.ribis          (CMW7772)  .......... .......... ....C..... -......... .......... ..........
B.ribis          (CMW7773)  .......... .......... ....C..... -......... .......... ..........
B.dothidea       (CMW8000)  ..C....... .......... .......T.. T......... C......... ..A.......
B.dothidea       (CMW7780)  ..C....... .......... .......T.. T......... C......... ..A.......
G.philoprina     (CMW7063)  ----T....C T?T..CC... .C.CAAAAA. -.AAT----- -.TTTTT.G. ..CCC....-

                                190        200        210        220        230        240
B.parva          (CMW8042)  CGACCGGCGT GCGACCGAAG CG--CGCCCC TCGCCAGA-- --CACGCCAC GCATGT----
B.parva          (CMW7500)  .......... .......... ..--...... ........-- --........ ......----
B.parva          (CMW7236)  .......... .......... ..--...... ........-- --........ ......----
B.parva          (CMW7238)  .......... .......... ..--...... ........-- --........ ......----
B.parva          (CMW9078)  .......... .......... ..--...... ........-- --........ ......----
B.parva          (CMW9077)  .......... .......... ..--...... ........-- --........ ......----
Unknown          (CMW8052)  .......... .......... ..--...... ........-- --........ ......----
Unknown          (CMW8041)  .......... .......... ..--...... ........-- --........ ......----
Unknown          (CMW7230)  .......... .......... ..--...... ........-- --........ ......----
Unknown          (CMW7231)  .......... .......... ..--...... ........-- --........ ......----
Unknown          (CMW8286)  .......... .......... ..--...... ........-- --........ ......----
B.ribis          (CMW7772)  .......... .......... ..--.A.... ........-- --........ ......----
B.ribis          (CMW7773)  .......... .......... ..--.A.... ........-- --........ ......----
B.dothidea       (CMW8000)  T......... ........C. ..AA.A.... ..A...ACGC TT.CA..... T..C..TCGT
B.dothidea       (CMW7780)  T......... ........C. ..AA.A.... ..A...ACGC TT.CA..... T..C..TCGT
G.philoprina     (CMW7063)  TAGTG..GCC A.A...CCGC .A--GAGTT. ...AT..C-- --AT.T.A.G .A.G.C----
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```
                                250        260        270        280        290        300
B.parva          (CMW8042)  ----GCGACC AGACGCTAAC A---GCCATC CCA---GGAA GCCACCGAGT TGATTCGAGC
B.parva          (CMW7500)  ----...... .......... .---...... ...---.... .......... ..........
B.parva          (CMW7236)  ----...... .......... .---...... ...---.... .......... ..........
B.parva          (CMW7238)  ----...... .......... .---...... ...---.... .......... ..........
B.parva          (CMW9078)  ----...... .......... .---...... ...---.... .......... ..........
B.parva          (CMW9077)  ----...... .......... .---...... ...---.... .......... ..........
Unknown          (CMW8052)  ----...... GA........ G---...... ...---.... .......... ..........
Unknown          (CMW8041)  ----...... .......... G---...... ...---.... .......... ..........
Unknown          (CMW7230)  ----...... .......... G---...... ...---.... .......... ..........
Unknown          (CMW7231)  ----...... .......... G---...... ...---.... .......... ..........
Unknown          (CMW8286)  ----....T. .......... G---...... ...---.... .......... ..........
B.ribis          (CMW7772)  ----...... .......... G---...... ...---.... .......... ..........
B.ribis          (CMW7773)  ----...... .......... G---...... ...---.... .......... ..........
B.dothidea       (CMW8000)  CTAT...... .T.T...... C---A..GC. A..ACA.... .......... .......G..
B.dothidea       (CMW7780)  CTAT...... .T.T...... CACC....CA A..---.... ....-..... .......G..
G.philoprina     (CMW7063)  ----A..CG. T...AG.C-- -----...AA AT.---.... ....AT...A .C..G.--C.

                                310        320        330        340        350        360
B.parva          (CMW8042)  TCCGGCTCGA CTCTCCCACC CTATGTGTAC C-TACCTCTG TTGCTTTGGC GGGCCGCGGT
B.parva          (CMW7500)  .......... .......... .......... .-........ .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .-........ .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .-........ .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .-........ .......... ..........
B.parva          (CMW9077)  .......... .......... .A........ .-........ .......... ..........
Unknown          (CMW8052)  .......... .......... .A........ .-........ .......... ..........
Unknown          (CMW8041)  .......... .......... .A........ .-........ .......... ..........
Unknown          (CMW7230)  .......... .......... .A........ .-........ .......... ..........
Unknown          (CMW7231)  .......... .......... .A........ .-........ .......... ..........
Unknown          (CMW8286)  .......... .......... .A........ .-........ .......... ..........
B.ribis          (CMW7772)  .......... .......... .A........ .-........ .......... ..........
B.ribis          (CMW7773)  .......... .......... .A........ .-........ .......... ..........
B.dothidea       (CMW8000)  ......C... TC........ ..T....... .-........ .......... ..........
B.dothidea       (CMW7780)  ......C... TC........ ..T....... .-........ .......... ..........
G.philoprina     (CMW7063)  .T...GGTAG AC........ ..T...T... AA.....T.. .......... ..C..----C
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                                370        380        390        400        410        420
B.parva          (CMW8042)  CCTCCGCA-C CGGCGCCCTT CG--GGGGGC TGGCCAGCGC CCGCCAGAGG ACCATAAAAC
B.parva          (CMW7500)  ........-. .......... ..--...... .......... .......... ..........
B.parva          (CMW7236)  ........-. .......... ..--...... .......... .......... ..........
B.parva          (CMW7238)  ........-. .......... ..--...... .......... .......... ..........
B.parva          (CMW9078)  ........-. .......... ..--...... .......... .......... ..........
B.parva          (CMW9077)  ........-. ........-. TCGG....-. .......... .......... ..........
Unknown          (CMW8052)  ........-. ........-. TCGG...... .......... .......... ..........
Unknown          (CMW8041)  ........-. ........-. TCGG....-. .......... .......... ..........
Unknown          (CMW7230)  ........-. ........-. TCGG....-. .......... .......... ..........
Unknown          (CMW7231)  ........-. ........-. TCGG....-. .......... .......... ..........
Unknown          (CMW8286)  ........-. ........-. TCGG....-. .......... .......... ..........
B.ribis          (CMW7772)  ........-. .......... ..G-...... .......... .......... ..........
B.ribis          (CMW7773)  ........-. ........-. TCGG...... .......... .......... ..........
B.dothidea       (CMW8000)  .......GG. ....C...C. .CCC.....G .......... .......... .....C....
B.dothidea       (CMW7780)  .......GG. ....C...C. .CCC.....G .......... .......... .....C....
G.philoprina     (CMW7063)  GTCG.AAG-A .AA.CGG.-. .CGG-----. ...T.....G .......... ..T-C.....

                                430        440        450        460        470        480
B.parva          (CMW8042)  TCCAGTCAGT GAACTTCGCA GTCTGAAAAA CAAGTTAATA AACTAAAACT TTCAACAACG
B.parva          (CMW7500)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8041)  .......... ......T... ......G... .......... .......... ..........
Unknown          (CMW7230)  .......... ......T... ......G... .......... .......... ..........
Unknown          (CMW7231)  .......... ......T... ......G... .......... .......... ..........
Unknown          (CMW8286)  .......... ......T... ......G... .......... .......... ..........
B.ribis          (CMW7772)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  .......... .......... .......... .......... .......... ..........
B.dothidea       (CMW8000)  .......... A...GAT... .......... ..T-...... .......... ..........
B.dothidea       (CMW7780)  .......... A...GAT... .......... ..T-...... .......... ..........
G.philoprina     (CMW7063)  ..ATA.T.T. A----.T.TC ......GT.C T.TA.-.... G-T....... ..........
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                                490        500        510        520        530        540
B.parva          (CMW8042)  GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG
B.parva          (CMW7500)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8041)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW7230)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW7231)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8286)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7772)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  .......... .......... .......... .......... .......... ..........
B.dothidea       (CMW8000)  .......... .......... .......... .......... .......... ..........
B.dothidea       (CMW7780)  .......... .......... .......... .......... .......... ..........
G.philoprina     (CMW7063)  .......... .......... .......... .......... .......... ..........

                                550        560        570        580        590        600
B.parva          (CMW8042)  CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC GCCCCTTGGT ATTCCGAGGG
B.parva          (CMW7500)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8041)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW7230)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW7231)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8286)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7772)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  .......... .......... .......... .......... .......... ..........
B.dothidea       (CMW8000)  .......... .......... .......... .......... ....T..... .......A..
B.dothidea       (CMW7780)  .......... .......... .......... .......... ....T..... .......A..
G.philoprina     (CMW7063)  .......... .......... .......... .......... .....C.... ......G...
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                                  610         620         630         640         650         660
B.parva          (CMW8042)  GCATGCCTGT TCGAGCGTCA TTTCAACCCT CAAGCTCTGC TTGGTATTGG GCCCCGTCCT
B.parva          (CMW7500)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .......... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  .......... .......... .......... .......... .......... ..T.......
Unknown          (CMW8041)  .......... .......... .......... .......... .......... ..T.......
Unknown          (CMW7230)  .......... .......... .......... .......... .......... ..T.......
Unknown          (CMW7231)  .......... .......... .......... .......... .......... ..T.......
Unknown          (CMW8286)  .......... .......... .......... .......... .......... ..T.......
B.ribis          (CMW7773)  .......... .......... .......... .......... .......... ..T.......
B.ribis          (CMW7772)  .......... .......... .......... .......... .......... ..T.......
B.dothidea       (CMW8000)  .......... .......... ..A....... .......... .......... ..A.......
B.dothidea       (CMW7780)  .......... .......... ..A....... .......... .......... ..A.......
G.philoprina     (CMW7063)  .......... .......... ..A....... .......... .......... .---....AC

                                  670         680         690         700         710         720
B.parva          (CMW8042)  CCACGGACGC GCCTTAAAGA CCTCGGCGGT GGCGTCTTGC CTCAAGCGTA GTAGAAAACA
B.parva          (CMW7500)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .......... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .......... ....C..... .......... .......... .......... .....--.A.
Unknown          (CMW8052)  .......... .......... .......... .......... .......... .....--.A.
Unknown          (CMW8041)  .......... .......... .......... .......... .......... .....--.A.
Unknown          (CMW7230)  .......... .......... .......... .......... .......... .....--.A.
Unknown          (CMW7231)  .......... .......... .......... .......... .......... .....--.A.
Unknown          (CMW8286)  .......... .......... .......... .......... .......... .....--.A.
B.ribis          (CMW7772)  .......... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  .......... .......... .......... .......... .......... .....--.A.
B.dothidea       (CMW8000)  TTG...G... ....C..... .......... .......... .......... ......C.T.
B.dothidea       (CMW7780)  TTG...G... ....C..... .......... .......... .......... ......C.T.
G.philoprina     (CMW7063)  ..C...GT.. ........AT .AGT...... .C.....G.. T......... ...----.T.
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```
                                730        740        750        760        770        780
B.parva          (CMW8042)  C--CTCGCTT TGGAGCGCAC GGCGTCGCCC GCCGGACGAA CCTTTGAATT ATTTCTCAAG
B.parva          (CMW7500)  .--..... .......... .......... .......... .......... ..........
B.parva          (CMW7236)  .--..... .......... .......... .......... .......... ..........
B.parva          (CMW7238)  .--..... .......... .......... .......... .......... ..........
B.parva          (CMW9078)  .--..... .......... .......... .......... .......... ..........
B.parva          (CMW9077)  .AC..... .......... .......... .......... .......... ..........
Unknown          (CMW8052)  .AC..... .......... .......... .......... .......... ..........
Unknown          (CMW8041)  .AC..... .......... .......... .......... .......... ..........
Unknown          (CMW7230)  .AC..... .......... .......... .......... .......... ..........
Unknown          (CMW7231)  .AC..... .......... .......... .......... .......... ..........
Unknown          (CMW8286)  .AC..... .......... .......... .......... .......... ..........
B.ribis          (CMW7772)  .--..... .......... .......... .......... .......... ..........
B.ribis          (CMW7773)  .AC..... .......... .......... .......... .......... ..........
B.dothidea       (CMW8000)  .AT..... C........G .......... .......... ....CTG.AC T.........
B.dothidea       (CMW7780)  .AT..... C........G .......... .......... ....CTG.AC T.........
G.philoprina     (CMW7063)  .TT..... .....TC.GG .CGAG..T.. TG.CA.--.. ..CCCA---. ....T.T...

                                790        800        810        820
B.parva          (CMW8042)  GTTGACCTCG GATCAGGTAG GGATACCCGC TGAACTTAAG CATAT
B.parva          (CMW7500)  .......... .......... .......... .......... .....
B.parva          (CMW7236)  .......... .......... .......... .......... .....
B.parva          (CMW7238)  .......... .......... .......... .......... .....
B.parva          (CMW9078)  .......... .......... .......... .......... .....
B.parva          (CMW9077)  .......... .......... .......... .......... .....
Unknown          (CMW8052)  .......... .......... .......... .........C .....
Unknown          (CMW8041)  .......... .......... .????????? ?????????? ?????
Unknown          (CMW7230)  .......... .......... .......... .......... .....
Unknown          (CMW7231)  .......... .......... .......... .......... .....
Unknown          (CMW8286)  .......... .......... .......... .......... .....
B.ribis          (CMW7772)  .......... .......... .?........ .????????? ?????
B.ribis          (CMW7773)  .......... ...??????? ?????????? ?????????? ?????
B.dothidea       (CMW8000)  .......... .......... .......... .????????? ?????
B.dothidea       (CMW7780)  .......... .......... .......... .......... .....
G.philoprina     (CMW7063)  .......... .......... .......... .......??? ?????
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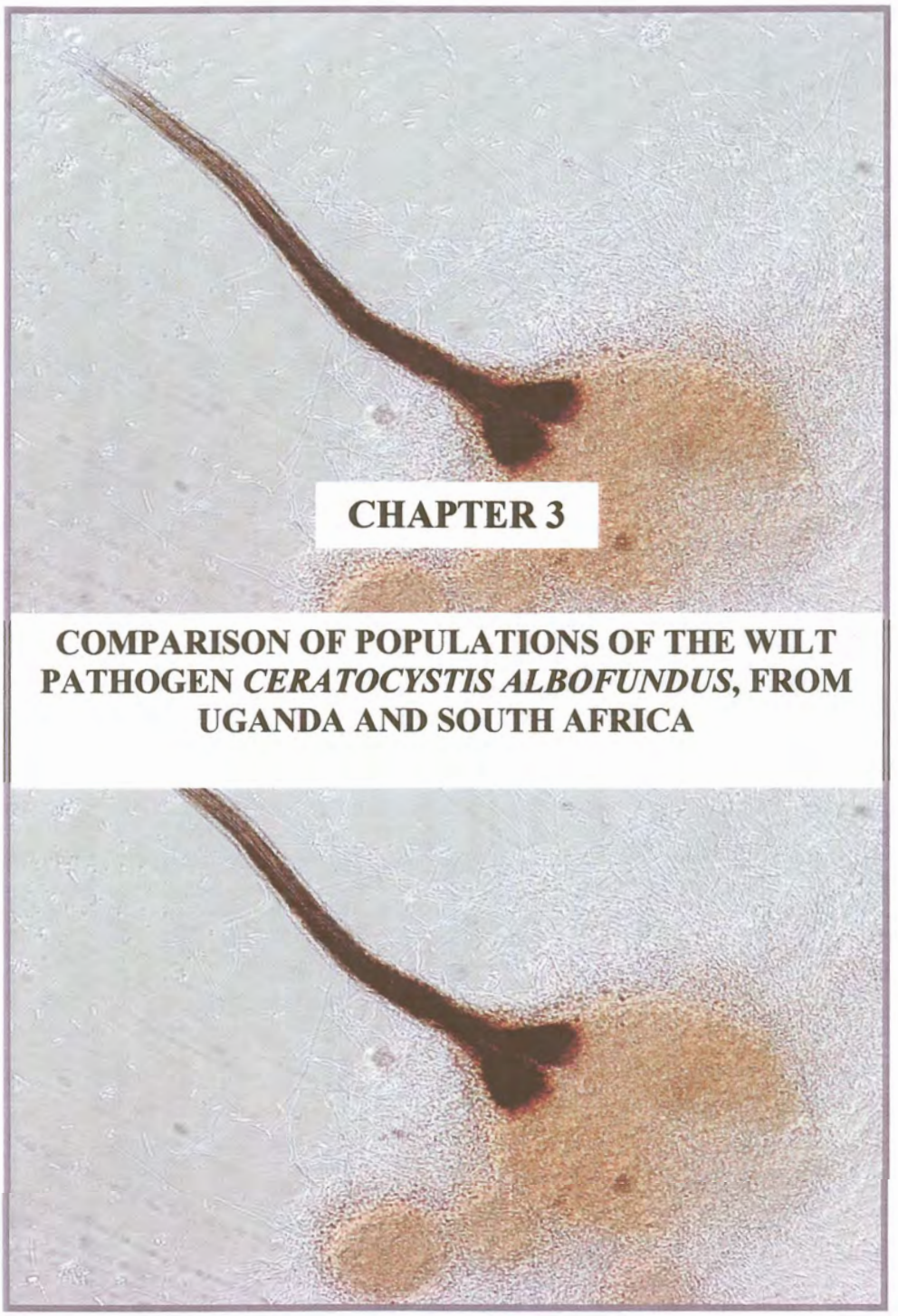

CHAPTER 3

COMPARISON OF POPULATIONS OF THE WILT PATHOGEN *CERATOCYSTIS ALBOFUNDUS*, FROM UGANDA AND SOUTH AFRICA

ABSTRACT

Ceratocystis albofundus is an important pathogen of *Acacia mearnsii* and *A. decurrens* in South Africa, where it causes stem cankers, die-back and death of these exotic trees. In 1999, *C. albofundus* was reported for the first time in South Western Uganda, where it is commonly associated with stem wounds on *A. mearnsii*, resulting from harvesting. It has previously been hypothesized that *C. albofundus* is native to South Africa. This is based on its occurrence on native *Protea* species and high gene diversity. The aim of this study was to determine the population diversity and structure of a Ugandan population of *C. albofundus* and to compare this with a South African population. Isolates were collected from 36 *A. mearnsii* stumps occurring in jungle stands in the South West of Uganda. Eight microsatellite primer pairs, previously developed for the related pathogen, *Ceratocystis fimbriata*, were used to amplify the microsatellite rich regions of the genome. Genescan analysis of the PCR amplicons showed that 7 of the loci were polymorphic and one was monomorphic, with a total of 26 alleles across all 8 loci. There were no common alleles within 2 of the 8 loci tested. Analysis of gene diversity showed that the Ugandan population is slightly more diverse than the South African population, with a gene diversity value of 0.407 compared to 0.378. Tests for gametic disequilibrium revealed clonality for both populations. Populations were highly sub-structured with very little gene flow. The high gene diversity values obtained in this study show that *C. albofundus* was probably not introduced into Uganda from South Africa and rather, that it is native to a wider area of Africa than previously assumed.

INTRODUCTION

The genus *Ceratocystis sensu stricto* Ell. & Halst. includes well-known and important canker and wilt pathogens of both forestry and agricultural crops (Kile 1993, Morris, Wingfield & De Beer 1993, Wingfield, Harrington & Solheim 1997, Roux *et al.* 2000a). Reports of *Ceratocystis* spp. causing serious disease problems on exotic trees grown intensively in plantations of the tropics and southern hemisphere have also increased in recent years. The first record of these fungi in this situation was of *C. fimbriata* Ell. & Halst., causing severe losses to *Acacia decurrens* Willd. (green wattle) in Brazil (Ribeiro *et al.* 1988). A year later, *C. albofundus* Wingfield, De Beer & Morris was recorded on *A. mearnsii* de Wild. (black wattle), *A. dealbata* Link. (silver wattle) and *A. decurrens* (green wattle) in South Africa, causing gummosis, wilting and death of trees (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux & Wingfield 1997). In 1998, disease surveys in the Republic of Congo revealed that *C. fimbriata* was responsible for wilt and death of *Eucalyptus* spp. in that country (Roux *et al.* 2000b). More recently, *C. fimbriata*, has been reported as a pathogen of *Eucalyptus* spp. in Brazil (Roux *et al.* 2000a & b), Uganda (Roux & Wingfield 2001) and Uruguay (Barnes *et al.* 2002b).

Ceratocystis wilt is characterised by formation of cankers and lesions on the bark of affected trees and exudation of gum from the lesions. Internal symptoms include extensive discoloration of the sapwood. In the final stages of disease development wilting, die-back and death of the trees occurs (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux, Wingfield & Dunlop 1999, Roux *et al.* 2000b), (Figure 1A & B).

Ceratocystis albofundus is distinguished from other species of *Ceratocystis* by its hyaline ascomatal bases, dark necks, and divergent ostiolar hyphae (Wingfield *et al.* 1996). In culture the fungus appears light cream colored in contrast to the green to grey appearance of other species, such as the closely related *C. fimbriata* (Wingfield *et al.* 1996). Phylogenetically, *C. albofundus* is most closely related to other *Ceratocystis* spp. with hat-shaped ascospores, such as *C. fimbriata* (Wingfield *et al.* 1996) and the newly described *C. pirilliformis* I. Barnes & M. J. Wingfield (Barnes *et al.* 2002a).

Ceratocystis spp. have a close association with insects (Kile 1993). *C. albofundus* produces a sweet aroma, which in other *Ceratocystis* spp., is known to attract insects

(De Vay, Davidson & Moller 1968, French & Stienstra 1978, Juzwik & French 1983, Kile 1993, Christen, Meza & Revah 1997). The sticky spore masses produced at the tips of the long necks of the ascomata facilitate dispersal of these spores (Hinds 1972, Juzwik & French 1983, Kile 1993).

Ceratocystis albofundus was discovered in Uganda for the first time in 1999 (Roux & Wingfield 2001). Prior to this, the fungus was known only in South Africa, where it is thought to be native (Roux *et al.* 2001, Barnes 2002). This was based partly on the fact that, apart from exotic Australian *Acacia* spp., the only other known hosts for *C. albofundus* are native South African *Protea* spp. (Gorter 1977). Molecular studies showing high levels of genetic diversity for a South African population of *C. albofundus* supported the view that the fungus is native to South Africa (Roux *et al.* 2001, Barnes 2002).

Discovery of *C. albofundus* in Uganda has raised the question as to whether it was introduced into that country from South Africa. An alternative hypothesis is that the fungus has a wide distribution in Africa, including Uganda. Our studies in Uganda have made it possible to collect a reasonably large set of isolates from this country. The aim of this study was thus to analyse the available population of *C. albofundus* in Uganda and to compare this with a population of the fungus from South Africa. This was achieved using polymorphic microsatellite DNA markers previously developed for *C. fimbriata* (Barnes *et al.* 2001).

MATERIALS AND METHODS

Isolates

Symptomatic tissue from *A. mearnsii* was collected from the Kabale district, South Western Uganda (Figure 1, Chapter 2). The area has an average temp of 17°C, which occasionally drops to 10°C at night, and the rainfall averages between 1000 – 1480mm per annum. Sampling was done randomly by collecting samples from as many trees as possible, from different woodlots. Collections were made from trees and stumps wounded during harvesting for fuelwood. Sampling involved chopping sections of symptomatic wood from the stems or branches of the trees, and wrapping these in paper bags for transportation to the laboratory and subsequent isolation of fungi.

Isolations were made on the same day as the collections. Two isolation techniques were used. Symptomatic wood pieces with streaked discoloration typical of *Ceratocystis* infections (Figure 1B) were placed in Petri dishes with moist filter paper, to induce sporulation. Wood in these moist chambers was inspected daily for the appearance of sexual fruiting structures, using a dissection microscope. In addition, small pieces of symptomatic wood were wrapped between slices of carrot and incubated for 7-14 days in plastic bags at room temperature (~ 25°C). This technique is known to be effective for isolating *Ceratocystis* spp. (Moller & De Vay 1968). After the development of sexual fruiting structures on the carrot or wood samples (Figure 1C), ascospore masses were transferred to Malt Yeast Agar (MYA) (2% malt extract, 1% yeast extract, Biolab, Midrand, Johannesburg) plates in order to obtain pure cultures (Figure 1D). All cultures, each from a different tree, are stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

DNA isolation

Cultures of the isolates for DNA based analyses (Table 1) were grown in sterile liquid media (2% malt extract, 1% yeast extract, Biolab, Midrand, Johannesburg) in 250 ml Erlenmyer flasks for a week at 25°C. Fungal mycelium was harvested by filtering and then freeze-dried overnight. A second set of mycelium for each isolate was stored at -20°C for later DNA isolation. Approximately 0.5 ml freeze dried mycelium for each isolate was placed in an Eppendorf tube, submerged in liquid nitrogen and ground into a fine powder using sterilized toothpicks. DNA was then extracted using the method described by Barnes *et al.* (2001).

Microsatellite Polymerase Chain Reaction (PCR) Amplification

PCR-based microsatellite DNA markers designed by Barnes *et al.* (2001) for *C. fimbriata* and used on different populations to determine their structure and diversity were used to study the Ugandan population of *C. albofundus*. The primers were first tested on a South African population collected by Roux *et al.* (2001). Eight of the eleven primers tested were effective and thus applied to the Ugandan population (Barnes 2002).

Polymerase chain reactions (PCR) were performed using 8 pairs of microsatellite primers (Table 2) following the protocol described by Barnes *et al.* (2001). The reactions were conducted in a total volume of 25 µl, comprising 2 ng DNA template (1 µl), 200 µM DNTPs (2 µl), 300 nM of each forward and reverse primer (0.75 µl) (Table 2), 5 U/µl expand high fidelity enzyme (0.1 µl) (Roche Diagnostics, Mannheim, Germany), 10 x PCR reaction buffer containing 1.5 mM $MgCl_2$ (Roche diagnostics, Mannheim, Germany) and 17.4 µl sterile distilled water. A 100 bp marker (Roche diagnostics, Mannheim, Germany) was used to estimate the DNA concentration and the size of the PCR products was determined on ethidium bromide-stained, 2% agarose gels, under UV light.

Genescan analysis

The agarose gel profiles obtained for the PCR products for all 8 microsatellite primer pairs were used to estimate DNA concentrations. Dilutions for each PCR sample were made for the genescan gel as described by Barnes *et al.* (2001). Each sample to be run on a single lane using Polyacrylamide Gel Electrophoresis (PAGE) comprised a mixture of 0.5 µl DNA (1.5 ng), 1.5 µl of loading buffer and 0.5 µl internal standard GENESCAN-TAMRA (Applied Biosystems, Warrington WA1 4SR, Great Britain). Samples were separated on a 4.25% PAGE gel, using an ABI Prism™ 377 DNA sequencer. The allele sizes for the different microsatellite regions were obtained using GeneScan 2.1 analysis software and Genotyper 2.0 (Applied Biosystems, Warrington WA1 4SR, Great Britain).

Genetic distance and population structure

The genetic distance between each isolate was calculated using allele size. A distance matrix based on total distance (D_{AD}) was obtained using the program MICROSAT (http: human.Stanford.edu/microsat). The emerging data matrix obtained was then analysed in MEGA version 2.1 (Kumar *et al.* 2001) and a dendrogram was constructed based on UPGMA (Unweighted Pair – Group Method with Arithmetic Mean). Analysis was done separately for the Ugandan population and then combined with the data obtained by Barnes (2002) for a South African population.

Genetic differentiation (G_{ST}) between the Ugandan and South African populations was calculated using the program POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh). This measure determines the level of population

differentiation by comparing the gene diversities within sub-populations with that of the total population. Thus, a high G_{ST} value implies that there is low gene diversity within the sub-populations compared to the total population and *vice versa*. A value of above 0.25 indicates a very high genetic differentiation, whereas a value in the range of 0 to 0.05 indicates very low differentiation (Wright 1978). Using the G_{ST} estimates, gene flow (Nm) between the two populations was estimated using the formula $Nm = 0.5(1-G_{ST})/G_{ST}$, illustrated by McDermott & McDonald (1993) and Slatkin & Barton (1989).

Gene diversity

The gene diversity, based on allele frequencies, was calculated with the formula $H = 1 - \sum_{\kappa} \chi_{\kappa}^2$, where χ_{κ} is the frequency of the k^{th} genotype (Nei 1973). The frequency for each allele was determined by scoring each locus for the presence or absence of an allele (0 ; 1), and calculating the number of times an allele appeared in the population, divided by the sample size.

Genotypic Diversity

Multiloci genotypes were determined based on the number and frequencies of combinations of alleles at multiple loci. Genotypic diversity was calculated for both the Ugandan and South African populations using the program Multilocus v1.2 (http: www.bio.ic.ac.uk/evolve/software/multilocus) (Agapow & Burt 2000). The program is based on the probability that two individuals taken at random have different genotypes. The formula $\frac{n}{n-1}\left(1-\sum_i pi^2\right)$ where pi is the frequency of the i^{th} genotype and n is the number of individuals sampled was used. The maximum value of 1 implies that every individual is different. Thus, the program samples all possible pairs of individuals, tests whether they are different and calculates the fraction of pairs that are different.

A graph showing the genotypic diversity versus the number of loci was plotted for the Ugandan population. This was done to determine whether scoring additional loci was likely to increase the genotypic diversity or whether a plateau had been reached (Agapow & Burt 2000).

Mode of reproduction

The mode of reproduction for the Ugandan population was determined by calculating the Index of Association (I_A) (Taylor, Jacobson & Fisher 1999) and by applying the Parsimony Tree Permutation Test (PTLPT) (Burt *et al.* 1996) using the same parameters previously calculated for the South African population (Barnes 2002). The PTLPT was calculated using PAUP 4.0b3 (Phylogenetic Analysis Using Parsimony) with 500 randomisations and I_A using the program multilocus with 1000 randomisations (Agapow & Burt 2000).

RESULTS

Isolates

The carrot baiting technique was the most effective technique for retrieving isolates of *C. albofundus*. Although more than 100 samples with typical *Ceratocystis* symptoms were collected from individual trees, *C. albofundus* was obtained from only 36 trees. These all originated from an area of approximately 45 km^2 (Table 1).

Microsatellite PCR amplification

DNA was extracted for all Ugandan isolates. Successful PCR amplifications were obtained for every isolate using the 8 pairs of microsatellite primers (Table 2).

Genescan analysis

For the Ugandan population, a total of 26 alleles were obtained for the 8 loci and these ranged from 160 to 384 bp (Table 3). A total of 7 alleles were obtained for locus A7/8. Loci C17/18 and C21/22 had a total of 5 alleles each and loci A17/18, C5/6 and C23/24 had 2 alleles each. Locus A15/16 was monomorphic with an allele size of 288 bp (Table 3). The South African population (40 isolates) had a total of 24 alleles (Barnes 2002), 9 of which were shared with the Ugandan population. No alleles were shared between the two populations at loci A17/18 and C5/6 (Table 4).

Genetic distance and population structure

The dendrogram obtained by UPGMA from the distance matrix, revealed that the Ugandan population is structured, although there are no significant sub-groups within the population (Figure 2). Isolates from different plantations/farms/plots had similar genotypes and thus were evenly distributed within the population. With the combined Ugandan and South African populations, isolates grouped according to geographical

origin. Three distinct groups (Figure 3) were thus obtained with South African isolates dominant in Group I and Ugandan isolates dominant in group III. Isolate CMW 4074 from Uganda occurred within the South African group comprised of 33 isolates, whereas isolate CMW 4107 from South Africa occurred within the Ugandan group, comprised of 27 isolates. Group II (Figure 3) included a mixture of isolates from the two countries, with 8 from Uganda and 6 from South Africa (Figure 3). Some of the isolates grouped separately from the three major main groups, residing in separate minor groups (Figure 3).

The measure of genetic differentiation revealed a high differentiation between the Ugandan and South African populations. A G_{ST} value of 0.3116 (Table 5) was obtained. Estimation of gene flow revealed a Nm value of 1.1043, indicating very low gene flow between the two populations.

Gene diversity

A diversity value of 0.407 was obtained for the Ugandan population, compared to a value of 0.378 for the South African population (Table 4 and 6). Ugandan locus C17/18, with 5 alleles had the highest gene diversity value of 0.6188 (Table 4). In the South African population, locus A7/8, with 9 alleles had the highest diversity (0.751) (Barnes 2002).

Genotypic diversity

A maximum genotypic value of 0.963, equivalent to 96% was obtained for the Ugandan population. For the South African population, a value of 0.977, equivalent to 98% was obtained, slightly higher than the Ugandan population.

A p-value of < 0.001 was obtained for the graph plotted for genotypic diversity versus the number of loci. A plateau had been reached indicating that additional sampling would not have changed the genotypic diversity obtained for the Ugandan population (Figure 4).

Mode of reproduction

For the Ugandan population, the observed index of association (I_A) was 0.46 ($P<0.001$), slightly beyond the normal distribution for a recombining population

(Figure 5). The test rejects the null hypothesis of recombination, and suggests that reproduction is predominantly clonal.

DISCUSSION

Results of this study have shown that *C. albofundus* in Uganda has a high genetic diversity and this reflects a fungus that is most likely native to the country. Previously, it was thought that the fungus was native only to South Africa (Roux *et al.* 2001), and one hypothesis on which the present study was based, was that *C. albofundus* had been introduced into Uganda. This is clearly not so and the fungus appears to have a much wider distribution in Africa, than previously assumed.

The higher gene diversity for the Ugandan population in contrast to that in South Africa, might give an impression that the fungus has been present in Uganda for an extended period of time. This would have allowed sufficient time for mutational events to occur. Populations that have been in an area for a long period of time are expected to have more alleles than recently introduced populations (McDonald 1997, McDonald & Linde 2002). The increase in allele number could also be due to genetic drift, which enhances the frequencies of new alleles to recognisable standards (McDonald 1997, McDonald & Linde 2002). Thus, a high level of gene diversity is expected at the centre of origin (McDonald & McDermott 1993, McDonald 1997, Hoegger *et al.* 2000). The high gene diversity revealed within both the South African and Ugandan populations gives an impression that the fungus is native to the African continent and not necessarily only to South Africa as previously hypothesized.

The occurrence of *C. albofundus* on native *Protea* species in South Africa (Gorter 1977) supports the view that the fungus is well established in that country, living on native species and causing disease on exotic trees such as *A. mearnsii* (Morris *et al.* 1993, Wingfield *et al.* 1996). Research pertaining to the occurrence of *C. albofundus* on native species, especially *Proteas* in Uganda and South Africa is necessary to give greater insight into the possible origin of the fungus.

Results of this study have shown that populations of *C. albofundus* in Uganda and South Africa are clonal. This could imply that sexual reproduction within the populations is dictated by the MAT 2 gene, which promotes selfing, resulting in clonal progeny (De Beer 1994, Witthunn *et al.* 2000). Our findings further support previous studies done on related *Ceratocystis* species, which have indicated that although members in the genus tend to produce sexual structures profusely, the offspring produced by these species are very similar, varying only in their mating types (Harrington & McNew 1997). The high gene diversities combined with the clonal reproductive mode of the two populations further, suggests that the fungus is well established in Africa.

The high level of genotypic diversity estimated for the Ugandan population (96%), similar to that of the South African population (98%), contradicts the indication of clonality revealed by measures of gametic disequilibrim. Measures of genotypic diversity provide information of the proportion of the population that results from sexual reproduction (McDonald 1997, McDonald & Linde 2002). In this context, recombination resulting in new genotypes is not expected due to the clonal nature of the populations. The higher genotypic diversities obtained in this study could be attributed to mutations. Because the fungus is haploid, mutation occurring at any locus will most likely change the genotypes. The high genotypic diversities, further supports the view that *C. albofundus* has been in Africa for a long time.

Analysis of genetic distance for isolates in the Ugandan population, showed a high structure within the Ugandan population, although no pure subgroups were formed. This could be due to the small geographic area from which the population was sampled. For the combined population (Ugandan and South African), sub-structuring was revealed. The fact that recently introduced pathogen populations are expected to have little genetic differentiation (Leung, Nelson & Leach 1993), implies that the Ugandan and South African populations have been present in their respective countries for an extended period of time. This view is supported by the fact that very few alleles are shared between the two populations. The high levels of genetic differentiation between these populations might imply that sufficient time has passed to allow for selection of different alleles in different locations, and genetic drift, which changes allele frequencies randomly (Leung *et al.* 1993).

The low levels of gene flow between the Ugandan and South African populations of *C. albofundus* is consistent with the fact that the two countries are very distant from each other. Populations from countries between Uganda and South Africa might provide a link between populations or make tracing the movement of the pathogen possible. The results we have obtained in this study are, therefore, insufficient to further elucidate the origin of *C. albofundus*. To fully explain the origin of this pathogen, attempts will need to be made to obtain populations from other African countries and from alternative, preferably native, hosts of *C. albofundus*.

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Table 1. Isolates of *C. albofundus* collected from diseased *Acacia mearnsii* in Uganda and used in this study.

[A]**Isolates**	**Area collected**
CMW 4074	Kagalama
CMW 4998	Kakarome
CMW 5329 -5364	Mafuga
CMW 7111-7112	Kakarome
CMW 7113-7114	Kachwekano
CMW 7115	Mafuga
CMW 7116	Karungu
CMW 7153-7162	Mafuga
CMW 7268	"
CMW 9173	Murutunga
CMW 9174	Kachwekano
CMW 9175-9176	Murutunga
CMW 9177	Kachwekano
CMW 9178	Mafuga
CMW 9179	Murutunga
CMW 9180	"
CMW 9181	Kakarome
CMW 9182-9184	Karungu
CMW 9375-9376	"
CMW 9377	Kachwekano

[A] Culture numbers refer to those in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 2. Microsatellite primer sequences used for Polymerase Chain Reaction amplifications[A].

Primers	Forward primer sequence	Reverse primer sequence
AG7/8	CGA GAC AGC AAC ACA AGC CC	GGG GCG GTG GTG CAA TTG TC
AG15/16	CTT GAC CGA CCT GCC GAT TG	GGA TAG CAG CGA CAA GGA CC
AG17/18	GTC GGT GGT GGA GAC GGT C	CGG CCC TGC CAA CGG ATG
CF5/6	GAC CAA AGA TGG TGG CGA GC	CAT GGG CAT GCC TAG CCT TG
CF15/16.2	CGT TTG CAA GGC AAG GCA GC	CAG GGA CTA GGG TCT GCC AG
CF17/18	GAA ACC GAG AGT CAT CGT CC	CGA GCC AAG ACG TTC ATT GAA G
CF21/22	GCG TTG AAA GAT GTG GCG TG	GCA CTA CGA GAA TAG AAT GCA G
CF23/24	CAT GAT CGA CAA GGG CGC TG	CAG GGA ATT CCC GAT GGC AG

[A]Information obtained from Barnes *et al.* 2001.

Table 3. Genescan results of eight microsatellite markers indicating allele sizes and genotypes obtained for each isolate in the Ugandan population.

CMW No.	A7/8							A15/16	A17/18		C5/6		C15/16.2				C17/18			C21/22					C23/24		Microsatellite	Genotype
	296	299	308	319	320	331	332	288	304	305	382	384	260	261	283	284	290	291	292	250	251	254	283	284	160	168	Profile	
4074	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
4998	1	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	1	0	11122241	B
7111	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	11212251	C
7112	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	51111352	D
7113	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	51112231	E
7114	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51222442	F
7116	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	31111442	G
7153	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	41111211	H
7154	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	51112251	I
7155	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	51212452	J
7156	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	51112251	I
7157	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	61111412	K
7115	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	41111241	L
7158	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	41112241	M
7159	0	0	0	1	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	41211442	N
7160	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	41112422	O
7161	0	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	31112452	P
7162	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	51212452	J
7268	0	1	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	21121442	Q
5329	0	0	0	0	1	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	51211151	R
5364	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	51221342	S
9178	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9181	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9173	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9180	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	51212441	U
9376	0	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	21222442	V
9184	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9183	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	51212141	W
9176	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	51212441	U
9175	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9177	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	51212242	X
9375	0	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	71212541	Y
9174	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9182	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9377	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9179	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A

Table 4. Comparison between the Ugandan and South African populations of *C. albofundus*. **A)** Number of polymorphic alleles present for each locus within the Ugandan and South African populations of *C. albofundus* and number of alleles shared between each population. **B)** Index of association, reproductive mode, gene diversity and maximum genotypic diversity of both the Ugandan and South African populations of *C. albofundus*.

A

Loci	A7/8	A15/16	A17/18	C5/6	C15/16.2	C17/18	C21/22	C23/24	Total Number of alleles
Uganda	7	1	2	2	2	5	5	2	26
S. Africa	9	2	2	2	1	3	3	2	24
No. of alleles shared	2	1	-	-	1	2	2	1	9

B

	Uganda	South Africa[B]
Sample size	36	40
Index of Association (observed value)	0.46	0.992
- **Minimum value**	-0.227	-0.246
- **Maximum value**	0.414	0.379
- **p-values[A]**	<0.001	< 0.001
Gene diversity	0.4072	0.3778
Maximum genotypic diversity	96%	98%
Reproductive mode	Clonal	Clonal

[A] p-Values reveal the relevance of linkage disequilibria. P-values greater than 0.05 are not significant, whereas, p-values less than 0.01 are highly significant.

[B] Information obtained from Barnes (2002).

Table 5. Results of Genetic differentiation (Gst) and geneflow (Nm) obtained for the combined Ugandan and South African populations.

Locus	**Gst**	**Nm[A]**
A7/8	0.2060	1.9267
A15/16	0.2121	1.8571
A17/18	0.3985	0.7546
C5/6	0.7137	0.2006
C15/16.2	0.6000	0.3333
C17/18	0.1031	4.3481
C21/22	0.0612	7.6638
C23/24	0.2360	1.6190
Mean	**0.3116**	**1.1047**
St. Dev	0.0281	0.0363

[A]Nm is the estimate of gene flow from G_{ST}. E.g., $Nm = 0.5(1 - G_{ST})/ G_{ST}$. A G_{ST} of 0-0.05 indicates very low differentiation whereas a value of 0.25 indicates high differentiation.

Table 6. Summary of genetic variation statistics for the Ugandan population for all 8 loci.

Locus	Sample Size	na*[A]	h*[B]
A7/8	36	7.00	0.5617
A15/16	36	1.00	0.0000
A17/18	36	2.00	0.4614
CF5/6	36	2.00	0.2392
C15/16.2	36	2.00	0.3750
C17/18	36	5.00	0.6188
C21/22	36	5.00	0.5015
C23/24	36	2.00	0.5000
Mean	36	3.25	**0.4072**

[A] Observed number of alleles.

[B] Nei's gene diversity.

Figure 1. Cultural characteristics and disease symptoms caused by *C. albofundus*. **A)** Wilt and death of *A. mearnsii* trees after infection by *C. albofundus*. **B)** Typical discoloration of the wood caused by *C. albofundus* infection. **C)** Light colored bases of ascomata and dark necks characteristic of *C. albofundus*. **D)** Creamy, whitish mycelium produced by *C. albofundus* grown on 2 % Malt Extract Agar.

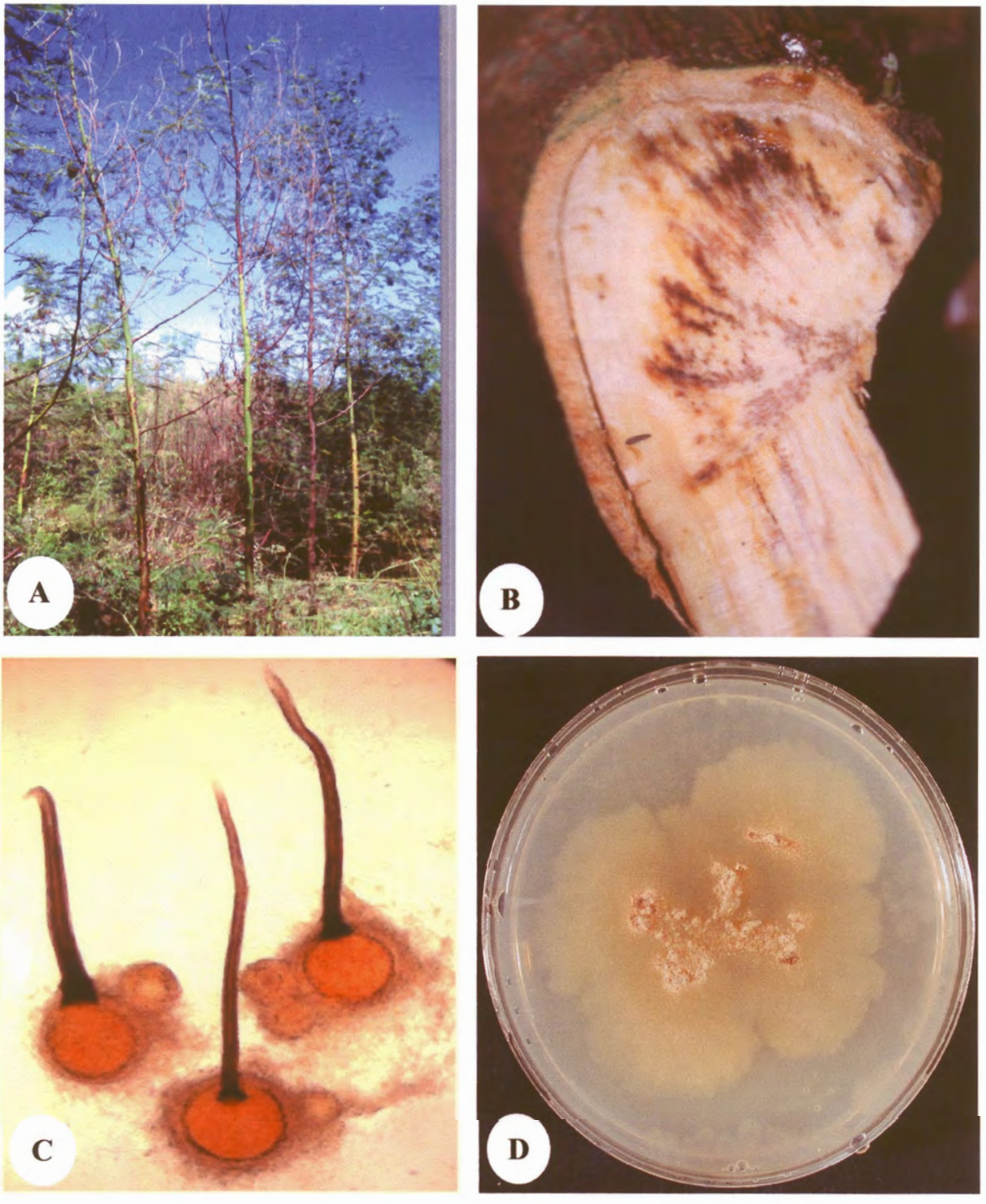

A
B
C
D

Figure 2. UPGMA dendrogram of the Ugandan population derived from genetic distances based on total nucleotide length calculated using total distance D_{AD}.

Ugandan population of *C. albofundus*

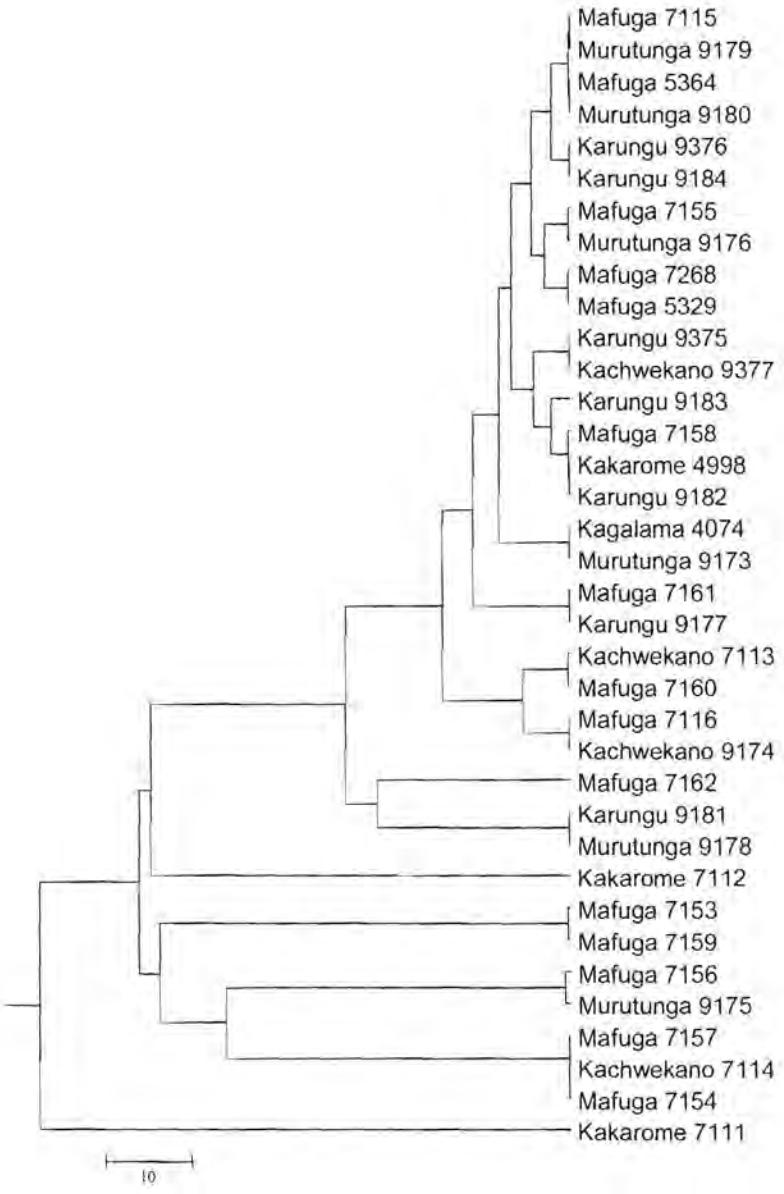


Figure 3. UPGMA dendrogram obtained using genetic distances for combined populations of Uganda and South Africa. Distances were calculated using D_{AD} based on total distance. A high differentiation within the populations was revealed with a G_{ST} value of 0.3116.

Ugandan and South African populations of *C. albofundus*

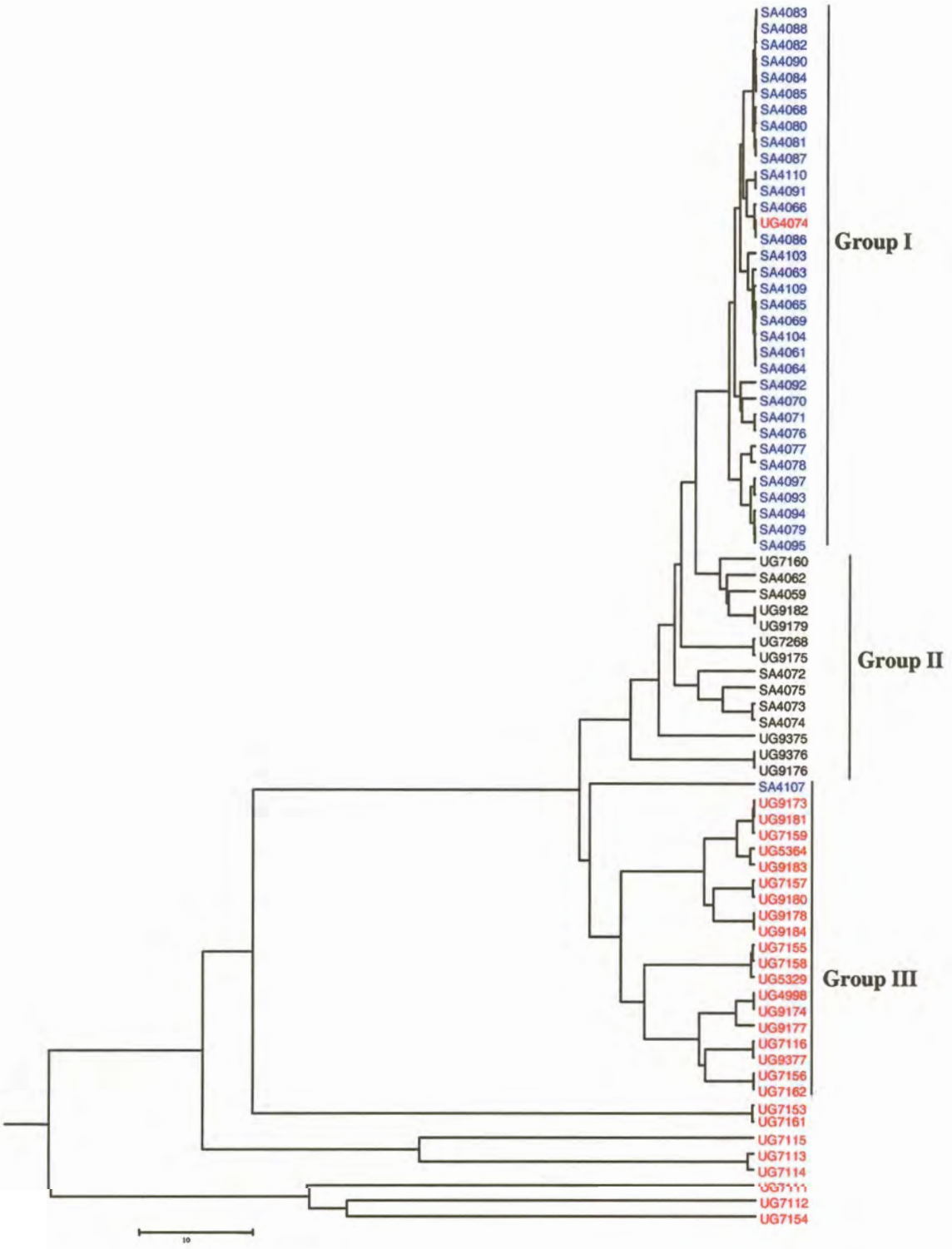


Figure 4. Curve of genotypic diversity against number of loci ($p<0.001$) for Ugandan population with 1000 sampling events.

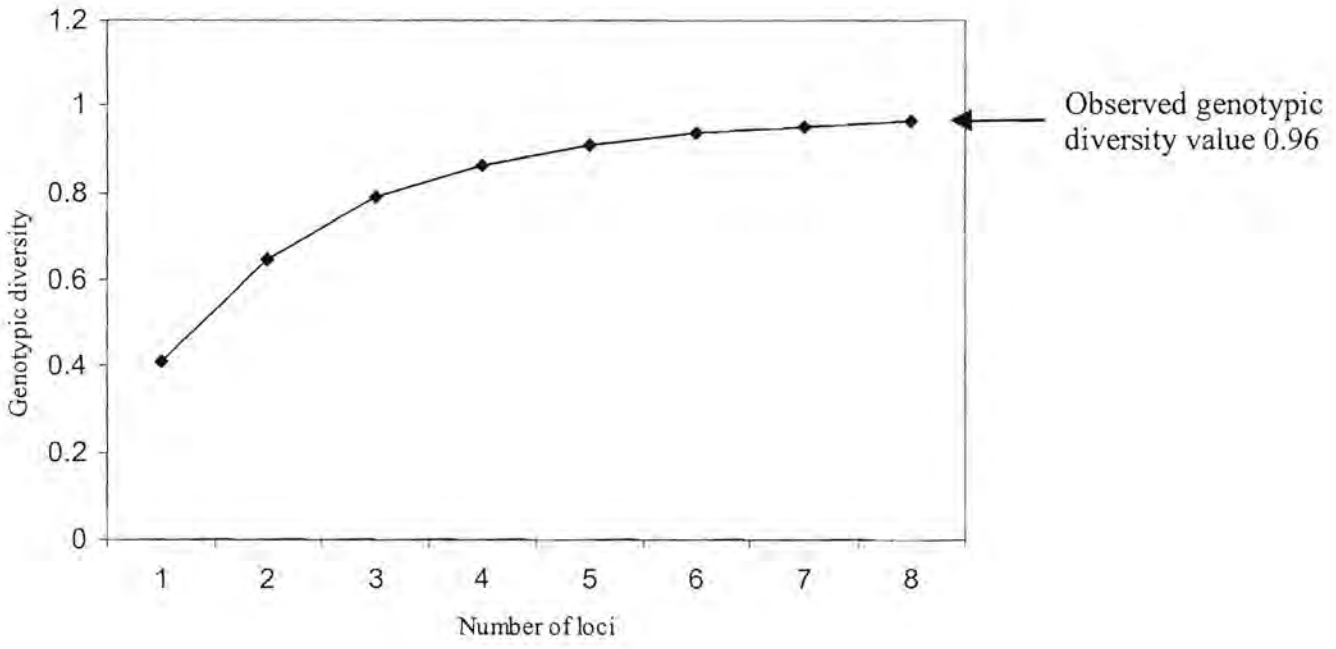
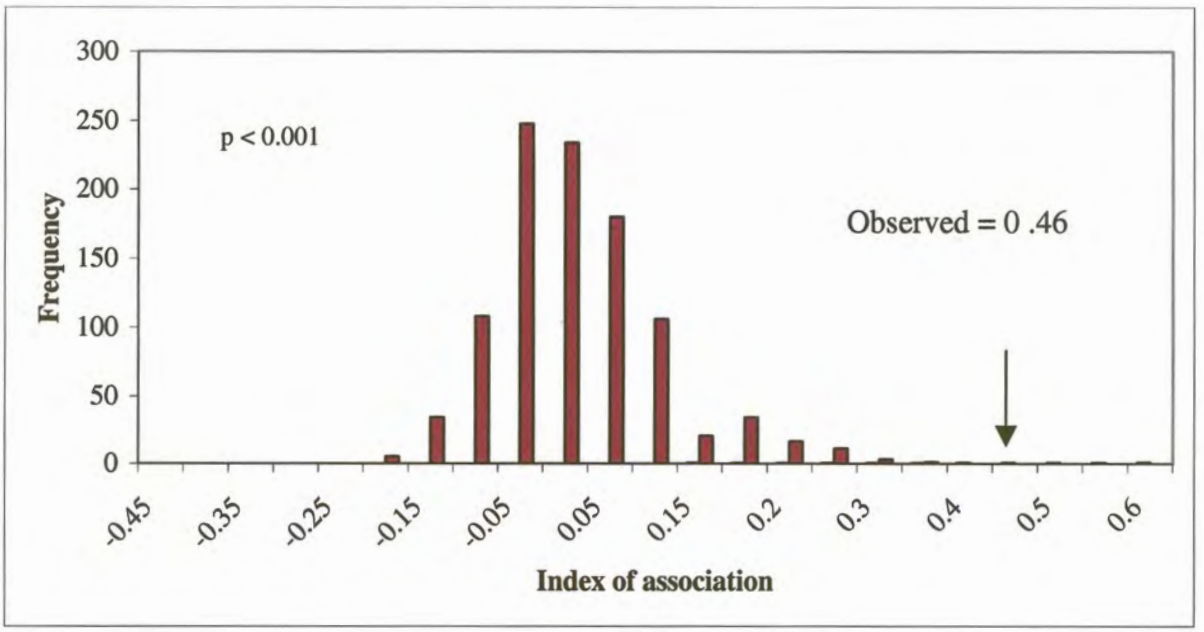
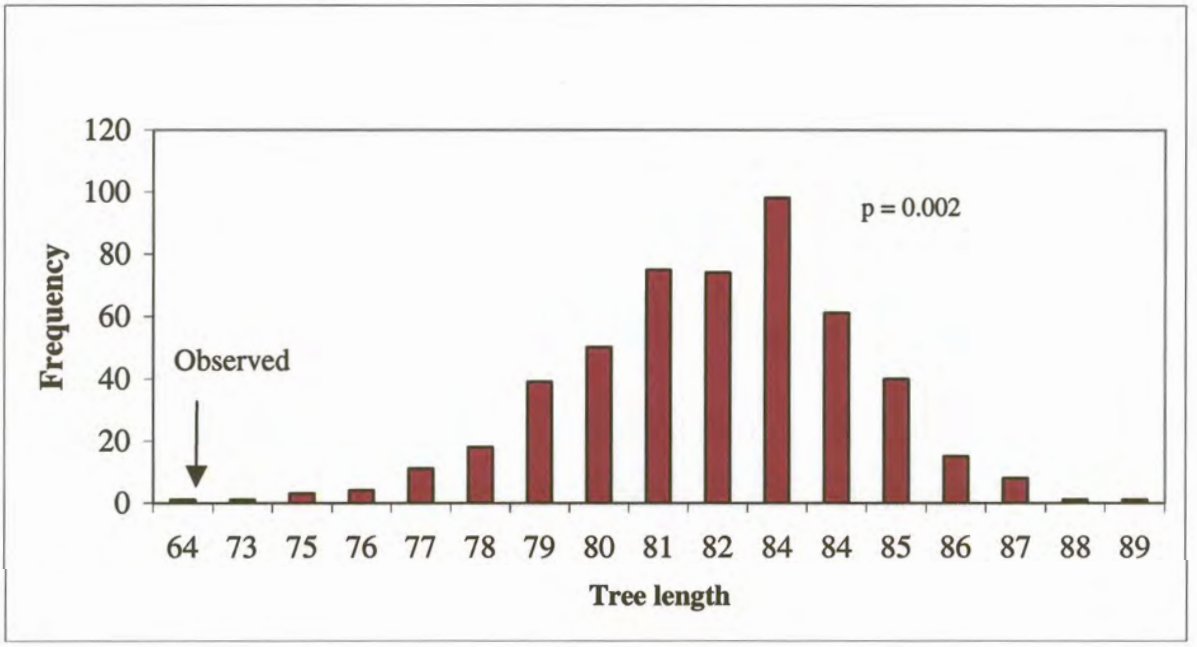

1.2
1
0.8
0.6
0.4
0.2
0
Genotypic diversity
1
2
3
4
5
6
7
8
Number of loci
Observed genotypic diversity value 0.96

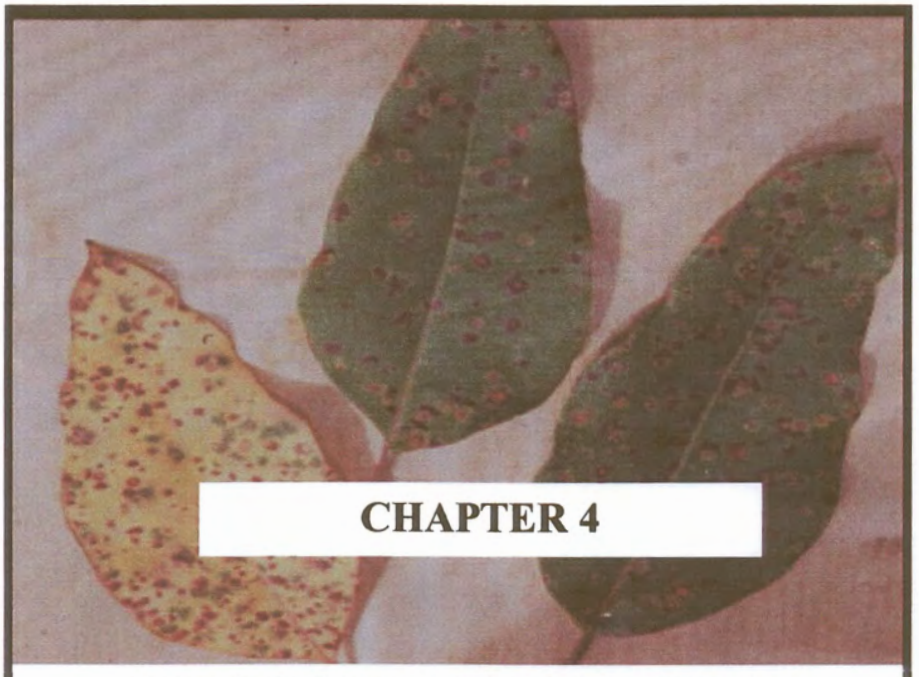
Figure 5. Histograms representing frequency distribution of pairwise distances among multiloci genotypes. **A)** Histogram representing the observed index of association for the Ugandan population, falling slightly beyond the distribution for randomised data sets ($P<0.001$, $I_A = 0.46$). **B)** Parsimony Tree Length Permutation Tests (PTLPT) based on 500 randomisation events. The tree length for the randomised data set is longer than that of the observed data. Both distributions indicate clonal reproduction in the Ugandan population of *C. albofundus*.

A


300
250
200
150
100
50
0
Frequency
p < 0.001
Observed = 0 .46
-0.45
-0.35
-0.25
-0.15
-0.05
0.05
0.15
0.2
0.3
0.4
0.5
0.6
Index of association

B


120
100
80
60
40
20
0
Frequency
p = 0.002
Observed
64
73
75
76
77
78
79
80
81
82
84
84
85
86
87
88
89
Tree length



CHAPTER 4

BACTERIAL BLIGHT OF *EUCALYPTUS* SPECIES IN UGANDA CAUSED BY *PANTOEA ANANATIS*.

Submitted as: Grace Nakabonge, Teresa Coutinho, Jolanda Roux & Michael Wingfield (2003) Bacterial blight of *Eucalyptus* species in Uganda. *Journal of Phytopathology.*

ABSTRACT

Dependency on exotic plantations for supplies of wood and wood products has increased significantly in the tropics and Southern Hemisphere. In Uganda, *Eucalyptus* species are amongst the most widely grown plantation trees, covering 40% of afforested areas. Diseases, however, reduce their growth rate, survival and the quality of products emerging from them. Most *Eucalyptus* diseases are caused by fungi, but bacterial pathogens are increasing in importance. During a disease survey in 2001, a leaf spot and blight disease was observed in Southern Uganda. Diseased trees showed leaf spots, lesions along the main leaf veins, premature abscission of the leaves, die-back and death. Isolations made from wilted shoots and leaves with water soaked lesions, consistently yielded bacterial isolates. The bacteria were characterized using Gram stain, Hugh-Leifson oxidation fermentation, oxidase and catalase tests as well as 16S rRNA gene sequences. The bacterium was identified as *Pantoea ananatis*, which is known to cause similar symptoms on *Eucalyptus* spp. in South Africa. This is the first record of bacterial blight caused by *P. ananatis* outside South Africa. The disease primarily damages young trees and it is thus of great concern in terms of plantation establishment. It will now be necessary to establish trials to select bacterial blight-tolerant planting stock.

INTRODUCTION

In Uganda, trees contribute to over 90% of the total energy consumed (Ruyooka 1999, Anonymous 2000a). *Eucalyptus* species, especially *Eucalyptus grandis* W. Hill, make up approximately 40% of afforested areas in the country (Ruyooka 1999). These plantations are owned by private companies, individual farmers as well as the Uganda Forest Department (Anonymous 2000a, Anonymous 2000b). The trees from these plantations are important for structural poles, transmission poles, fuelwood and sawn timber (Ruyooka 1999, Anonymous 2000a).

Diseases pose a significant threat to exotic plantation forestry. In Uganda, diseases such as Botryosphaeria canker and Ceratocystis wilt of *Eucalyptus* have already been recognised as serious threats to the emerging and important plantation forestry industry (Roux *et al.* 2001). This is similar to the situation in other countries, such as South Africa, where new diseases continue to be discovered and increase the overall costs of forestry. Despite intensive research, diseases such as Mycosphaerella leaf blotch caused by *Mycosphaerella* spp., Cryphonectria canker caused by *Cryphonectria cubensis* (Bruner) Hodges and Coniothyrium canker caused by *Coniothyrium zuluense* Wingfield, Crous & Coutinho continue to threaten exotic plantation forestry, especially in the Southern Hemisphere (Conradie, Swart & Wingfield 1990, Crous & Wingfield 1996, Wingfield, Crous & Coutinho 1997). Despite the importance of diseases in plantation forestry, little is known regarding the situation in Uganda.

Most serious diseases of exotic plantation trees are caused by fungal pathogens. However, in recent years a number of bacterial diseases have had a serious negative impact on *Eucalyptus* plantations. Bacterial wilt caused by *Ralstonia solanacearum* (synonyms *Pseudomonas solanacearum* and *Burkholderia solanacearum*) Yabuuchi *et al.* has, for example, caused damage to plantations in many parts of the world (Dianese 1986, Wu & Liang 1988, Akiew & Trevorrow 1994, Coutinho *et al.* 2000, Roux *et al.* 2000). Crown gall caused by *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942 and shoot blight caused by *Xanthomonas campestris* (Pammel 1895) Dowson 1939 pv. *eucalypti* (Truman 1974) Dye 1978, are less common and are considered to be of minor importance (Wardlaw, Kile & Dianese 2000). The most recently described bacterial disease of *Eucalyptus* spp. is bacterial

blight caused by *Pantoea ananatis* Serrano (synonyms *Erwinia ananas* and *Erwinia uredovora*). It is currently only known from South Africa (Coutinho *et al.* 2002). Very little is known regarding this newly discovered pathogen, but its damage to young trees at establishment is of considerable concern (Coutinho *et al.* 2002).

During surveys of *Eucalyptus* diseases in Uganda, a leaf blight disease was commonly observed on young trees, in several areas of the country. The aim of this study was to identify the causal agent of this leaf disease. Use was made of both biochemical and molecular techniques to identify the pathogen and to compare it with isolates from South Africa.

MATERIALS AND METHODS

Disease incidence and isolations

In February 2001, a survey of *Eucalyptus* plantations was conducted in the Southern parts of Uganda to identify the distribution and cause of diseases of these trees. A leaf disease was identified on young trees with symptoms similar to those of bacterial blight in South Africa (Coutinho *et al.* 2002). Samples were collected from leaves of diseased *E. grandis* trees in Bweyogerere, Entebbe (central Uganda) and Tororo (eastern Uganda) (Figure 1, Chapter 2).

Isolations were made from wilted shoots and leaves with water soaked lesions after surface disinfestation of the tissue by dipping it in 70% ethanol and placing small leaf pieces on a drop of sterile water on a microscope slide. These samples were viewed under a phase contrast microscope (Nikon SMZ645) for bacterial exudates. Excised lesions from diseased leaves were macerated in 1ml of sterile distilled water using a mortar and pestle and the resulting macerate was streaked on to nutrient agar [3.0 g beef extract (Difco), 5.0 g peptone (Difco) and 15 g agar in 1000 ml H_2O]. Pure cultures were obtained from single bacterial colonies appearing from these primary isolations. Bacterial cultures were preserved using the Microbank™ Bacterial Preservation System according to the manufacturer's protocols (http://www.pro-lab.com/Microbank.html) (Prolab diagnostics, Canada). All isolates are stored at –70°C in the bacterial culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Physiological and Biochemical tests

Gram staining was performed using the Hucker's modification Gram stain protocol (http://biology.clc.uc.edu/Fankhauser/Labs/Microbiology/Gram_Stain/Gram_stain.htm). Hugh Leifson, oxidative and catalase tests were also conducted once pure bacterial cultures were obtained (Hugh & Leifson 1953, Richard 1994, Schaad, Jones & Chun 2001).

Molecular characterisation

DNA Extraction

Bacterial isolates were grown overnight in 5 ml nutrient broth (2.0 g Yeast extract, 5.0 g Peptone, 5.0 g NaCl in 1000 ml distilled water) (Biolab Diagnostics, Midrand, Johannesburg) and a pellet was obtained by centrifugation. Nucleic acids were extracted and purified as described by Hauben *et al.* (1998). DNA pellets were dried and suspended in 50 µl H_20. The concentration of nucleic acid extracts was estimated on a 2% agarose gel containing ethidium bromide, exposed to UV light.

Amplification of the 16S rRNA gene.

The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene. The reaction was done in a volume of 25 µl comprising of a 2 ng DNA template, 800 µM dNTPs, 300 nM of universal forward primer (W3) (5'- AGA GTT TGA TCC TGG CTC AG- 3') and reverse primer (W4) (5' - AAG GAG GTG ATC CAG CCG CA -3') (Coutinho *et al.* 2002), 5U/µl Taq polymerase (Roche Diagnostics, Mannheim, Germany) and sterile distilled water (17.4 µl). The reaction consisted of an initial denaturation step at 94°C for 2 minutes. This was followed by 30 amplification cycles consisting of 1 min at 92°C and 30 seconds of annealing at 56°C. The PCR products were visualised under UV light on a 2% agarose gel containing Ethidium bromide, to determine the presence or absence of bands. The PCR products were purified using the High pure PCR product purification kit according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

The purified PCR products were ligated overnight into pGEM-T Easy vector at 10°C. The total volume was 10 µl, consisting of 2X–rapid buffer, T4 DNA ligase (5 µl), pGEM®-T Easy vector (50 ng) (1 µl), PCR product (3 µl), and T4 DNA ligase (1 µl) (Promega, Madison, USA). The ligation products were transformed by mixing 2 µl of

ligation product with 25 µl of competent *Escherichia coli* JM109 (Promega) in sterile Eppendorf tubes. These were kept on ice for 20 minutes, heat shocked for 45 seconds in a water bath (42°C), transferred to test tubes containing 475 µl Luria–Bertani (LB) broth (10 g Bacto®-Tryptone, 5 g Bacto®-Yeast Extract, 5 g NaCl) and incubated for 2 hrs at 37°C with shaking (~150 rpm). 150 µl of each transformation culture was incubated overnight at 37°C on LB medium [10 g Bacto®-Tryptone, 5 g Bacto®-Yeast Extract, 5 g NaCl, 80 µg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 100 µg ampicillin]. The positive clones (without blue colour) were transferred to LB broth containing 100 µg ampicillin and incubated overnight at 37°C with shaking (~150 rpm). Plasmid DNA was recovered by alkaline lysis as described by Sambrook, Fritsch & Maniatis (1989). To release the insert (16S rRNA gene) and to determine its size, plasmid DNA was digested with *EcoR*1, run on an ethidium bromide stained 2% gel and visualized under UV illumination.

DNA sequencing and analysis

The 16S rRNA gene was sequenced from the undigested plasmid DNA. The reaction was done in a total volume of 10 µl consisting of 5x dilution buffer (1 µl), 4.5 µl H_2O, 2 µl DNA (50ng PCR product), 10x reaction mix (2 µl), ~ 2 pmol/µl of one of either reverse or forward universal primers, T7 and Sp6 (pGEM®-T Easy vector systems, Promega, Madison, USA) or one of the internal primers 0T1 (5' - GAA GAA GGC CTT CGG GTT G -3') and 0T2 (5'- CAC GAC ACG AGC TGA CGA C - 3') (Coutinho *et al.* 2002). The DNA was precipitated by addition of 2µl sodium acetate (3 M) and 50 µl of 100% ethanol. The mixture was agitated using a vortex mixer and centrifuged for 30 min (10000 rpm). The pellet was then washed with 70% ethanol and vacuum dried for 5 minutes. The PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, California) on an ABI Prism™ 3100 DNA sequencer.

The 16S rRNA gene sequences were analyzed and edited using the Sequence Navigator programme (Applied Biosystems). The Blast program (National Center for Biotechnology Information, United States National Institutes of Health, Bethesda, MD) was used for homology searches using GenBank/EMBL databases (http://www.ncbi.nlm.nih.gov/BLAST/). An assortment of 16S rRNA gene sequences was obtained using blast searches. These were aligned with sequences obtained from

the Ugandan bacterial isolates using the program Clustal X (Thompson, Higgins & Gibson 1994).

Phylogenetic analysis

Phylogenetic analysis of aligned sequences was achieved by using PAUP 4.0b3 (Phylogenetic Analysis Using Parsimony) (Swofford 1998). Heuristic searches with maximum parsimony, using tree bisection-reconstruction and simple stepwise addition was used to produce phylogenetic trees. Gaps were treated as missing data. Support for branching was determined by performing 1000 bootstrap replicates (Felsenstein 1985). The tree was rooted to the outgroup taxon *Klebsiella pneumoniae* (Schroeter) Trevisan., which was treated as a monophyletic sister group to the other taxa (Coutinho *et al.* 2002).

RESULTS

Disease incidence and isolations

The disease was detected in all the areas surveyed (Table 1) on two to four-year-old *E. grandis* trees. Most severe damage was, however, observed in the Entebbe area (Wakiso district). Symptoms included tip die-back and leaf spots on young leaves. Leaf spots first appeared as water soaked lesions, which finally became corky. Infections appeared to begin from the leaf petioles spreading into the main veins and subsequently to adjacent tissue. In some cases, the infected trees appeared bush-like due to the formation of numerous new growth leader shoots (Figure 1).

Numerous rod shaped bacteria were observed streaming from leaf tissues macerated in sterile distilled water, on microscope slides. A total of 20 isolates, each obtained from different symptomatic trees, were used in physiological and biochemical tests. Four of these isolates (BCC0107, BCC0109, BCC0105, BCC0160) were subsequently selected for DNA-based characterisation (Table 2).

Physiological and biochemical tests

Isolates formed yellow-pigmented round colonies on nutrient agar. All cells were Gram negative, straight rods varying between 0.45 to 0.6 µm in width and 1.0 to 2.0 µm in length. All were catalase positive, oxidase negative, exhibiting a fermentative metabolism (Table 3). The physiological and biochemical characteristics confirmed

that the bacterial isolates belong to the family *Enterobacteriaceae* (*Erwinia – Pantoea* group).

Molecular characterization

PCR amplification and DNA Sequence analysis

The 16S rRNA gene was successfully amplified and all Ugandan isolates produced a ~1500bp fragment (Figure 2). The fragments were successfully cloned and sequenced. A blast search revealed that the Ugandan isolates were most similar to *Pantoea ananatis* (Z96081). The sequences obtained for isolates from Uganda were aligned with other related *Enterobacteriaceae* in the genus *Erwinia* and *Pantoea* obtained from Genebank (http://www.ncbi.nlm.gov). Manual alignment of these sequences resulted in a total of 1337 base pairs for final analysis (Figure 3).

Phylogenetic analysis of the aligned sequences resulted in 7 most parsimonious trees after 8686 rearrangements. A consistency index (CI) of 0.905 and retention index (RI) of 0.839 was obtained. The trees had the same topologies, differing only in the internal position of isolates. The total tree length obtained was 142. Of the 1337 characters, 1257 were constant, 51 parsimony uninformative and 29 parsimony informative. The most parsimonious tree had the same topology as the consensus tree and clearly showed that the Ugandan isolates group together with *P. ananatis* isolated from *Eucalyptus* in South Africa and with the type species isolated from pineapples (*P. ananatis*, Z96081). Isolates of *P. ananatis* (U80209 and U8019), which are hyperparasites on uredia of *Puccinia graminis* Pers.: Pers also grouped in this clade, separate from closely related species such as *P. agglomerans* (Kwon *et al.* 1997) (Figure 4).

DISCUSSION

In this study, we have established that the bacterium associated with bacterial blight and die-back on young *E. grandis* trees in Uganda is *Pantoea ananatis*. Given the association of this bacterium with a similar disease in South Africa (Coutinho *et al.* 2002), we believe that *P. ananatis* is the cause of the disease in Uganda. This represents the first report of *P. ananatis* associated with leaf and shoot blight of *Eucalyptus* spp., outside South Africa. This disease appears to be widespread in

Eucalyptus growing areas of Uganda and it is clearly an important constraint to tree establishment.

Using physiological and biochemical tests, we were able to identify Ugandan bacterial isolates only as belonging to the family *Enterobacteriaceae*. By sequencing the 16S rRNA gene, we were able to align the sequences from Ugandan isolates with those of other *Pantoea* species and related *Erwinia* species. DNA sequence data clearly showed that the Ugandan isolates are *P. ananatis*, making this the second report of bacterial blight of *Eucalyptus* caused by *P. ananatis* in the world.

The genus *Pantoea* contains a number of pathogenic speceis, including *P. stewartii* (Smith 1898) and *P. ananatis*. Before its establishment in 1989, these pathogens resided in *Erwinia* (Mergaert, Verdonck & Kersters 1993). *P. ananatis* Serrano (Synonyms: *Erwinia uredovora*, *Erwinia ananas*) was first described in 1928, where it was responsible for fruitlet rot on pineapples (Serrano 1928). Since then, there have been various reports of its occurrence on other crops. These include leaf blotch on sorghum (Azad, Holmes & Cooksey 2000), cantaloupe post harvest diseases (Bruton *et al.* 1991), honeydew melon rot (Wells *et al.* 1987) and bulb decay of onions (Gitaitis & Gay 1997). In 1998, it was reported as the cause of bacterial blight of *Eucalyptus* clones (Coutinho *et al.* 2002). The association of *P. ananatis* with disease is enigmatic, as it is commonly known as an epiphyte on the fungal smut pathogen *Ustilago maydis* and on the panicles of barley, buckwheat and rice (Watanabe, Kawakita & Sato 1996, Paccola-Meirelles *et al.* 2001).

In Uganda, bacterial blight has been reported in *Eucalyptus* plantation areas, previously occupied by natural forests. The plantations are also in close proximity to agricultural crops such as cassava, maize, beans and sweet potatoes. At the time of the survey in which this disease was discovered, damage was not observed on other crops. It is possible, however, that this bacterium originated on other crops which might act as alternative hosts. The possibility of seed transmission can also not be ruled out, since some farmers might have purchased *Eucalyptus* seed from suppliers in South Africa.

Since this disease report originates from a limited survey, more detailed surveys will be required to establish the relationships between *P. ananatis*, *Eucalyptus* trees,

agricultural crops and weeds growing in proximity. In this way a method of developing future recommendations for commercial activities, in the absence of disease losses might be developed.

The occurrence of bacterial blight on young *Eucalyptus* species may pose a serious problem to the Ugandan forestry industry. The disease leads to the stunting of trees and eventual death. The extent of losses in Uganda has not been quantified and detailed studies are needed in this regard. However, during the current survey, the levels of damage appeared to be heavy. In South Africa, for example, susceptible clones had to be removed from breeding programmes, as infection by *P. ananatis* resulted in severe stunting and malformation of trees, especially in nurseries (Coutinho *et al.* 2002).

Long-term control through the selection and development of clones resistant to bacterial blight shows the most potential for reducing the impact of bacterial blight of *Eucalyptus* spp. Matching trees to sites might also be considered, since trees planted on inappropriate sites are subject to severe stress, thus enhancing their chance of being affected by diseases. In cases where seedlings are used for propagation, care should also be taken to ensure that pathogen-free seeds are planted. This is especially since *P. ananatis* has been shown to be seed borne and seed-transmitted in onions and Sudangrass (Azad *et al.* 2000, Walcott *et al.* 2002). These strategies will ensure reduced losses due to bacterial blight and other diseases in Uganda.

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Table 1. Weather conditions and altitude of areas sampled[A].

Sampling area	Average temperature (°C)	Average rainfall (mm/yr^{-1})	Altitude (m)
Entebbe (Central Uganda)	21.5	1538	1155
Bweyogerere (Central Uganda)	21.5	1538	1155
Tororo (Eastern Uganda)	22.5	1427	1170
Kabale (Western Uganda)	15.3	1180	2241

[A]Information obtained from Uganda Meteorological Department, Entebbe.

Table 2. List of bacterial isolates used in this study.

Species	Host	Culture number	Origin	Accession No.
P. ananatis[a]	*E. grandis*	BCC0107	Entebbe, Uganda	AY173020
"	"	BCC0109	"	AY173023
"	"	BCC0105	"	AY173021
"	"	BCC0160	"	AY173022
"	*Eucalyptus* clones	LMG 20106	"	AF364844
"	"	LMG 20103	South Africa	AF364847
"	"	LMG 20104	South Africa	AF364846
"	"	LMG 20105	"	AF364845
"	*Ananas comosus*	LMG 2665	Brazil	Z96081
"	*Puccinia graminis f. sp. tritici*			U80209
"	*Puccinia graminis f. sp. tritici*			U8019
P. agglomerans				AJ251466
P. agglomerans				U80183
Klebsiella pneumoniae				Y17656

[a] Ugandan isolates characterized in this study

- Isolates designated by BCC are maintained in the bacterial culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.
- Isolates designated by LMG are deposited in the culture collection at the Universiteit Gent, K. L. Ledegranckstraat 35, B-9000 Gent, Belgium.

Table 3. Characteristics of *P. ananatis* strains isolated from diseased *Eucalyptus* species from Uganda.

Characteristic	**Result**
Cell dimensions	0.45 to 0.6 x 1.0 to 2.0 µm
Colony characteristics	Entire, round
Pigmentation	Yellow
Mucoid growth	Yes
Cell morphology	Rods & motile
Gram stain	Negative
Hugh Leifson test	Fermentative
Oxidase test	Negative
Catalase	Positive

Figure 1. Symptoms associated with bacterial blight and dieback. **A)** Leaf spots, which appear water soaked in the initial stages and eventually become corky. **B)** Manifestation of lesions along the main leaf vein. The arrow shows bacteria exuding out of the leaf. **C)** Bushy appearance of an infected tree due to formation of numerous new shoots. **D)** Stunted *Eucalyptus* tree and death of main shoot.

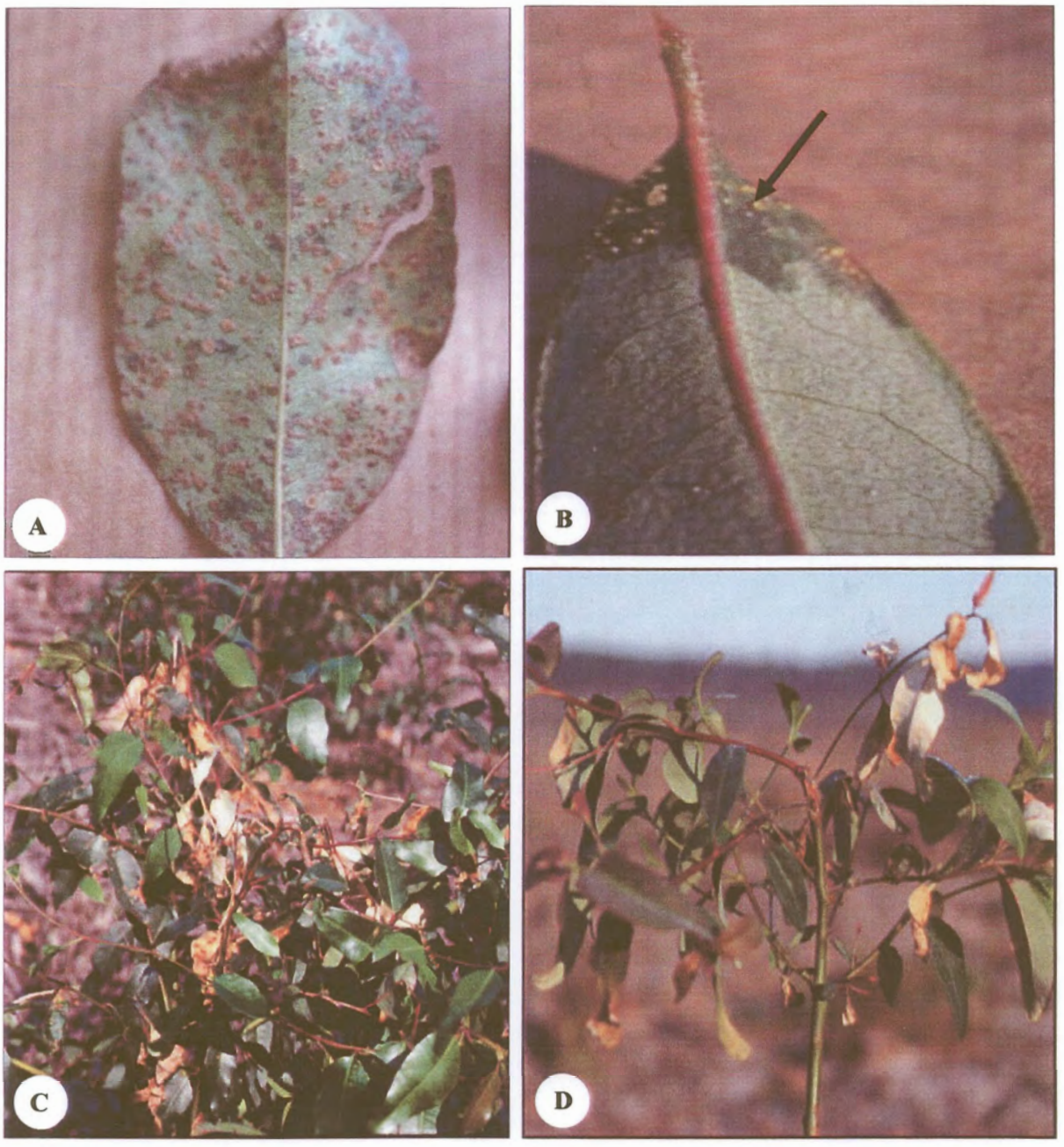

Figure 2. Gel profile (on a 2% agarose gel) obtained after amplifying the 16S rRNA gene. A fragment size of approximately 1500 was obtained.

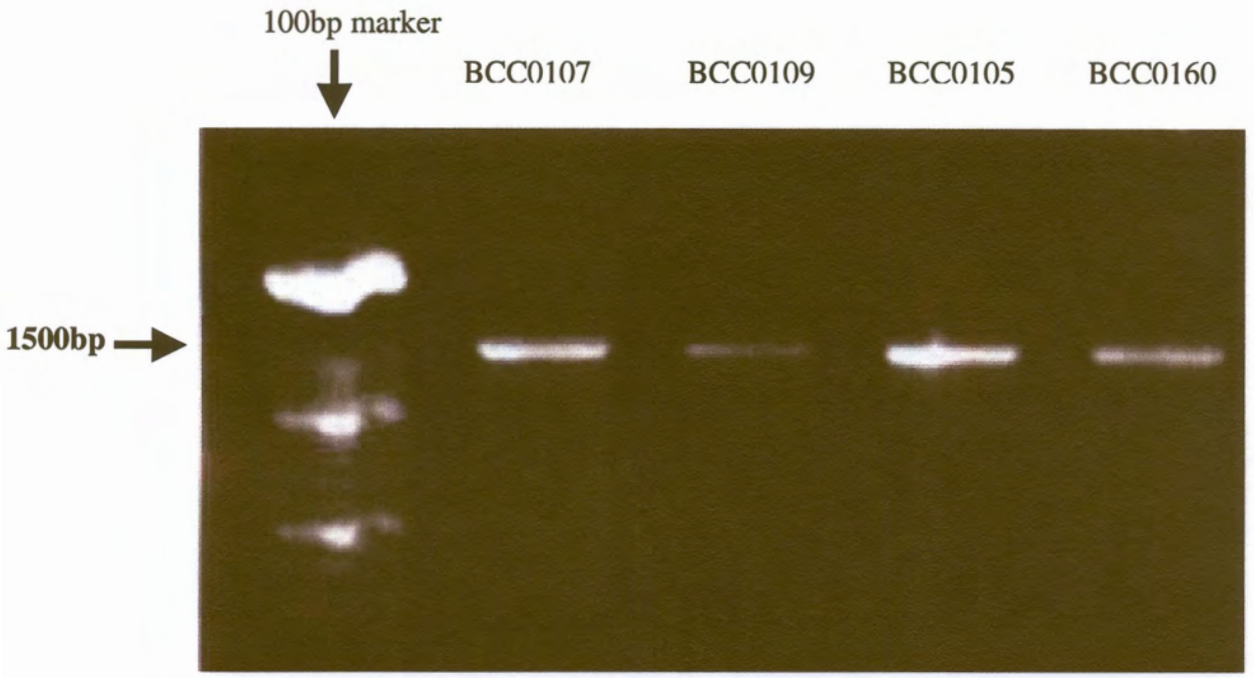

Figure 3. DNA sequence data for the partial 16S rRNA gene of Ugandan isolates, aligned against sequences of *Pantoea* species obtained from gene bank.
(- represents gaps, N represents missing data, . represents identical bases)

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                                    10         20         30         40         50
P.stewartii     (U80208)    GGGTGACGAG TGGCGGACGG GTGAGTAATG TCTGGGAAAC TGCCCGATGG
P.agglomerans   (U80183)    .......... .......... .......... ......G.T. ........A.
P.agglomerans   (AJ251466)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364844)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364845)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364847)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364846)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (U80209)    .T........ .......... .......... ......G.T. ........A.
P.ananatis      (U8019)     .......... .......... .......... ......G.T. ........A.
P.ananatis      (Z96081)    .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173020)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173021)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173022)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173023)  .......... .......... .......... ......G.T. ........A.
K. pneumoniae   (Y17656)    .......... C......... .......... .......... ....T.....

                                    60         70         80         90        100
P.stewartii     (U80208)    AGGGGGATAA CTACTGGAAA CGGTAGCTAA TACCGCATAA CGTCGCAAGA
P.agglomerans   (U80183)    .......... .C........ ....G..... .......... ..........
P.agglomerans   (AJ251466)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364844)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364845)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364847)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364846)  .......... .C........ ....G..... .......... ..........
P.ananatis      (U80209)    .......... .C........ ....G..... .......... ..........
P.ananatis      (U8019)     .......... .C........ ....G..... .......... ..........
P.ananatis      (Z96081)    .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173020)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173021)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173022)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173023)  .......... .C........ ....G..... .......... ..........
K. pneumoniae   (Y17656)    .......... .......... .......... .......... ..........
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                                  110        120        130        140        150
P.stewartii      (U80208)    CCAAAGTGGG GGACCTCCGG GCCTCACACC ATCGGATGTG CCCAGATGGG
P.agglomerans    (U80183)    ......A... ......T... .....T...T ........AA ..........
P.agglomerans    (AJ251466)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364844)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364845)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364847)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364846)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (U80209)    ......A... ......T... .....T...T ........AA ..........
P.ananatis       (U8019)     ......A... ......T... .....T...T ........AA ..........
P.ananatis       (Z96081)    ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173020)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173021)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173022)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173023)  ......A... ......T... .....T...T ........AA ..........
K. pneumoniae    (Y17656)    .......... ......T... ......TG.. ...A...... ..........

                                  160        170        180        190        200
P.stewartii      (U80208)    ATTAGCTAGT AGGCGGGGTA ACGGCCCACC TAGGCGACGA TCCCTAGCTG
P.agglomerans    (U80183)    .......... .......... .T........ .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .T........ .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    .......... ...T...... .....T.... .......... ..........
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                                    210        220        230        240        250
P.stewartii      (U80208)     GTCTGAGAGG ATGACCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
P.agglomerans    (U80183)     .......G.. .......... .......... .......... .........-
P.agglomerans    (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis       (AF364844)   .......... .......... .......... .......... ..........
P.ananatis       (AF364845)   .......... .......... .......... .......... ..........
P.ananatis       (AF364847)   .......... .......... .......... .......... ..........
P.ananatis       (AF364846)   .......... .......... .......... .......... ..........
P.ananatis       (U80209)     .......... .......... .......... .......... ..........
P.ananatis       (U8019)      .......... .......... .......... .......... ..........
P.ananatis       (Z96081)     .......... .......... .......... .......... ..........
P.ananatis       (AY173020)   .......... .......... .......... .......... ..........
P.ananatis       (AY173021)   .......... .......... .......... .......... ..........
P.ananatis       (AY173022)   .......... .......... .......... .......... ..........
P.ananatis       (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)     .......... .......... .......... .......... ..........

                                    260        270        280        290        300
P.stewartii      (U80208)     TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC
P.agglomerans    (U80183)     .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis       (AF364844)   .......... .......... .......... .......... ..........
P.ananatis       (AF364845)   .......... .......... .......... .......... ..........
P.ananatis       (AF364847)   .G........ .......... .......... .......... ..........
P.ananatis       (AF364846)   .......... .......... .......... .......... ..........
P.ananatis       (U80209)     .......... .......... .......... .......... ..........
P.ananatis       (U8019)      .......... .......... .......... .......... ..........
P.ananatis       (Z96081)     .......... .......... .......... .......... ..........
P.ananatis       (AY173020)   .......... .......... .......... .......... ..........
P.ananatis       (AY173021)   .......... .......... .......... .......... ..........
P.ananatis       (AY173022)   .......... .......... .......... .......... ..........
P.ananatis       (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)     .......... .......... .......... .......... ..........
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                                  310        320        330        340        350
P.stewartii       (U80208)    AGCCATGCCG CGTGTATGAA GAAGGCCTTC GGGTTGTAAA GTACTTTCAG
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .......... .....G.... .......... .......... .C........

                                  360        370        380        390        400
P.stewartii       (U80208)    C-GGGGAGGA AGGTGGT--G AGGTTAATAA CCTC-ATCAA TTGACATTAC
P.agglomerans     (U80183)    .-........ ...C.AC--. -......... ..CT-G..G. .....G....
P.agglomerans     (AJ251466)  .-........ ...C.A.--. G......... ...T-...G. .....G....
P.ananatis        (AF364844)  .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (AF364845)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AF364847)  .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (AF364846)  .-........ ...C.A.-GT -......... ..G.-G.TG. .....G....
P.ananatis        (U80209)    .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (U8019)     .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (Z96081)    .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173020)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173021)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173022)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173022)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
K. pneumoniae     (Y17656)    .-........ ...C.A.--. ..............-...G. .....G....
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                                  410        420        430        440        450
P.stewartii      (U80208)    CCG-CAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
P.agglomerans    (U80183)    ...-...... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  ...-...... ....-..... .......... .......... ..........
P.ananatis       (AF364844)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364845)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364847)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364846)  ...-...... .......... .......... .......... ..........
P.ananatis       (U80209)    ...-...... .......... .......... .......... ..........
P.ananatis       (U8019)     ...-...... .......... .......... .......... ..........
P.ananatis       (Z96081)    ...-...... .......... .......... .......... ..........
P.ananatis       (AY173020)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173021)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173022)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173023)  ...-...... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    ...-...... .......... .......... .......... ..........

                                  460        470        480        490        500
P.stewartii      (U80208)    GAGGGTGCAA GCGTTAATCG GAATTACTGG GCGTAAAGCG CACGCAGGCG
P.agglomerans    (U80183)    .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    .......... .......... .......... .......... ..........
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                                  510        520        530        540        550
P.stewartii      (U80208)    GTCTGTTAAG TCAGATGTGA AATCCCCGGG CTTAACCTGG GAACTGCATT
P.agglomerans    (U80183)    .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    ......C... ..G..... .......... ..C..... ..........

                                  560        570        580        590        600
P.stewartii      (U80208)    TGAAACTGGC AGGCTTGAGT CTCGTAGAGG GGGGTAGAAT TCCAGGTGTA
P.agglomerans    (U80183)    .......... .......... ..T..... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... ..T..... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    C......... .....A.... ..T..... .......... ..........
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                                    610        620        630        640        650
P.stewartii     (U80208)        GCGGTGAAAT GCGTAGAGAT CTGGAGGAAT ACCGGTGGCG AAGGCGGTCC
P.agglomerans   (U80183)        .......... .......... .......... .......... .......C..
P.agglomerans   (AJ251466)       .......... .......... .......... .......... .......C..
P.ananatis      (AF364844)       .......... .......... .......... .......... .......C..
P.ananatis      (AF364845)       .......... .......... .......... .......... ..........
P.ananatis      (AF364847)       .......... .......... .......... .......... ..........
P.ananatis      (AF364846)       .......... .......... .......... .......... ..........
P.ananatis      (U80209)         .......... .......... .......... .......... .......C..
P.ananatis      (U8019)          .......... .......... .......... .......... .......C..
P.ananatis      (Z96081)         .......... .......... .......... .......... .......C..
P.ananatis      (AY173020)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173021)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173022)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173023)       .......... .......... .......... .......... .......C..
K. pneumoniae   (Y17656)         .......... .......... .......... .......... .......C..

                                    660        670        680        690        700
P.stewartii     (U80208)        CCTGGACGAA GACTGACGCT CAGGTGCGAA AGCGTGGGGA GCAAACAGGA
P.agglomerans   (U80183)        .......A.. .......... .......... .......... ..........
P.agglomerans   (AJ251466)       .......A.. .......... .......... .......... ..........
P.ananatis      (AF364844)       .......... .......... .......... .......... ..........
P.ananatis      (AF364845)       .......... .......... .......... .......... ..........
P.ananatis      (AF364847)       .......... .......... .......... .......... ..........
P.ananatis      (AF364846)       .......... .......... .......... .......... ..........
P.ananatis      (U80209)         .......... .......... .......... .......... ..........
P.ananatis      (U8019)          .......... .......... .......... .......... ..........
P.ananatis      (Z96081)         .......... .......... .......... .......... ..........
P.ananatis      (AY173020)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173021)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173022)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173023)       .......A.. .......... .......... .......... ..........
K. pneumoniae   (Y17656)         .......A.. .......... .......... .......... ..........
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                                    710        720        730        740        750
P.stewartii     (U80208)    TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGTCGACT TGGAGGTTGT
P.agglomerans   (U80183)    .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis      (AF364844)  .......... .......... .......... .......... ..........
P.ananatis      (AF364845)  .......... .......... .......... .......... ..........
P.ananatis      (AF364847)  .......... .......... .......... .......... ..........
P.ananatis      (AF364846)  .......... .......... .......... .......... ..........
P.ananatis      (U80209)    .......... .......... .......... .......... ..........
P.ananatis      (U8019)     .......... .......... .......... .......... ..........
P.ananatis      (Z96081)    .......... .......... .......... .......... ..........
P.ananatis      (AY173020)  .......... .......... .......... .......... ..........
P.ananatis      (AY173021)  .......... .......... .......... .......... ..........
P.ananatis      (AY173022)  .......... .......... .......... .......... ..........
P.ananatis      (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)   .......... .......... .......... ........T. ..........

                                    760        770        780        790        800
P.stewartii     (U80208)    TCCCTTGAGG AGTGGCTTCC GGAGCTAACG CGTTAAGTCG ACCGCCTGGG
P.agglomerans   (U80183)    .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis      (AF364844)  .......... .......... .......... .......... ..........
P.ananatis      (AF364845)  .......... .......... .......... .......... ..........
P.ananatis      (AF364847)  .......... .......... .......... .......... ..........
P.ananatis      (AF364846)  .......... .......... .......... .......... ..........
P.ananatis      (U80209)    .......... .......... .......... .......... ..........
P.ananatis      (U8019)     .......... .......... .......... .......... ..........
P.ananatis      (Z96081)    .......... .......... .......... .......... ..........
P.ananatis      (AY173020)  .......... .......... .......... .......... ..........
P.ananatis      (AY173021)  .......... .......... .......... .......... ..........
P.ananatis      (AY173022)  .......... .......... .......... .......... ..........
P.ananatis      (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)   G......... C......... .......... ......A... ..........
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                                  810        820        830        840        850
P.stewartii     (U80208)     GAGTACGGCC GCAAGGTTAA AACTCAAATG AATTGACGGG GGCCCGCACA
P.agglomerans   (U80183)     .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis      (AF364844)   .......... .......... .......... .......... ..........
P.ananatis      (AF364845)   .......... .......... .......... .......... ..........
P.ananatis      (AF364847)   .......... .......... .......... .......... ..........
P.ananatis      (AF364846)   .......... .......... .......... .......... ..........
P.ananatis      (U80209)     .......... .......... .......... .......... ..........
P.ananatis      (U8019)      .......... .......... .......... .......... ..........
P.ananatis      (Z96081)     .......... .......... .......... .......... ..........
P.ananatis      (AY173020)   .......... .......... .......... .......... ..........
P.ananatis      (AY173021)   .......... .......... .......... .......... ..........
P.ananatis      (AY173022)   .......... .......... .......... .......... ..........
P.ananatis      (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)     .......... .......... .......... .......... ..........

                                  860        870        880        890        900
P.stewartii     (U80208)     AGCGGTGGAG CATGTGGTTT AATTCGATGC AACGCGAAGA ACCTTACCTA
P.agglomerans   (U80183)     .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis      (AF364844)   .......... .......... .......... .......... ..........
P.ananatis      (AF364845)   .......... .......... .......... .......... ..........
P.ananatis      (AF364847)   .......... .......... .......... .......... ..........
P.ananatis      (AF364846)   .......... .......... .......... .......... ..........
P.ananatis      (U80209)     .......... ...A...... .......... .......... ..........
P.ananatis      (U8019)      .......... ...A...... .......... .......... ..........
P.ananatis      (Z96081)     .......... .......... .......... .......... ..........
P.ananatis      (AY173020)   .......... .......... .......... .......... ..........
P.ananatis      (AY173021)   .......... .......... .......... .......... ..........
P.ananatis      (AY173022)   .......... .......... .......... .......... ..........
P.ananatis      (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)     .......... .......... .......... .......... .........G
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                                      910        920        930        940        950
P.stewartii     (U80208)      CTCTTGACAT CCAGCGAACT TGGC----AG AGATGCCTTG GTGCCTTC-G
P.agglomerans   (U80183)      .......... ...CG...T. ....----.. .........A ........-.
P.agglomerans   (AJ251466)    .......... ...-G...T. ....----.. .........A ........-.
P.ananatis      (AF364844)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364845)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364847)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364846)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (U80209)      .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (U8019)       .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (Z96081)      .......... ....A..... ....----.. ......T... ........-.
P.ananatis      (AY173020)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173021)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173022)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173023)    .......... ....A..... .A..----.. ......T... ........-C
K. pneumoniae   (Y17656)      G......... ...CA..... .TC.----.. .....GA... ........-.

                                      960        970        980        990       1000
P.stewartii     (U80208)      GGAACGCTGA GACAGGTGCT GCATGGCTGT CGTCAGCTCG TGTTGTGAAA
P.agglomerans   (U80183)      ..G..CG... .......... .......... .......... ..........
P.agglomerans   (AJ251466)    .....CG... .......... .......... .......... ..........
P.ananatis      (AF364844)    .....T.... .......... .......... .......... ..........
P.ananatis      (AF364845)    ...G.T.... .......... .......... .......... ..........
P.ananatis      (AF364847)    .....T.... .......... .......... .......... ..........
P.ananatis      (AF364846)    .....T.... .......... .......... .......... ..........
P.ananatis      (U80209)      .....T.... .......... .......... .......... ..........
P.ananatis      (U8019)       .....T.... .......... .......... .......... ..........
P.ananatis      (Z96081)      ...G.C.... .......... .......... .......... ..........
P.ananatis      (AY173020)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173021)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173022)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173023)    .....C.... .......... .......... .......... ..........
K. pneumoniae   (Y17656)      .....TG... .......... .......... .......... ..........
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                                  1010       1020       1030       1040       1050
P.stewartii       (U80208)    TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTATCCTT TGTTGCCAGC
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .......... .......... .......... .......... ..........

                                  1060       1070       1080       1090       1100
P.stewartii       (U80208)    GATTCGGTCG GGAACTCAAA GGAGACTGCC GGTGATAAAC CGGAGGAAGG
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .G..A..C.. .......... .......... A......... T.........
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                                1110       1120       1130       1140       1150
P.stewartii      (U80208)   TGGGGATGAC GTCAAGTCAT CATGGCCCTT ACGAGTAGGG CTACACACGT
P.agglomerans    (U80183)   .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466) .......... .......... .......... .......... ..........
P.ananatis       (AF364844) .......... .......... .......... .......... ..........
P.ananatis       (AF364845) .......... .......... .......... .......... ..........
P.ananatis       (AF364847) .......... .......... .......... .......... ..........
P.ananatis       (AF364846) .......... .......... .......... .......... ..........
P.ananatis       (U80209)   .......... .......... .......... .......... ..........
P.ananatis       (U8019)    .......... .......... .......... .......... ..........
P.ananatis       (Z96081)   .......... .......... .......... .......... ..........
P.ananatis       (AY173020) .......... .......... .......... .......... ..........
P.ananatis       (AY173021) .......... .......... .......... .......... ..........
P.ananatis       (AY173022) .......... .......... .......... .......... ..........
P.ananatis       (AY173023) .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)   .......... .......... .......... ....CC.... ..........

                                1160       1170       1180       1190       1200
P.stewartii      (U80208)   GCTACAATGG CGCATA-CAA AGAGAAGCGA CCTCGCGAGA GCAAGCGGAC
P.agglomerans    (U80183)   .......... ......-... ........A. .......... ..........
P.agglomerans    (AJ251466) .......... ......-... .......... .......... ..........
P.ananatis       (AF364844) .......... ......-... .......... .......... ..........
P.ananatis       (AF364845) .......... ......-... .......... .......... ..........
P.ananatis       (AF364847) .......... ......-... .......... .......... ..........
P.ananatis       (AF364846) .......... ......-... .......... .......... ..........
P.ananatis       (U80209)   .......... ......-... .......... .......... ..........
P.ananatis       (U8019)    .......... ......-... .......... .......... ..........
P.ananatis       (Z96081)   .......... ......-... .......... .......... ..........
P.ananatis       (AY173020) .......... ......-... .......... .......... ..........
P.ananatis       (AY173021) .......... ......-... .......... .......... ..........
P.ananatis       (AY173022) .......... ......-... .......... .......... ..........
P.ananatis       (AY173023) .......... ......-... .......... .......... ..........
K. pneumoniae    (Y17656)   .......... .AT...-... .......... .......... ..........
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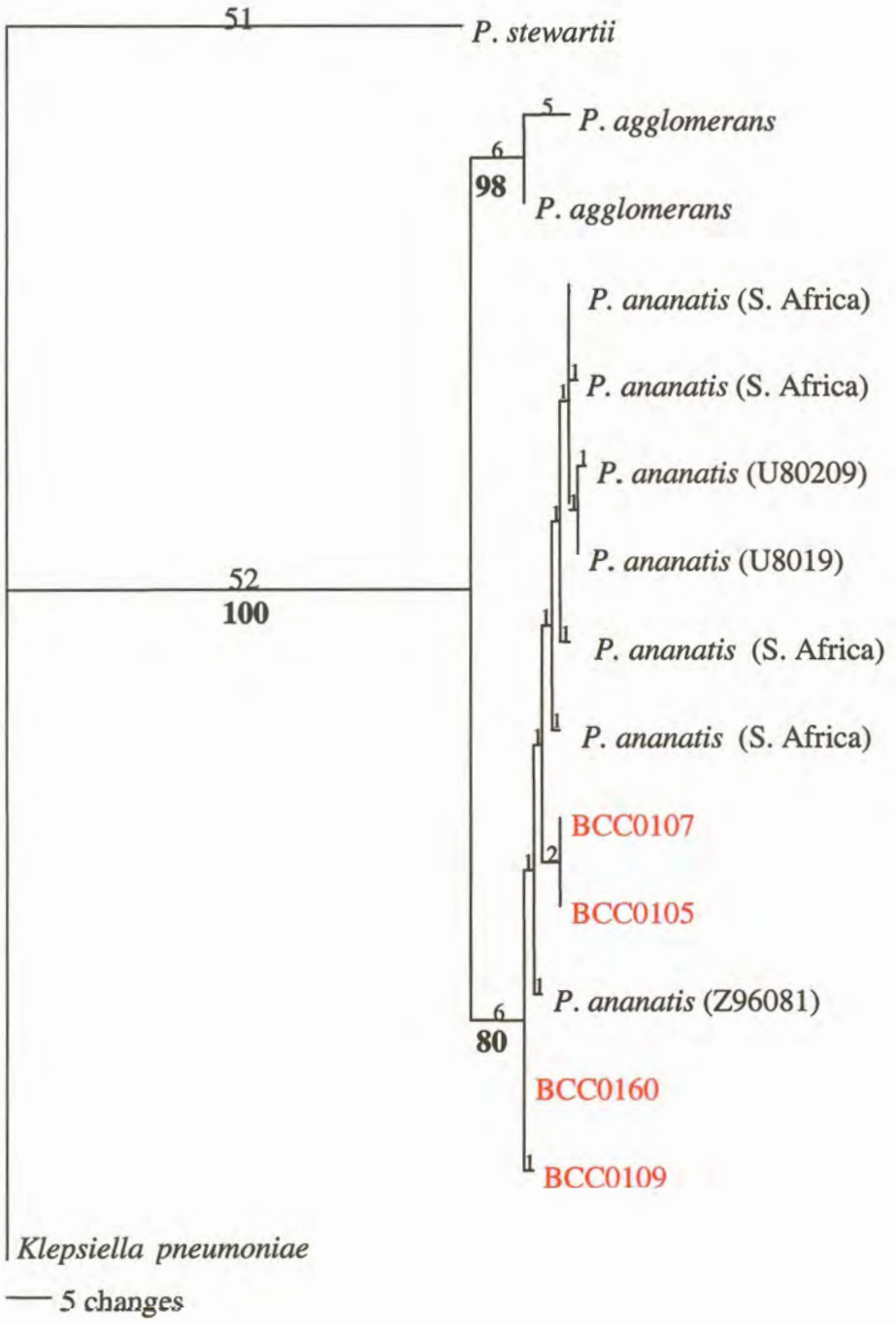
                                1210       1220       1230       1240       1250
P.stewartii       (U80208)      CTCATAAAGT GCGTCGTAGT CCGGATCGGA GTCTGCAACT CGACTCCGTG
P.agglomerans     (U80183)      ....C..... .......... .......... .......... ..........
P.agglomerans     (AJ251466)    ....C..... .......... .......... .......... ..........
P.ananatis        (AF364844)    .......... .......... .......... .......... ..........
P.ananatis        (AF364845)    .......... .......... .......... .......... ..........
P.ananatis        (AF364847)    .......... .......... .......... .......... ..........
P.ananatis        (AF364846)    .......... .......... .......... .......... ..........
P.ananatis        (U80209)      .......... .......... .......... .......... ..........
P.ananatis        (U8019)       .......... .......... .......... .......... ..........
P.ananatis        (Z96081)      .......... .......... .......... .......... ..........
P.ananatis        (AY173020)    ....C..... .......... .......... .......... ..........
P.ananatis        (AY173021)    ....C..... .......... .......... .......... ..........
P.ananatis        (AY173022)    .......... .......... .......... .......... ..........
P.ananatis        (AY173023)    .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)      .......... AT........ ......T... .......... .......A..

                                1260       1270       1280       1290       1300
P.stewartii       (U80208)      AAGTCGGAAT CGCTA-GTAA TCGTGGATCA GAATGCCACG GTGAATACGT
P.agglomerans     (U80183)      .......... .....-.... .......... .......... ..........
P.agglomerans     (AJ251466)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364844)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364845)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364847)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364846)    .......... .....-.... .......... .......... ..........
P.ananatis        (U80209)      .......... .....-.... .......... .......... ..........
P.ananatis        (U8019)       .......... .....-.... .......... .......... ..........
P.ananatis        (Z96081)      .......... .....-.... .......... .......... ..........
P.ananatis        (AY173020)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173021)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173022)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173023)    .......... .....-.... .......... .......... ..........
K. pneumoniae     (Y17656)      .......... .....-.... ....A..... ......T... ..........
```

		1310	1320	1330	1340
P.stewartii	(U80208)	TCCCGGGCCT	TGTACACACC	GCCCGTCACA	CCATGGG
P.agglomerans	(U80183)	....	....	....	....
P.agglomerans	(AJ251466)	....	....	....	....
P.ananatis	(AF364844)	....	....	....	....
P.ananatis	(AF364845)	....	....	....	....
P.ananatis	(AF364847)	....	....	....	....
P.ananatis	(AF364846)	....	....	....	....
P.ananatis	(U80209)	....	....	....	....
P.ananatis	(U8019)	....	....	....	....
P.ananatis	(Z96081)	....	....	....	....
P.ananatis	(AY173020)	....	....	....	....
P.ananatis	(AY173021)	....	....	....	....
P.ananatis	(AY173022)	....	....	....	....
P.ananatis	(AY173023)	....	....	....	....
K. pneumoniae	(Y17656)	....	....	....	....

Figure 4. One of the 7 most parsimonious phylogenetic trees obtained after aligning the 16S rRNA gene for *P. ananatis*. *P. stewartii*, *P. agglomerans* and Ugandan isolates (in red). *Klepsiella pneumoniae* is the out-group taxon, rooted at internal nodes as a monophyletic sister group to ingroup. Total length of the tree = 142, CI = 0.905, RI = 0.839. Bootstrap values are typed in bold and appear below the branches.


51
P. stewartii
5
P. agglomerans
6
98
P. agglomerans
P. ananatis (S. Africa)
1
P. ananatis (S. Africa)
1
P. ananatis (U80209)
1
P. ananatis (U8019)
52
100
P. ananatis (S. Africa)
1
P. ananatis (S. Africa)
BCC0107
2
BCC0105
1
P. ananatis (Z96081)
6
80
BCC0160
1
BCC0109
Klepsiella pneumoniae
5 changes

SUMMARY

Plantation forestry is important to Uganda as it represents a future resource of timber for fuel and structural purposes. Diseases, however, pose a serious threat to the productivity and sustainability of this emerging industry. A number of serious diseases have already been reported from surveys recently conducted in Uganda. The aim of the present study was to expand on the current knowledge of some of the diseases and to gain additional information regarding their causal agents.

The first chapter of this thesis reviewed the literature pertaining to diseases of plantation trees in Uganda. The observation is made that diseases were recognised as problematic, as early as the 1950's and trials were conducted at that time to select disease resistant planting stock. Due to political unrest, these initiatives ceased and a reliance on natural forests for the supply of wood and wood products persisted. Over exploitation of natural forests has now resulted in awareness that this resource is threatened and plantation forestry is widely recognised as the only alternative for the supply of wood and wood products. The Uganda Forestry Department is now actively promoting the establishment of plantations to reduce destruction of natural forests. This initiative has included surveys to identify the diseases affecting forest plantations in Uganda. These surveys have shown that diseases pose a serious threat to the establishment of plantation forests in the country.

Botryosphaeria canker is the most common disease of plantation *Eucalyptus* in Uganda. In chapter two of this thesis, I have shown that *B. parva*, *L. theobromae* and an undescribed species are associated with Botryosphaeria canker of *Eucalyptus* spp. in Uganda. Pathogenicity trials revealed statistically significant differences in virulence between different species, with *L. theobromae* being the least pathogenic and the undescribed species the most pathogenic. Further investigations are now needed to evaluate the pathogenicity of these fungi in the field and to consider their biology and relative importance.

Ceratocystis albofundus is an important pathogen of *Acacia mearnsii* and *A. decurrens* in South Africa. In 1999, *C. albofundus* was reported for the first time in south western Uganda. The results obtained in the current study indicate that the Ugandan population has high gene diversity similar to that of a South African

population. The results further show that the fungus reproduces clonally in both countries, with very little gene flow occurring between them. The high gene diversity values obtained in this study indicate that *C. albofundus* is native to Africa and not only South Africa, as previously hypothesized. The high gene diversity of *C. albofundus* revealed within the two populations gives a clear indication that management of Ceratocystis wilt should focus on breeding for resistance. Selection of resistant varieties is in progress in South Africa and in future, it will be useful in management of the disease.

Most *Eucalyptus* diseases are caused by fungi, but bacterial pathogens are increasing in importance. In the fourth chapter of this thesis, a bacterium known as *Pantoea ananatis* was identified as the causal agent of blight and dieback on *Eucalyptus* spp. in Uganda. This is the first report of the disease outside South Africa. The disease primarily damages young trees and it is thus of great concern in terms of plantation establishment. It will now be necessary to establish trials to select bacterial blight-tolerant planting stock. The bacterium has also been reported to infect agricultural crops which is especially important in the Ugandan situation, where trees are grown in close proximity to agricultural crops.

The results obtained in this thesis will help to manage diseases affecting plantation forest species in Uganda. The study will hopefully also provide farmers and foresters with an elevated understanding of the importance of diseases in plantation forestry. This should impact strongly on the capacity of Ugandans to deal with diseases, not only those caused by the pathogens identified in the study, but also other diseases, particularly of forest and fruit crops.

OPSOMMING

Plantasiebosbou is belangrik in Uganda, omdat dit 'n toekomstige bron van hout vir energie en strukturele behoeftes bied. Siektes is egter 'n ernstige bedreiging vir die produktiwiteit en volhoubaarheid van hierdie opkomende industrie. 'n Aantal ernstige siektes is alreeds aangemeld tydens vorige opnames wat in Uganda gedoen is. Die doelwit van die huidige studie is om die kennis oor sommige van hierdie siektes te verbreed en om verdere inligting oor siekteveroorsakende agente te verkry.

Die eerste hoofstuk van hierdie verhandeling gee 'n oorsig van die literatuur i.v.m. die siektes van plantasiespesies in Uganda. Dit word uitgewys dat siektes as problematies beskou is van so vroeg as die 1950's en dat proewe gedoen is om siekteweerstandbiedende plantmateriaal te selekteer. As gevolg van politieke oproer is hierdie inisiatiewe gestaak en het die land afhanklik gebly van natuurlike woude vir die verskaffing van hout en houtprodukte. Huidiglik is daar 'n bewuswording dat oorbenutting die voortbestaan van natuurlike woude bedrieg, en dat plantasiebosbou die enigste alternatief bied vir die verskaffing van hout en houtprodukte. Die Uganda Bosbou Departement promofeer nou aktief die vestiging van plantasies om die vernietiging van natuurlike woude te verminder. Hierdie inisiatiewe sluit in pogings om die siektes wat bosbouplantasies in Uganda beïnvloed, te identifiseer. Vroër het ondersoeke het getoon dat siektes 'n ernstige bedreiging inhou vir die vestiging van plantasiebosbou in die land.

Botryosphaeria kanker is die mees algemene siekte van *Eucalyptus* spp. in Uganda. In hoofstuk twee van die verhandeling, wys ek dat *B. parva*, *L. theobromae* en 'n onbekende *Botryosphaeria* sp. geassosieer word met Botryosphaeria kanker van *Eucalyptus* in Uganda. Patogenisiteitstoetse het gewys dat daar statisties betekenisvolle verskille in die virulensie van die genoemde spesies is. *Lasiodiplodia theobromae* was die mins virulente spesie, terwyl die onbekende spesie die mees virulent was. Verdere ondersoek is nodig om die virulensie van hierdie swamme in die veld te toets en om hulle biologie en relatiewe belang te bepaal.

Ceratocystis albofundus is 'n belangrike patogeen van *Acacia mearnsii* en *A. decurrens* in Suid-Afrika. In 1999, is *C. albofundus* die eerste keer geidentifiseer in die suid weste van Uganda. Die resultate in die huidige studie wys daarop dat die

Ugandese populasie hoë geendiversiteit het, wat vergelykbaar is met die Suid Afrikaanse populasie van die swam. Die resultate wys verder dat die swam klonaal voortplant in beide bg. lande, met baie min genetiese vloei tussen hulle. Die hoë geendiversiteits-waardes wat in hierdie studie verkry is, wys dat *C. albofundus* inheems is aan Afrika en nie Suid-Afrika soos 'n vroeëre hipotese voorstel nie. Die hoë geendiversiteit van *C. albofundus* in die twee populasies, wys daarop dat daar gefokus moet word op die kweek van weerstandbiedende variëteite as 'n beheerstrategie. Die seleksie van weerstandbiedende variëteite is reeds aan die gang in Suid-Afrika en sal baie bruikbaar wees vir beheer van die siekte in die toekoms.

Meeste siektes op *Eucalyptys* word veroorsaak deur swamme, maar bakteriese patogene word al meer belangrik. In die vierde hoofstuk van die verhandeling word die bakterium, *Pantoea ananatis,* geïdentifiseer as die oorsaak van skroeisiekte en terugsterwing van *Eucalyptus* spesies in Uganda. Dit is die eerste aanmelding van die siekte buite Suid-Afrika. Die siekte affekteer hoofsaaklik jong boompies en is daarom 'n bron van ernstige kommer vir die vestiging van plantasies. Dit is nou nodig om proewe op te stel om plantmateriaal te selekteer wat weerstanbiedend is teen bakteriese skroeisiekte. Die bakterium infekteer ook ander landbou-gewasse. Laasgenoemde is belangrik in Uganda, aangesien bome dikwels naby aan ander landbou gewasse geplant word.

Die resultate van hierdie verhandeling sal help met die beheer van siektes van bosbouplantasies in Uganda. Die studie sal hopelik ook dien as hulpmiddel om land- en bosbouers 'n beter begrip te gee van die belang van siektes in bosbouplantasies. Dit sal ook 'n wesenlike impak hê op die kapasiteit van die mense van Uganda om plantasiesiektes te hanteer, nie net die wat veroorsaak word deur die patogene wat in hierdie verhandeling geïdentifiseer is nie, maar ook ander siektes in die bosbou- en vrugtebedrywe.