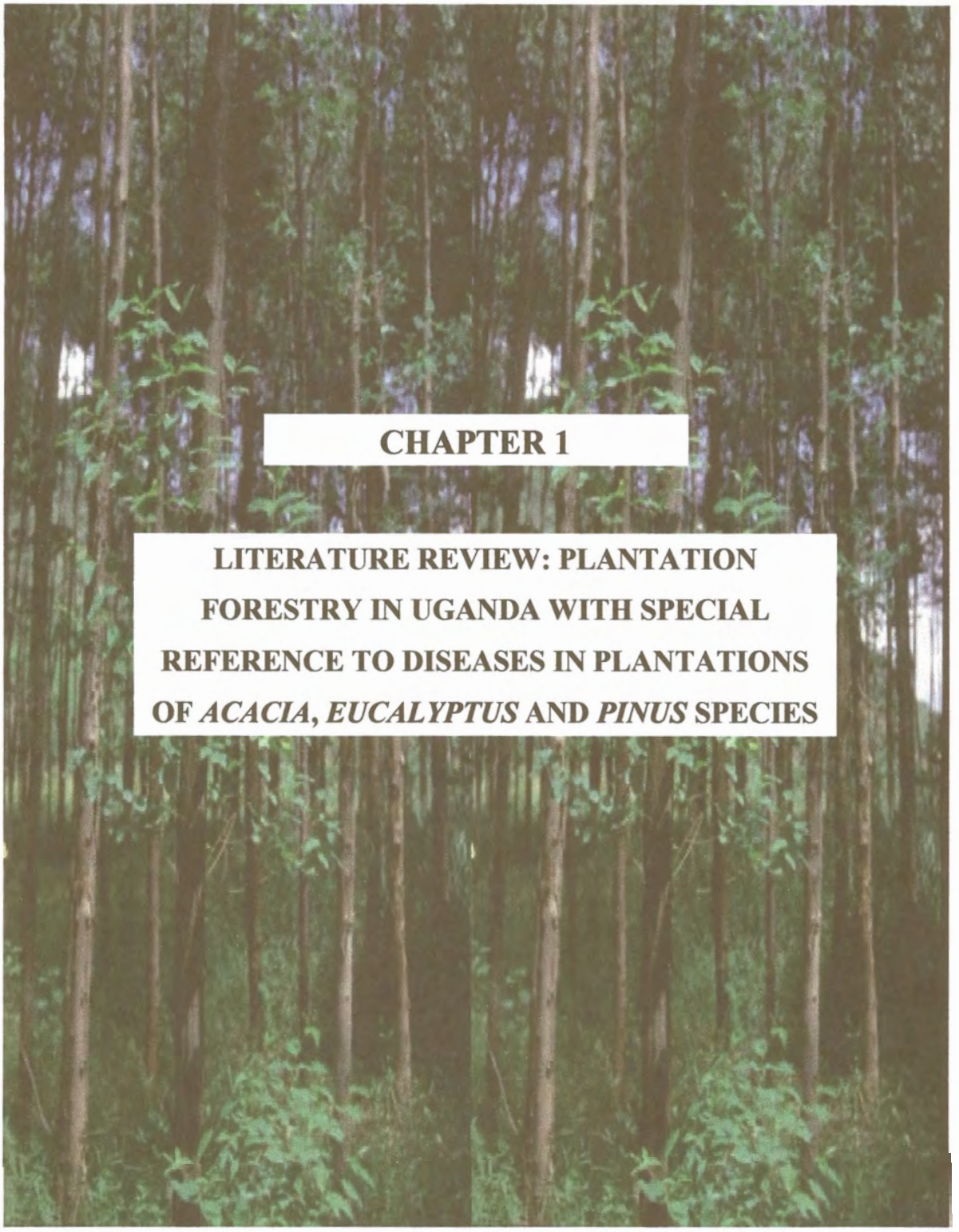

CHAPTER 1

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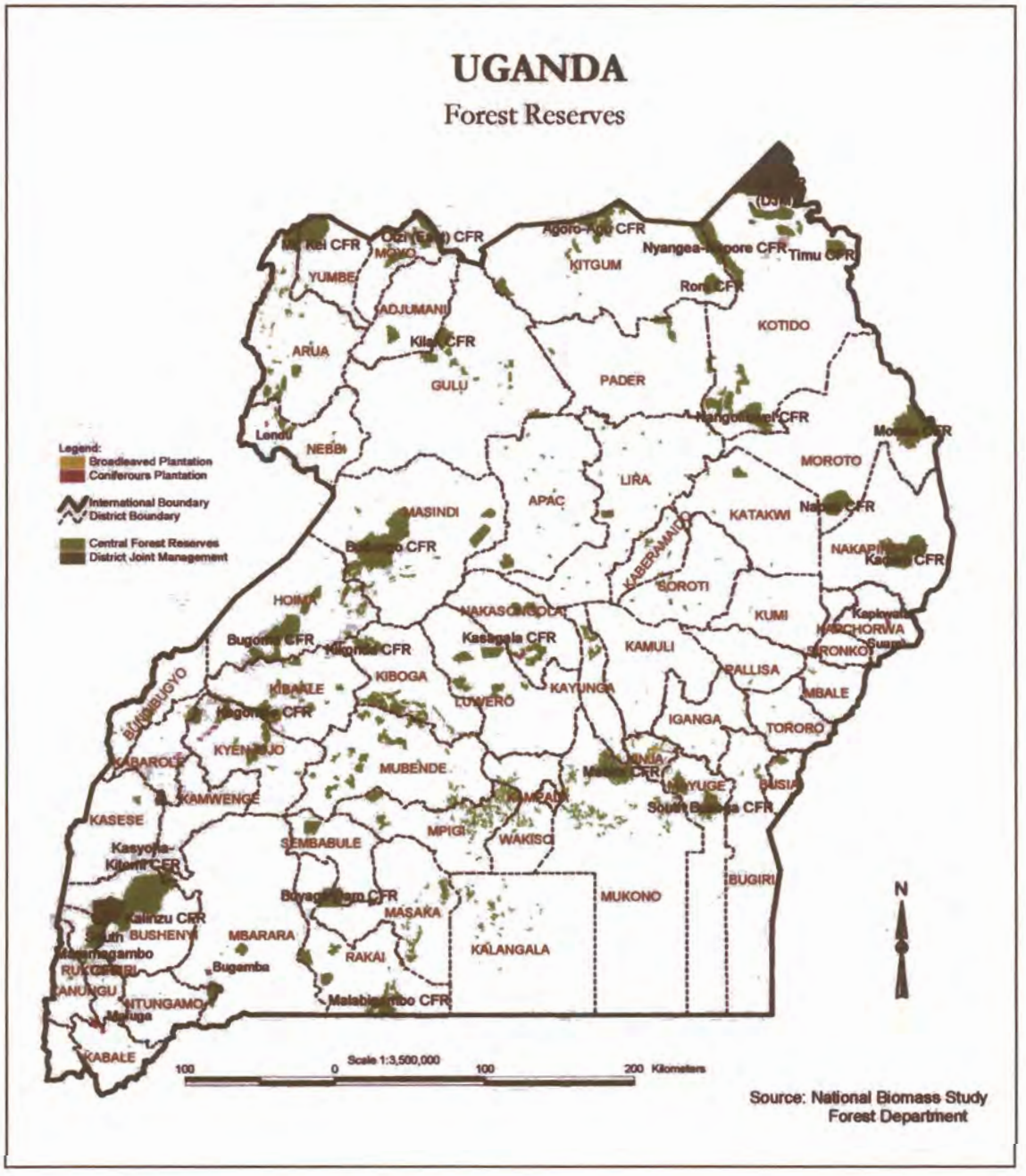
Table 1. Old and newly proposed Uganda government prices for tree poles of four different size classes (Ruyooka 1999).

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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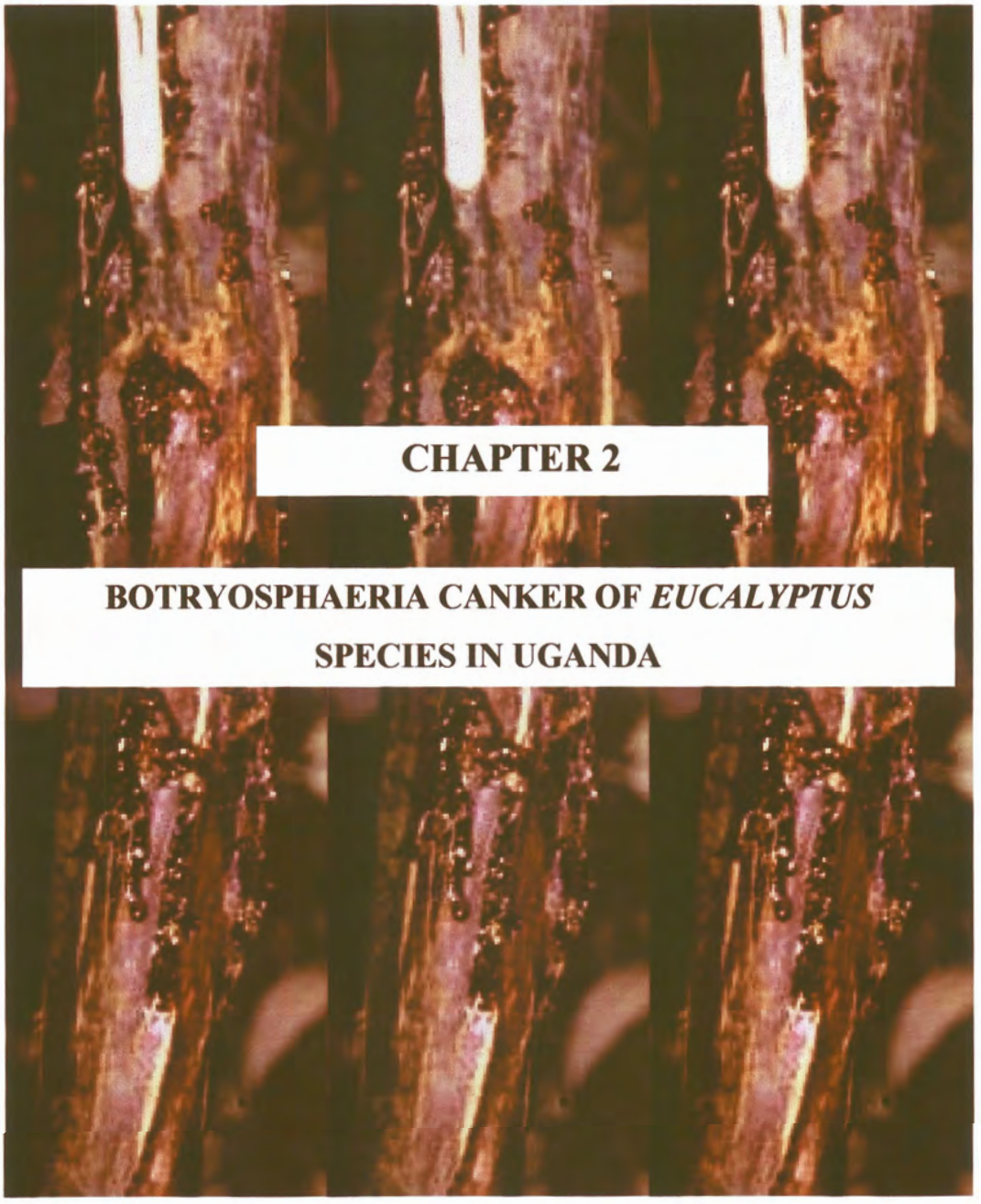

CHAPTER 2

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.

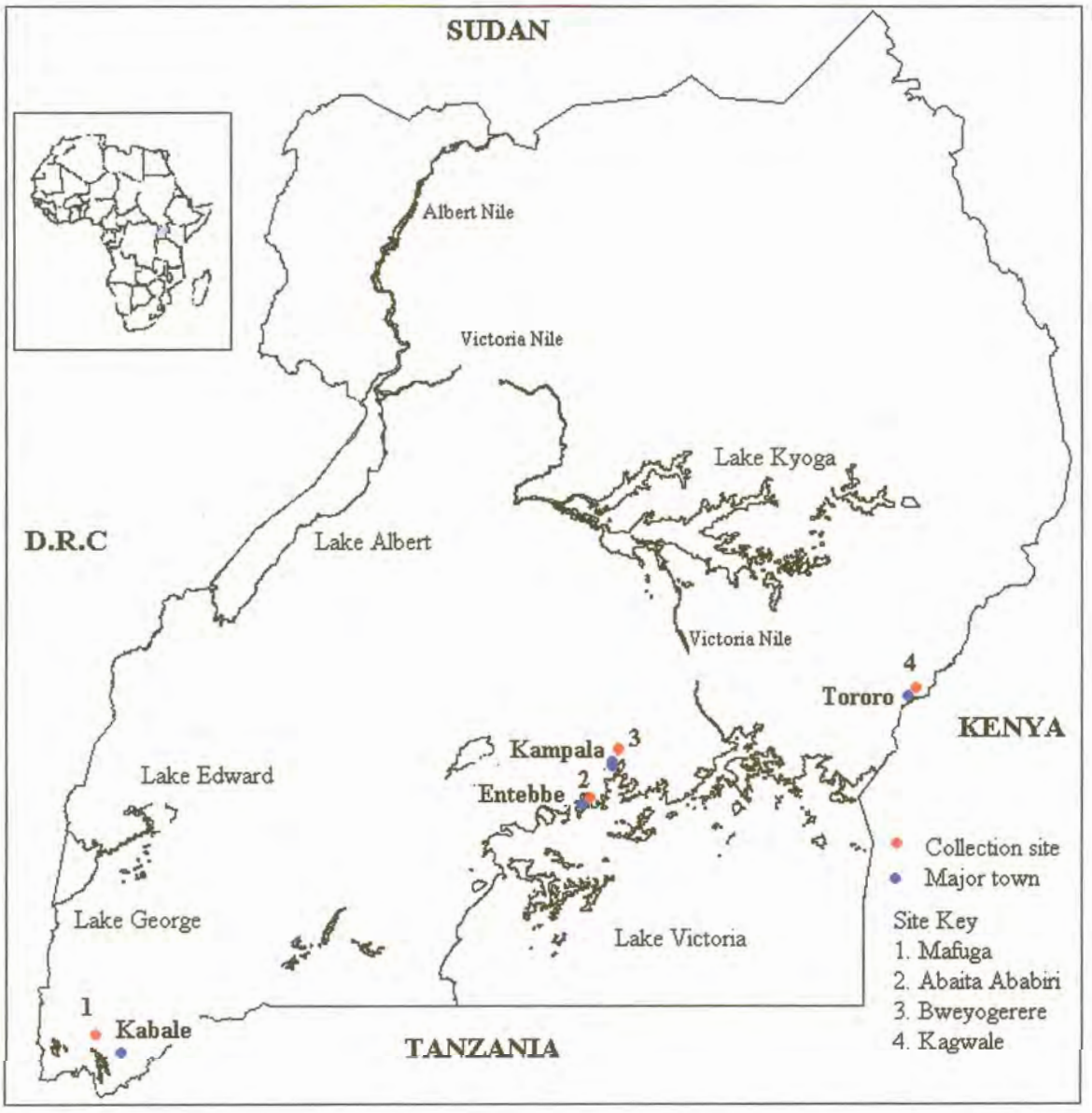
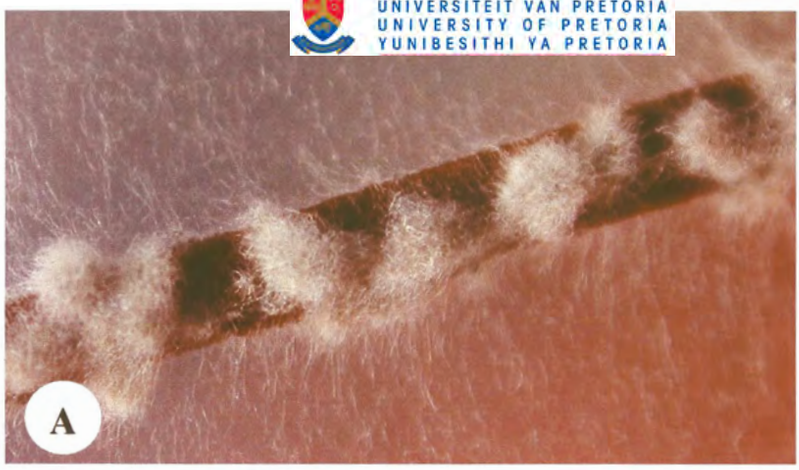
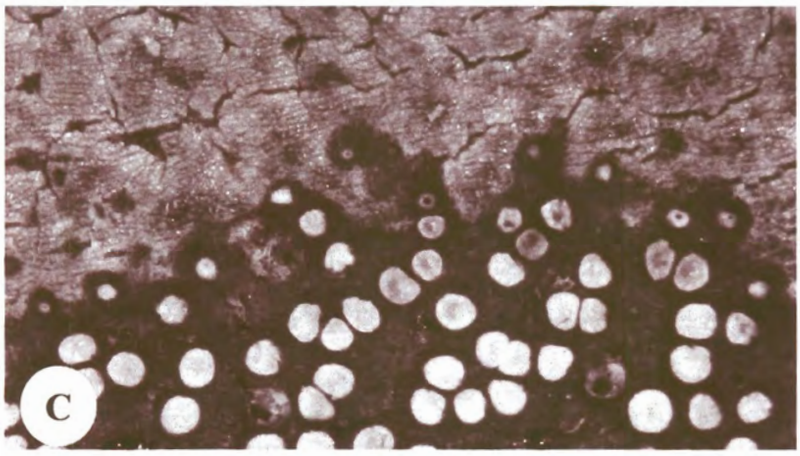

SUDAN
Albert Nile
Victoria Nile
Lake Kyoga
D.R.C
Lake Albert
Victoria Nile
4
Tororo
KENYA
Kampala
3
Lake Edward
Entebbe
2
Collection site
Major town
Site Key
1. Mafuga
2. Abaita Ababiri
3. Bweyogerere
4. Kagwale
Lake George
Lake Victoria
1
Kabale
TANZANIA

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.





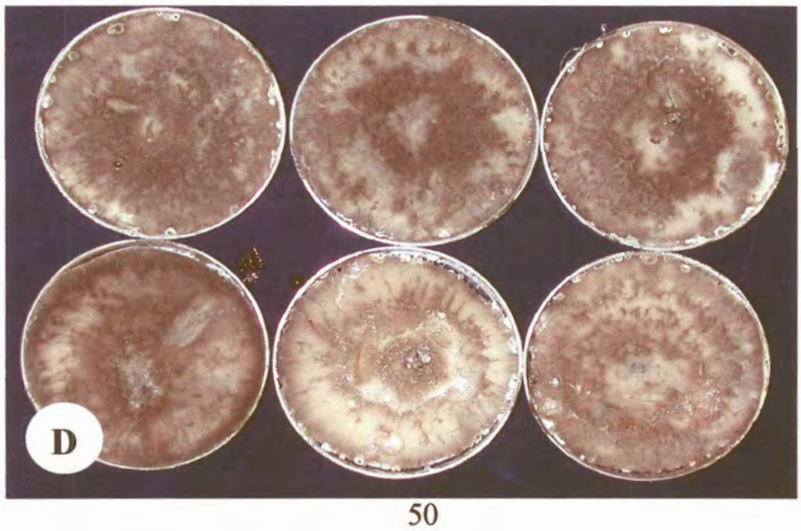


Figure 4. Morphological structures of *Botryosphaeria* spp. from *Eucalyptus* in Uganda. **A)** Teleomorph showing asci and ascospores. **B)** *Fusicoccum* state of *Botryosphaeria* sp. **C)** *Fusicoccum* state with granular appearance of conidia. **D)** *Lasiodiplodia theobromae* with typical septa.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

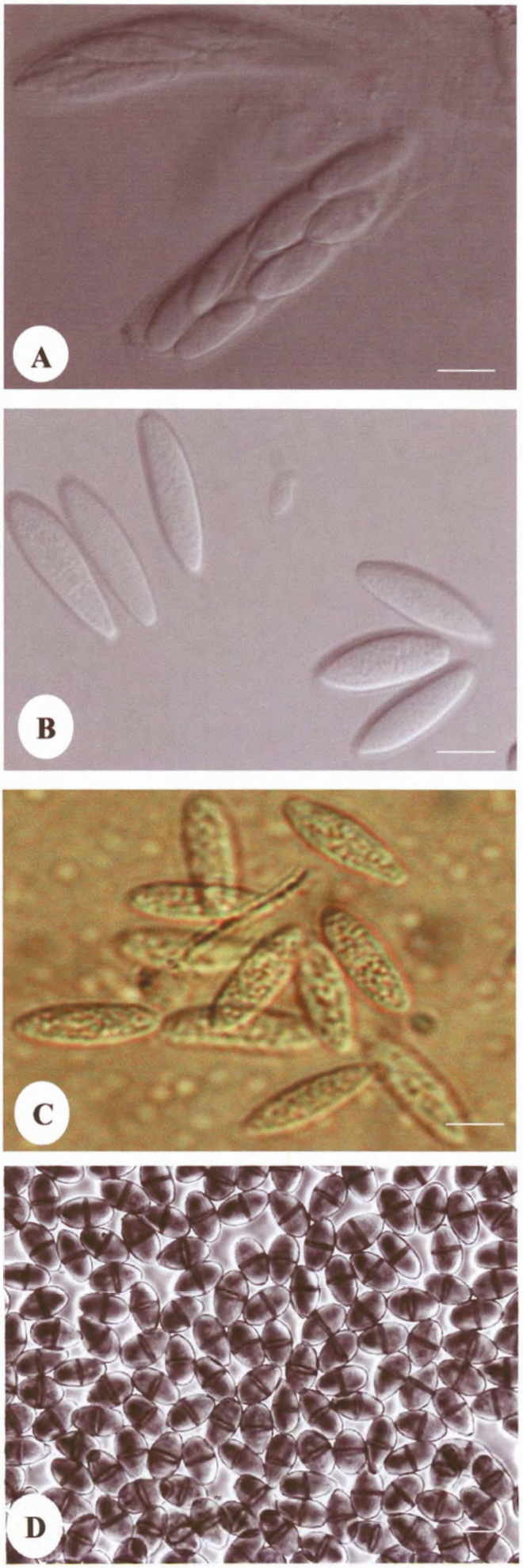
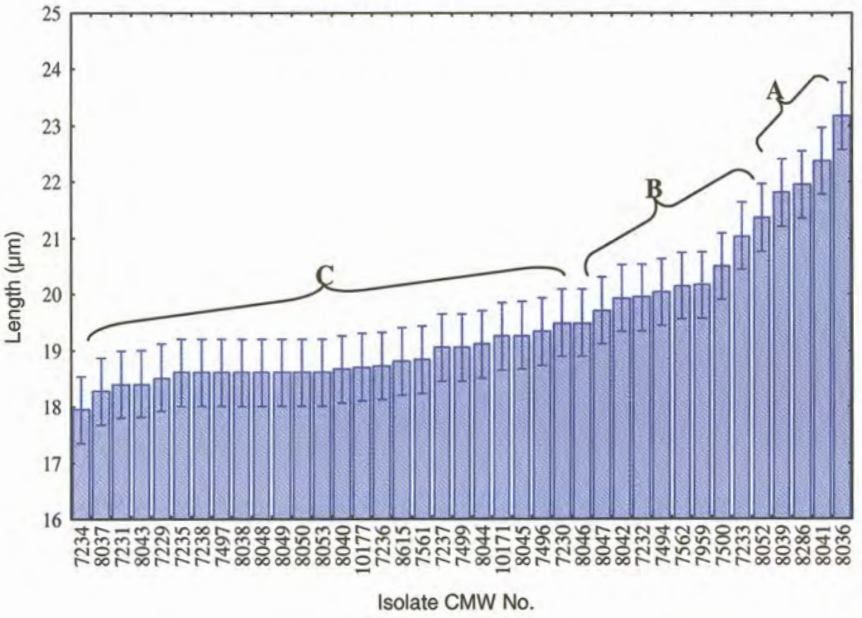

A
B
C
D

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

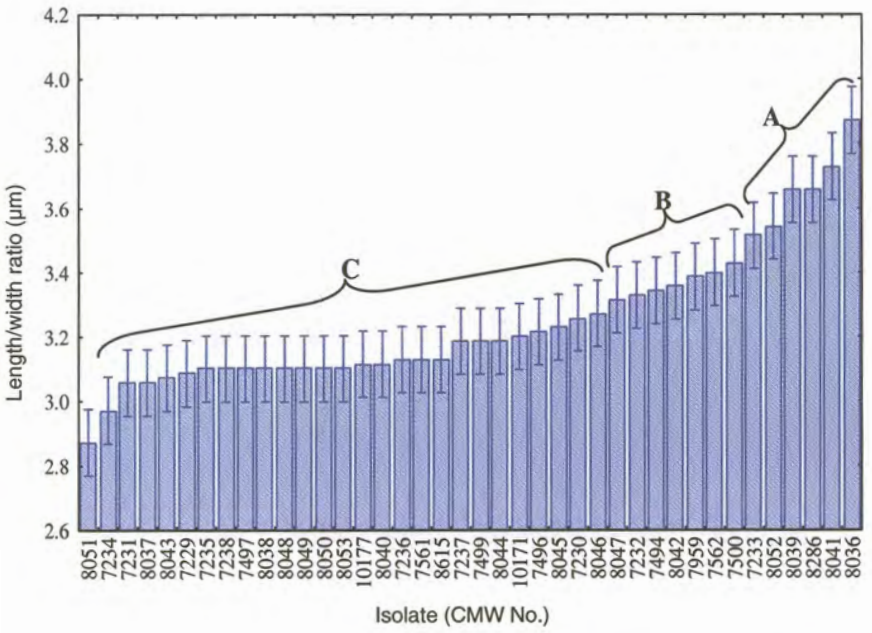

Length/width ratio (µm)
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
A
B
C
8051 7234 7231 8037 8043 7229 7235 7238 7497 8038 8048 8049 8050 8053 10177 8040 7236 7561 8615 7237 7499 8044 10171 7496 8045 7230 8046 8047 7232 7494 8042 7959 7562 7500 7233 8052 8039 8286 8041 8036
Isolate (CMW No.)

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).

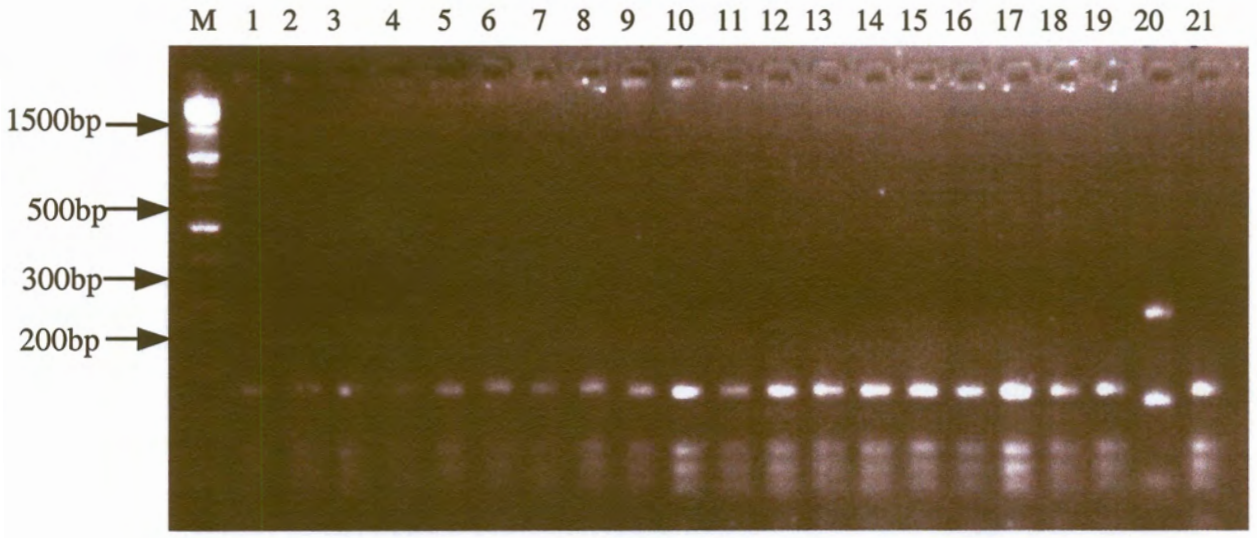

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
1500bp
500bp
300bp
200bp

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.

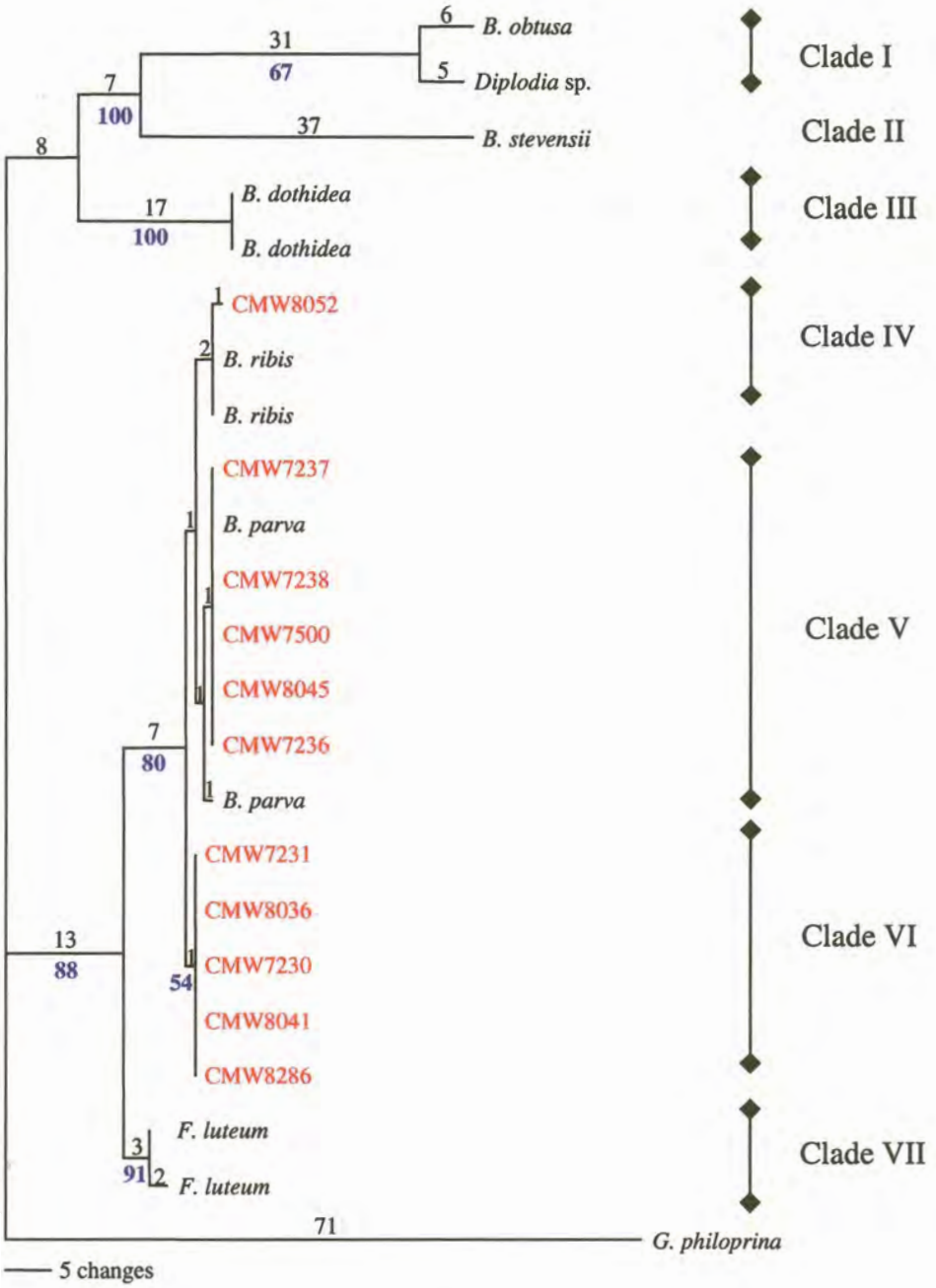


Figure 8. Most parsimonious phylogenetic tree obtained from a heuristic search of the EF1-α sequence data of *Botryosphaeria* isolates from Uganda (red font) compared to other known isolates (black font). Numbers above and below the branches are distances and bootstrap values respectively.

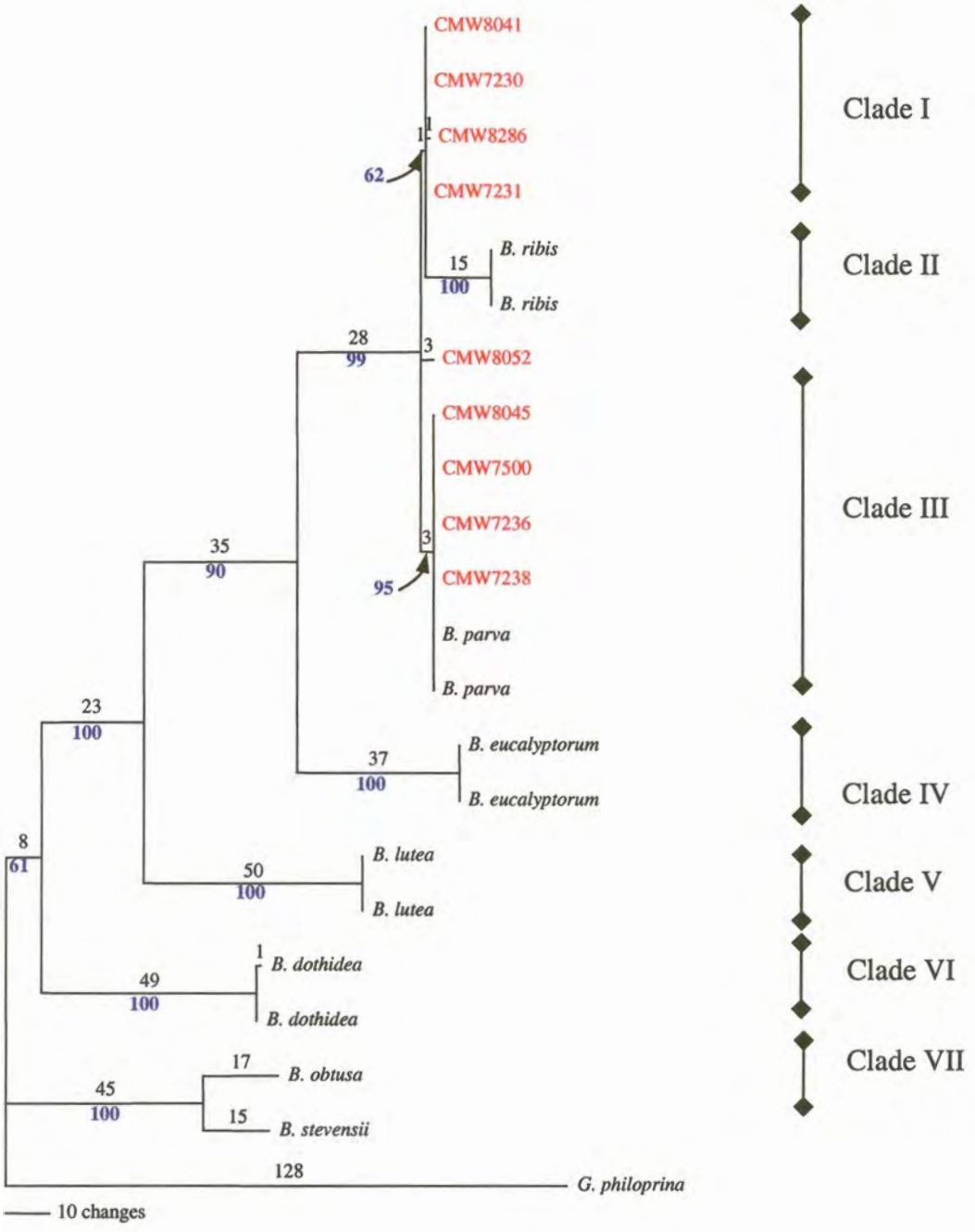

CMW8041
CMW7230
1
1
CMW8286
62
CMW7231
B. ribis
15
100
B. ribis
28
99
3
CMW8052
CMW8045
CMW7500
CMW7236
3
35
90
95
CMW7238
B. parva
B. parva
23
100
B. eucalyptorum
37
100
B. eucalyptorum
8
61
B. lutea
50
100
B. lutea
1
B. dothidea
49
100
B. dothidea
17
B. obtusa
45
100
15
B. stevensii
128
G. philoprina
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.

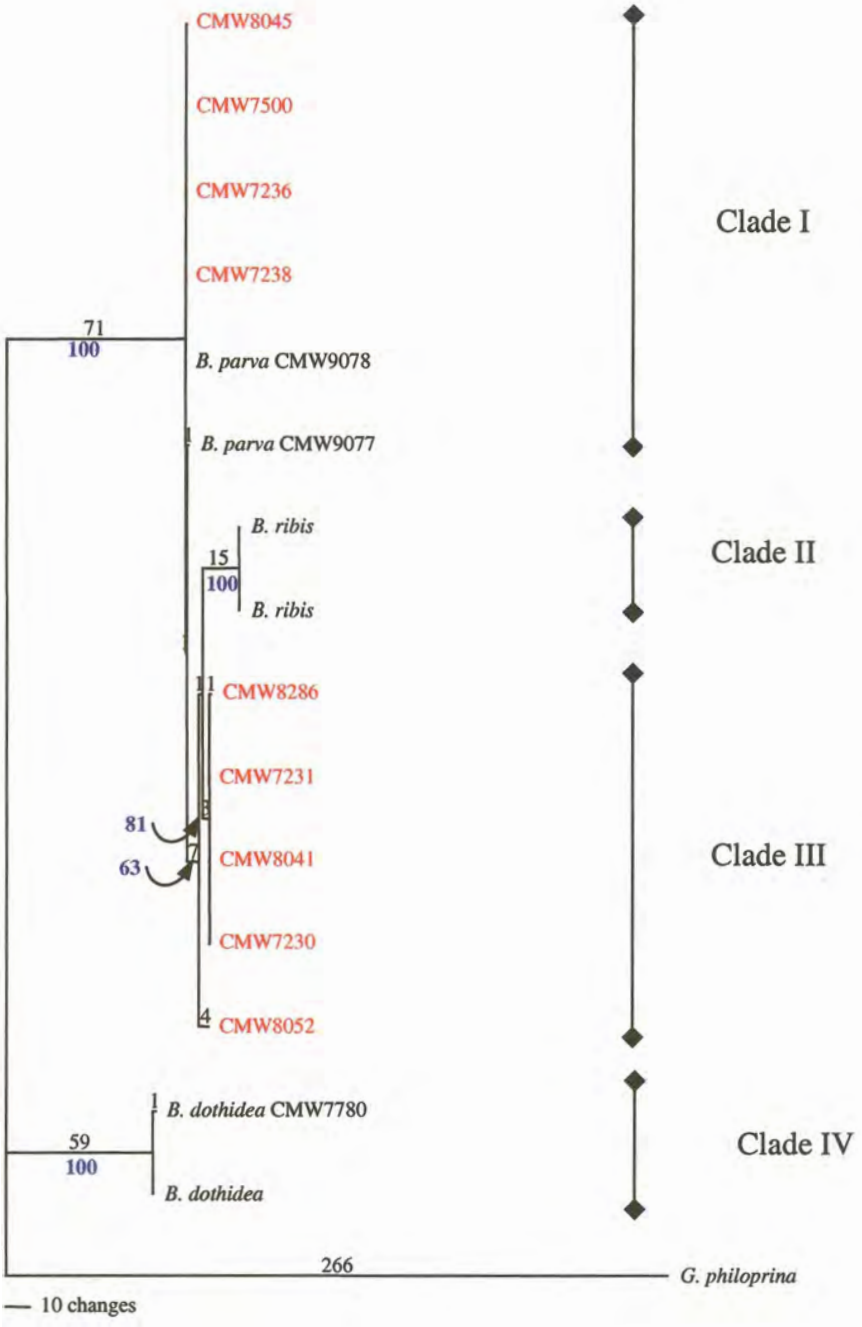

CMW8045
CMW7500
CMW7236
CMW7238
71
100
B. parva CMW9078
B. parva CMW9077
B. ribis
15
100
B. ribis
11
CMW8286
CMW7231
81
63
CMW8041
CMW7230
4
CMW8052
1
B. dothidea CMW7780
59
100
B. dothidea
266
G. philoprina
10 changes
Clade I
Clade II
Clade III
Clade IV

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).

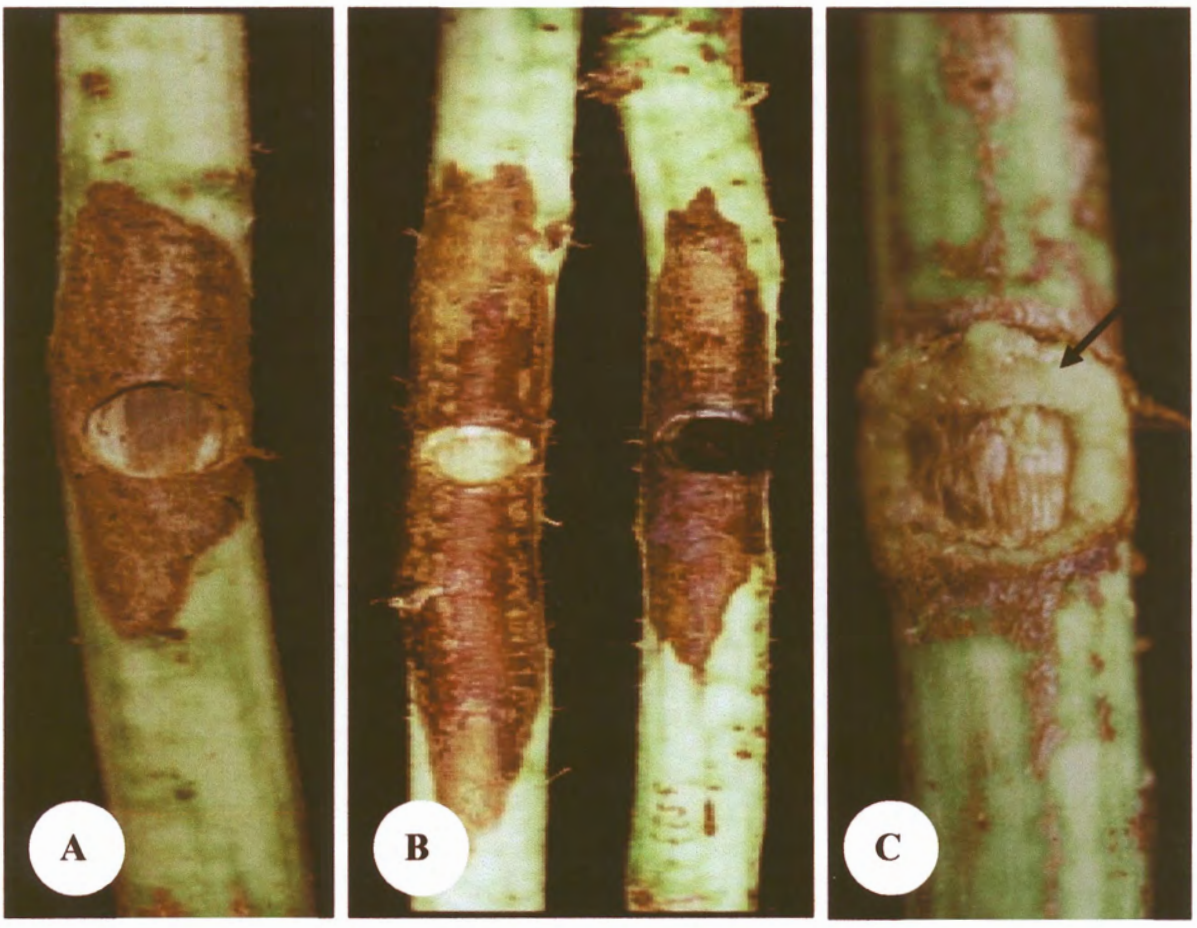
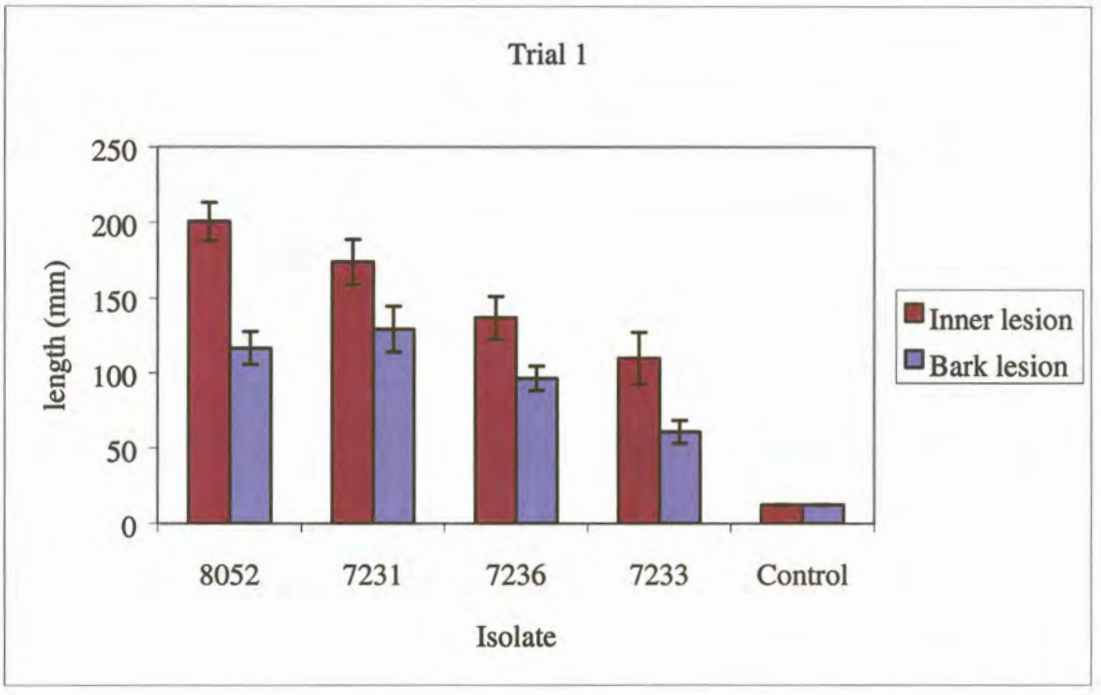

A
B
C

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

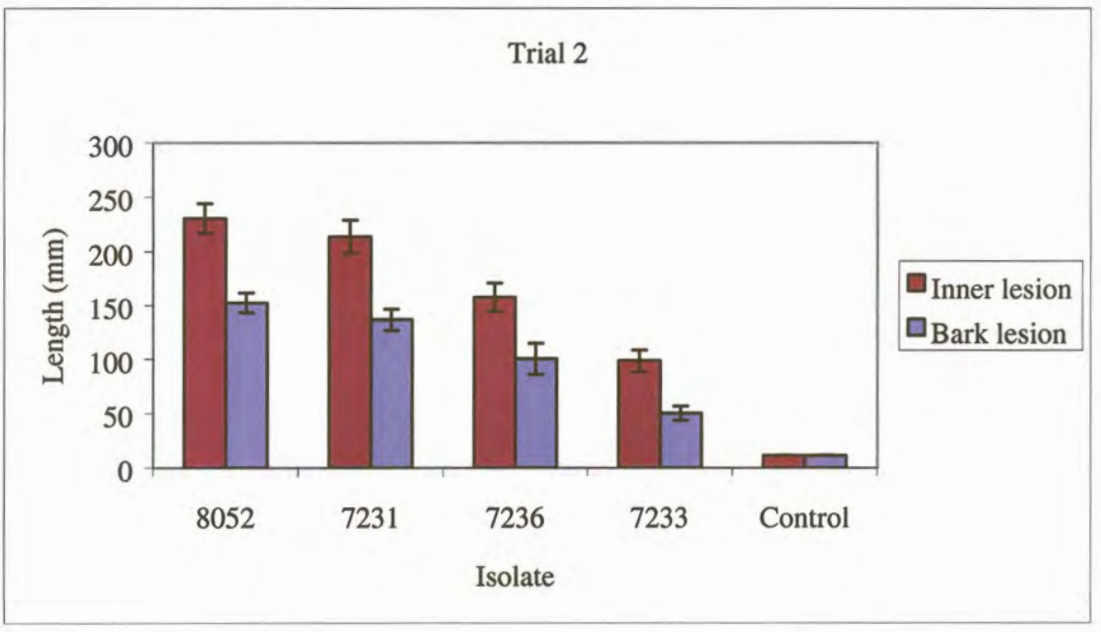

Trial 2
300
250
200
150
100
50
0
Length (mm)
8052
7231
7236
7233
Control
Isolate
Inner lesion
Bark lesion

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
```

```
                                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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