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MOLECULAR CHARACTERISATION OF *GANODERMA* SPECIES

BY

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DECLARATION

I the undersigned hereby declare that the thesis submitted herewith for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work which has hitherto not been submitted for any degree at any other university or faculty.

Vuledzani Gloria Muthelo

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PREFACE

Ganoderma species are important wood decaying fungi occurring throughout the world. Most species of *Ganoderma* are pathogenic, causing root and stem rot on a variety of monocots, dicots and gymnosperms, which results in the death of affected trees. Some species are saprophytic and cause white rot of wood. The genus is particularly diverse in the tropics where it affects plantation crops such as oil palm, coconut, rubber, betelnut, tea and forest trees such as *Acacia.* The taxonomy of the species within the genus has been considered to be in disarray due to the use of morphological characters as an identification tool. Currently, there are several synonyms, species complexes and misidentification of species within the genus.

A literature review concerning the taxonomy and characters used in identification of *Ganoderma* species is presented in Chapter One of this thesis. A brief overview of *Ganoderma* and the characters used in species identification is provided. Different characters such as morphological characters, sexual compatibility tests, as well as the molecular techniques that have been used to identify *Ganoderma* species are discussed in this chapter. The advantages and disadvantages of using such characters are discussed.

Chapter Two of this thesis deals with the identification and characterisation of *Ganoderma* species responsible for root rot disease on *Jacaranda mimosifolia* in South Africa. Identification of *Ganoderma* species has traditionally been based on the morphological features of the basidiocarps. Identification based on these features, however, is prone to problems due to the absence of basidiocarps during certain times of the year, their morphological plasticity and presence of cryptic species. For this reason,

identification of the causal species was based on a combination of both morphological characteristics and DNA sequence data. Results from this study showed that the disease is caused by a single species that resides in the *G. lucidum* complex.

The identity of the causal agent of *Ganoderma* root rot of *Acacia mangium* in Indonesia was investigated. Results from this research are presented in the final chapter of this thesis. During the time of field surveys in Indonesia, basidiocarps were not present on diseased trees. However, the trees showed typical symptoms of *Ganoderma* root rot such as reddish-brown rhizomorphic skins covering the roots as well as crown dieback, foliage discoloration, and a white mottled mycelium under the bark. The absence of basidiocarps made it impossible to identify the *Ganoderma* species that is causing the disease using morphology. Identification of the isolates collected was therefore based on DNA sequence comparisons. The results indicated that the disease is caused by *G. philippii* and that this species is a major pathogen on *A. mangium* in the surveyed area.

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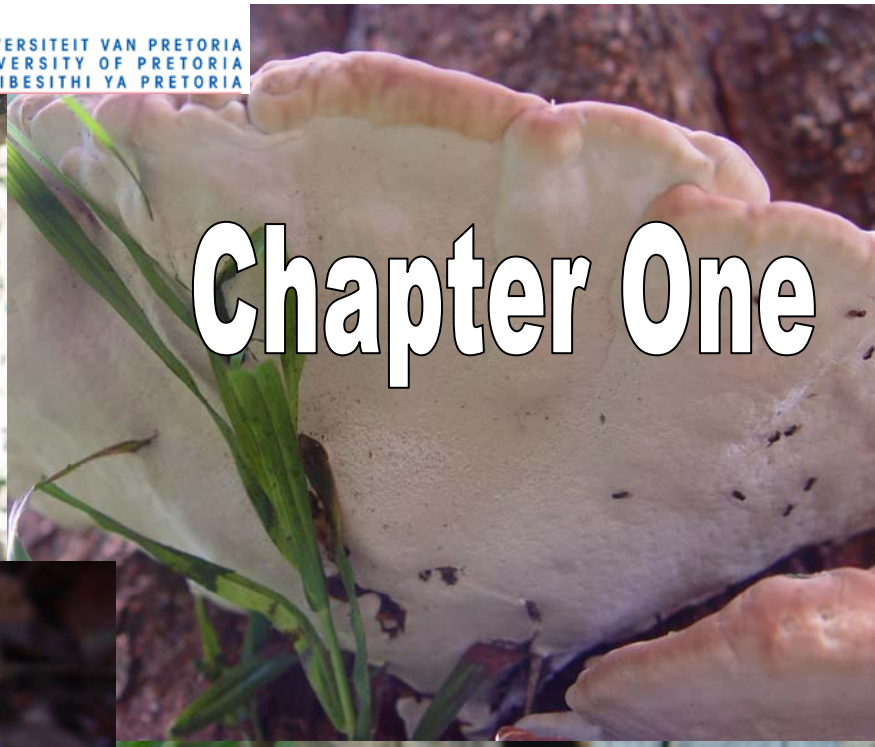

Chapter One

The root rot fungus *Ganoderma*: A review

The root rot fungus *Ganoderma*: A review

1.1 INTRODUCTION

Ganoderma is a Basidiomycete fungal genus including of both saprophytic and phytopathogenic species that occur worldwide. Most species of *Ganoderma* are pathogenic, causing root rot disease on a variety of monocots, dicots and gymnosperms (Seo and Kirk, 2000) which results in the death of affected trees. Some species act as saprophytes, causing white rot of hardwoods such as oak, maple, and ash by decomposing lignin as well as cellulose (Adaskaveg *et al.,* 1991, 1993). The genus is particularly diverse in the tropics where it affects plantation crops such as oil palm, coconut, rubber, betelnut, tea and forest trees such as *Acacia* (Chee, 1990; Singh 1991; Ariffin *et al.,* 2000; Lee, 2000; Flood *et al.,* 2000). The rot disease has become epidemic in certain regions of the world, incurring significant economic losses to forestry and the oil palm industries. For example, *G. boninense* is the major pathogen of oil palm in Malaysia and Papua New Guinea (Ho and Nawawi 1985; Pilotti *et al.*, 2003).

Although *Ganoderma* species are economically important plant pathogens that cause disease in different crops and trees, some species are well known, especially in Asian countries including China, Japan and Korea, for their medicinal value. The *G. lucidum* complex, which is known in China as *Lingzhi,* has been considered a symbol of good fortune and prosperity and has been used as a medicinal herb (Zhao and Zhang, 1994). The medicinal value of these species include treating diseases such as gastric ulcer, chronic hepatitis, hypertension, nephritis, asthma, arthritis, bronchitis, insomnia, cancer, diabetes and anorexia (Jong and Birmingham, 1992). *Ganoderma* species are also

recognised for the steroids, lignins, lectins, ganomycins, vitamins, nucleosides, nucleotides, alkaloids, amino acids and triterpenes that they produce. These compounds have antitumor, antibacterial, immuno-modulatory, anti-inflammatory, antiviral and antioxidative activities (Jong and Birmingham, 1992).

The taxonomy of the genus *Ganoderma* is considered to be in disarray (Ryvarden, 1994). This is due to the fact that basidiocarp morphological characters have been used to differentiate the species and many species within this genus share the same characteristics which make it difficult to distinguish between them (Adaskaveg and Gilbertson, 1986, 1988; Gottlieb and Wright, 1999a, b; Smith and Sivasithamparam, 2003; Pilotti *et al.*, 2004). Several studies have used different alternative methods such as cultural characters (Adaskaveg and Gilbertson, 1989), sexual compatibility tests (Adaskaveg and Gilbertson, 1986; Pilotti *et al.*, 2002, 2003), isozyme analysis (Gottlieb *et al*., 1998; Smith and Sivasithamparam, 2000b) and DNA-based techniques (Moncalvo *et al*., 1995a, b; Gottlieb *et al*., 2000; Smith and Sivasithamparam, 2000a; Hong and Jung, 2004) to determine the identity of *Ganoderma* species. Consequently, molecular techniques have become a powerful diagnostic tool to elucidate the taxonomy of *Ganoderma* species. Application of these techniques has resolved some of these taxonomic problems but names in the genus are still poorly understood.

The studies presented in this dissertation deal with the identification and characterisation of *Ganoderma* species from infection sites. This review provides a brief overview of *Ganoderma* as well as the taxonomic characters used in identification and characterisation of species within this genus.

1.2 THE GENUS *GANODERMA*

The genus *Ganoderma* was introduced by Finnish mycologist Peter Adolf Karsten in 1881 (Karsten 1881), with *G. lucidum* (Curtis:Fr.) P. Karst from England as the type species. The genus belongs to the family Ganodermataceae that resides in the order Polyporales of the Basidiomycetes. The family includes eight genera that are distinguished by their unique double-walled basidiospores. The genus *Ganoderma* was further subdivided into two subgenera: subgenus *Ganoderma* based on *G. lucidum* for the laccate species and subgenus *Elfvingia* based on *G. applanatum* for the species with a non-laccate fruiting body (Moncalvo and Ryvarden, 1997). Thus *G. lucidum* and *G. applanatum* are the two important species complexes in the history and nomenclature of the genus. These are two of the most poorly understood species of *Ganoderma* and most frequently with misapplied names (Seo and Kirk, 2000).

Ganoderma species are important wood decaying fungi occurring throughout the world. They are diverse in the tropics affecting plantation crops such as oil palm (Singh 1991; Ariffin *et al.,* 2000; Flood *et al.,* 2000; Pilotti *et al.,* 2003; Pilotti, 2005) and they also affect ornamental and forest trees in tropical and temperate areas causing disease and wood rots of timber (Lee, 2000). These fungi cause white-rot of hardwoods through delignification (Adaskaveg *et al.,* 1991, 1993; Schwarze *et* al., 1995). In the early stages of decay caused by *Ganoderma* species, bleached zones usually appear in the wood, as a result of selective delignification. As the decay progresses, the wood becomes softer and loses tensile strength until a late stage where the wood disintegrates and becomes soft or spongy (Schwarze and Ferner, 2003).

Species of *Ganoderma* cause root and stem rot disease resulting in losses of crops such as oil palm, rubber and other trees worldwide (Chee, 1990; Singh 1991; Ariffin *et al.,* 2000; Lee, 2000; Sankaran *et al.*, 2005). Presence of the fungus can easily be detected when fruiting bodies (basidiocarps) appear on the stem of a tree. *Ganoderma* is spread primarily through the basidiospores (Pilotti *et al*., 2003; Pilotti, 2005; Sanderson, 2005) or by root to root contact among trees (Turner, 1965; Ariffin *et al.,* 2000). The spores become incorporated into the soil, germinate and then the hyphae grow over the roots. The fungus simply moves from the roots to the woody trunk tissue where it destroys the wood affecting the xylem (water conducting tissue). Primary symptoms that may be observed in infected trees include a mild to severe wilting of leaves, loss of foliage, and die-back of some branches. Depending on the environmental conditions, infected trees die gradually (Schwarze and Ferner, 2003).

More than 250 *Ganoderma* species have been described worldwide, and most of these descriptions have been based on pleomorphic characters (Ryvarden, 1994). However, uncertainty exists about the taxonomic status of many of these species, and the taxonomy of the genus is thus considered to be chaotic and one of the most difficult genera amongst the polypores (Ryvarden, 1994). Such taxonomic problems stem from the misuse of names, absence of type specimens, the large number of synonymies and differences in morphological characters that are often tenuous (Ryvarden, 1994; Smith and Sivasithamparam, 2003). Due to such problems, Ryvarden (1994) has proposed that no new species be described in *Ganoderma* in the decade to 2005. For these reasons, studies have focused on developing methods to determine the identity of *Ganoderma* species. These include cultural characters (Adaskaveg and Glibertson, 1989), sexual compatibility studies (Adaskaveg and Gilbertson, 1986; Pilotti *et al.,* 2002; 2003),

isozyme studies (Gottlieb *et al*., 1998; Smith and Sivasithamparam, 2000b) and DNA-based techniques (Moncalvo *et al,* 1995a, b; Smith and Sivasithamparam, 2000a; Gottlieb *et al,* 2000; Hong and Jung, 2004). These techniques have played an important role in elucidating the taxonomy of *Ganoderma* since problems such as synonymies and misidentification of species was largely reduced.

1.3 TAXONOMIC CHARACTERS USED TO IDENTIFY *GANODERMA* SPECIES

The taxonomy of basidiomycetes has traditionally been based on the morphological features of the basidiocarps. Identification based on these basidiocarp features, however, is prone to problems such as absence of basidiocarps during certain times of the year, their morphological plasticity and presence of cryptic species (Moncalvo and Ryvarden, 1997; Gottlieb and Wright, 1999a, b). For these reasons, contemporary taxonomy and identification of *Ganoderma* species employ morphological studies, mating tests, analyses of biochemical and DNA sequence information, or combinations of these.

1.3.1 Morphological characters

Basidiocarps, which are the sexual structures in *Ganoderma* and other polypores, grow from a living, or more commonly, from a dead trunk or branch of a tree in the form of bracket (Figure 1). One of two types of basidiocarps is produced, depending on the species: a laccate fruiting body (Figure 1A) with a shiny upper surface, or a non-laccate (Figure 1B) fruiting body with a dull upper surface. Historically samples with a laccate pileus were placed in the *G. lucidum* complex, within the sub-genus *Ganoderma*. Those

with non-laccate pilei were grouped in the *G. applanatum* complex, sub-genus *Elfvingia* (Moncalvo and Ryvarde, 1997).

Basidiome features such as the size, context colour, shape of the brackets as well as host specificity and geographical distribution have often been used in the taxonomy of *Ganoderma* species (Furtado, 1962; Bazzalo and Wright, 1982; Gottlieb and Wright, 1999a, b; Smith and Sivasithamparam, 2003; Pilotti *et al*., 2004). However, a number of studies have demonstrated that basidiocarp morphology should be used with caution due to its plasticity and pleomorphic nature in some species (Steyaert, 1975; Ryvarden, 1994, 2000).

Basidiocarps of *Ganoderma* species have various morphological characteristics such as sessile, stipitate, imbricate and non-imbricate (Adaskaveg and Gilbertson, 1988; Seo and Kirk, 2000). The attachment of the stipe to the pileus as well as host range varies with species. Normally the pileus is laterally attached to the stipe, but eccentric, central, imbricate, and sessile fruit bodies are also produced (Seo and Kirk, 2000). The colour of the pileus surface varies from deep red, laccate, non-laccate and yellow to white. The laccate character of the pileus does not play an important role in the segregation of the species within the genus but it has been widely employed and remains available as an identification aid. Context colour varies from white to deep brown and may change with the age of the fruit body or upon drying. The morphology of the basidiocarps may differ between the isolates due to different environmental conditions during development (Seo and Kirk, 2000). Table 1 summarises basidiocarp morphological characteristics of different *Ganoderma* species from North America (Adaskaveg and Gilbertson, 1988) to exemplify character differences among species.

Micro-morphological characters of the basidiocarps such as the size and morphology of basidiospores, type of hyphal system, as well as the structure of the pileal crust/ cuticle surface have been used in the taxonomy of *Ganoderma* (Adaskaveg and Gilbertson, 1986; Gottlieb and Wright, 1999a, b; Smith and Sivasithamparam, 2003). The family Ganodermataceae is characterised by unique double walled basidiospores. Basidiospores of *Ganoderma* species are brown in colour, ovoid or ellipsoid-ovoid, occasionally cylindric-ovoid in shape with a truncated apex (Seo and Kirk, 2000; Figure 2). The size and morphology of basidiospores have been considered to be the most important characteristic micro-morphological feature for the taxonomy of *Ganoderma* (Furtado, 1962; Seo and Kirk, 2000). Differences in basidiospore morphology have been reported for different species within this fungal genus (Adaskaveg and Gilbertson, 1986, 1988; Gottlieb and Wright, 1999a, b; Smith and Sivasithamparam, 2003). However, Steyaert (1972, 1975) noted that the basidiospore size of some species, e.g. *G. tornatum,* varies with humidity and geographical latitude. Due to that, Ryvarden (1994) stated that the size of the basidiospores should be used carefully in distinguishing *Ganoderma* species.

The hyphal system in the Ganodermataceae is usually trimitic and some occasionally dimitic with the generative hyphae hyaline, thin walled, branched, clamped and septate or not. Clamp connections are difficult to observe in dried specimens but are easily observed in the context of fresh specimens and young parts of fresh hymenium. This character is not important in separating *Ganoderma* species because the hyphal system in most species is consistently trimitic and they are also influenced by environmental factors (Ryvarden, 1994; Seo and Kirk, 2000).

The structure of the pileal crust or cuticle surface has been used in the taxonomy of *Ganoderma* (Furtado, 1965; Adaskaveg and Gilbertson, 1989; Gottlieb and Wright, 1999a, b). Fruitbodies of *Ganoderma* species mostly have hymenioderm or characoderm pileal crust while *Elfivingia* species have a trichoderm or an irregular tissue. Some authors who have used cuticle anatomy as the criteria for the taxonomy of *Ganoderma* have failed to define unambiguously the observed structures (Furtado, 1965; Adaskaveg and Gilbertson, 1989) and this contributes to the difficulty of studying these fungi, especially using this characteristic.

The sole use of basidiocarps macro- and micro-morphology in the taxonomy of *Ganoderma* species has resulted in many synonyms, species complexes and possible misidentifications of species (Steyaert, 1972; Bazzalo and Wright, 1982; Adaskaveg & Gilbertson, 1986). This is because different species often share the same basidiocarp characteristics which make it difficult to distinguish between them. Therefore such species appear similar while they represent different species. For example, *G. tsugae* was considered to be a synonym of *G. lucidum* based on basidiospore morphology and host specificity (Steyaert, 1972). Later studies, however, have shown that these are not only separate species, but also that *G. lucidum* does not occur in North America (Steyaert, 1980).

The major disadvantage of using basidiocarps morphology in species identification is that they appear seasonally and in fast grown plantations basidiocarps may be absent on diseased trees (Lee 2000) which makes identification of the fungus very difficult or impossible. Most basidiocarp characters are affected by environmental conditions during development which result in high level of intra-species variation in some species caused

by this morphological plasticity. This adds to the disadvantages of using such characters in species identification as it causes confusion and misidentification of such species. For example, the fruiting bodies of *G. applanatum* and *G. australe* from Europe have been confused with each other (Leonard, 1998). Overlapping morphological characters have been observed in species such as *G. lucidum* and *G. tsugae*, which, as a result, cannot be differentiated based on these features (Steyaert, 1972).

1.3.2 Cultural characters

In addition to basidiocarp morphology, cultural characteristics such as chlamydospore production, growth rate and thermophily have been used to differentiate *Ganoderma* species (Adaskaveg and Gilbertson, 1986, 1989). The culture colony of *Ganoderma* species is white to pale yellow and even, felty to floccose and they become more yellowish under the exposure to light. The cultures grow at different optimum temperatures depending on the species. *Ganoderma* species produce various hyphal structures in culture such as generative hyphae with clamp connections, fibre or skeletal hyphae, stag-horn hyphae, cuticular cells and vesicles, and hyphal rosettes as well as chlamydospores (Adaskaveg and Gilbertson, 1989; Figure 3). Chlamydospore production, growth rate and thermophily of the cultures are the most cultural characters that have been used for distinguishing *Ganoderma* species (Adaskaveg and Gilbertson, 1989; Seo and Kirk, 2000).

Most researchers have attempted the use of cultural characteristics to differentiate *Ganoderma* species. For example, North American and European isolates of species in the *G. lucidum* complex were differentiated based on cultural characteristics.

Ganoderma lucidum and *G. tsugae* from North America have different cultural morphologies; *G. lucidum* produces chlamydospores while *G. tsugae* does not. Consequently, these were considered to be different species (Adaskaveg and Gilbertson, 1986). In culture, *G. resinaceum* and *G. valesiacum* from Europe appear to be similar to *G. lucidum* and *G. tsugae*, respectively. As a result, *G. resinaceum* was considered to be a synonym to *G. lucidum*, and *G. valesiacum* to *G. tsugae* (Adaskaveg and Gilbertson, 1986). Adaskaveg and Gilbertson (1989) reported that *G. colossum, G. zonatum, G. oregonense, G. meridithiae*, *G. lucidum* and *G. tsugae* could be distinguished from each other based on cultural characteristics. The only exceptions to this were *G. tsugae* and *G. oregonense*, which shared similar features in culture. These examples illustrate that the sole use of cultural morphology in the taxonomy of *Ganoderma* can lead to flawed conclusions and has caused more confusion as most of them were different from identifications based on morphological characters.

1.3.3 Sexual compatibility tests

Sexual compatibility tests have been used in the taxonomy of various fungi, including *Ganoderma* (Adaskaveg and Gilbertson, 1986; Pilotti *et al,* 2002, 2003). These tests are used to determine if two isolates belong to the same biological species, and/or to establish the identity of unknown isolates, assuming known tester strains are available for a particular biological species. Two isolates that successfully exchange nuclei are sexually compatible and are considered to belong to the same biological species (Adaskaveg and Gilbertson, 1986). At least one of the two isolates used in a compatibility test must be haploid while the other could be diploid or dikaryotic (Adaskaveg and Gilbertson, 1986). The best results, however, are obtained between haploid isolates.

Sexual compatibility between isolates of *Ganoderma* is determined by the absence or presence of a demarcation line between strains paired on a culture medium. The sexual system of some *Ganoderma* species has been shown to be tetrapolar (i.e., these species have different mating type alleles at two unlinked loci) and heterothallic (Adaskaveg & Gilbertson, 1986; Pilotti *et al.*, 2002, 2003). Therefore, three different mating interactions (Figure 4) can be observed when haploid isolates are paired: weak antagonism (Figure 4A) indicating a compatible reaction; and medium (Figure 4B) or strong antagonism (Figure 4C) indicating semi compatible or incompatible reactions, respectively (Pilotti *et al*., 2002).

Sexual compatibility tests have been useful in some taxonomic studies on *Ganoderma*. Adaskaveg and Gilbertson (1986) found that homokaryons of *G. resinaceum* from Europe were interfertile with homokaryons of *G. lucidum* from North America. Consequently, they considered *G. resinaceum* to be synonymous with *G. lucidum. Ganoderma tsugae* and *G. lucidum* were found to be bifactorial (i.e., they had a heterothallic tetrapolar mating system), which confirmed that the two were separate species (Adaskaveg and Gilbertson, 1986).

The use of sexual compatibility tests alone for the identification of *Ganoderma* species has several limitations. Tester strains of known identity are needed in order to identify species. It is very difficult to obtain haploid strains in *Ganoderma* because of the low percentage of basidiospore germination *in vitro* (Adaskaveg and Gilbertson, 1986, 1987). It is also possible for phylogenetically distinct species to be sexually compatible, complicating their identification using sexual compatibility tests. For example, North American *G. lucidum* and the European isolate of *G. resinaceum* belong to the same

biological species (Adaskaveg and Gilbertson, 1986), though later studies showed that these are phylogenetically distinct species (Moncalvo *et al*., 1995a, b; Hong and Jung, 2004).

1.3.4 Protein and DNA based characters

Protein and DNA-based characters have been used widely in the taxonomy of fungi at various levels (Bruns *et al.,* 1991; Mitchell *et al*., 1995). Analyses of isozyme and protein profiles (Bonde *et al*., 1993; Harrington *et* al., 1996; Mwenje and Ride, 1996, 1997); DNA–DNA hybridization (Bruns *et al*., 1991); restriction fragment length polymorphisms (RFLPs) of ribosomal DNA (rDNA), mitochondrial DNA (mtDNA), and PCR-amplified regions (Bruns *et* al., 1991; Bunyard *et al.,* 1996; Nicholson *et al*., 1997; Gomes *et al.,* 2000); as well as nucleotide sequence data (Bruns *et al*., 1991) have been used to differentiate taxa. Of these, analyses of isozymes, PCR-RFLPs and nucleotide sequences have been employed in the taxonomy of *Ganoderma*.

1.3.4.1 Isozymes

Isozymes were first described by Hunter and Markert (1957), who defined them as variants of the same enzyme that have different amino acid sequences, but identical functions, and are present in the same organism (Hunter and Markert, 1957; Bonde *et al.,* 1993). Isozymes usually result from gene duplication, but can also arise from polyploidisation or nucleic acid hybridization (Hunter and Markert, 1957). Isozymes have been widely used as molecular markers in taxonomic studies in fungi (Bonde *et al.,* 1993; Harrington *et al*., 1996; Mwenje and Ride, 1996, 1997; Georgios *et al.*, 2001).

Isozyme analysis involves separating total protein extracts using gel electrophoresis, where differences in electrophoretic profiles indicate genetic variability. Similar profiles indicate isolates of the same species, while isolates of different species produce profiles that are different (Bonde *et al.,* 1993). Numerous loci can be detected using this technique and a large number of isolates can be screened. It is also less expensive than conventional immunological tests. Co-dominance of enzyme expression is also an advantage of the technique (Bonde *et al*., 1993; Smith and Sivasithamparam, 2000b).

Isozyme analyses have been used in taxonomic studies of *Ganoderma* species, where cellulose acetate gel electrophoresis (CAGE) or polyacrylamide gel electrophoresis (PAGE) have been used. Smith and Sivasithamparam (2000b) successfully used this technique to distinguish amongst Australian species. These authors differentiated among three laccate Australian species, *G. weberianum, G. cupreum,* and *Ganoderma* sp., based on PAGE profiles of their pectic isozymes. However, the non-laccate *G. australe* and *G. incrassatum* could not be distinguished from each other using these isozymes. CAGE profiles of isozyme glucose-6-phosphate dehydrogenase were used to resolve species identities of Australian *Ganoderma* isolates, and based on this; the authors concluded that CAGE is a simple diagnostic tool that can be used to identify *Ganoderma* species with a small number of isozyme loci (Smith and Sivasithamparam, 2000b).

The relationship of laccate and non-laccate *Ganoderma* species from South America has been examined by horizontal PAGE using eight enzyme systems codified for the presence or absence of bands (Gottlieb *et al*., 1998). The results of that study were inconclusive in resolving species boundaries. Gottlieb *et al*. (1998) concluded that some of the specimens studied may be very closely related species, and therefore, suggested

that a combination of mating tests, morphological and isoenzymic characters need to be employed for the classification of species within *Ganoderma*.

Isozyme analyses as an aid to the taxonomy of *Ganoderma* have some limitations. For example, isolates of the *G. lucidum* complex share the banding patterns of some enzyme systems with isolates of *G. applanatum* complex rendering identification difficult (Gottlieb *et al.,* 1998). This may be a reflection of the fact that proteins do not reveal much variation at lower taxonomic levels. Introns, which house most of the variability in genes, are spliced out during transcription (Tourasse and Kolstø, 2008). Thus much of the variability in genes is lost in proteins. Proteins with different amino acid sequences may not necessarily differ in their electrophoretic mobility due to neutrality of some amino acids in an electric field. Moreover, identical amino acid sequences may not imply identical DNA sequences as third base differences in codons do not necessarily result in changes in amino acid residues due to degeneracy of the genetic code. As such, similar bands may have different amino acid sequences. The fact that a large amount of fresh material is needed for isozyme analysis is an added disadvantage of the technique (Bonde *et al.,* 1993; Smith and Sivasithamparam, 2000b).

1.3.4.2 PCR-RFLPs

Techniques that explore nucleotide variation among isolates have been useful in the systematic and phylogenetic studies of fungi (Bruns *et al*., 1991; Mitchell *et al.,* 1995; Kohn, 1992). Molecular techniques such as PCR-RFLPs and nucleotide sequence analysis have been useful in taxonomic studies of different fungi (Nicholson *et al*., 1997; Gomes *et al*., 2000; Thomas *et al*., 2002; Llorens *et al*., 2006; Moreau *et al*., 2006). PCR-RFLP is an easy method to differentiate and/or identify species. For this purpose, a

particular region of the DNA is PCR-amplified and cut with an endonuclease(s). The resulting fragments, which depend on the presence of the specific recognition sites for the enzyme as well as the number of recognition sites present, are then separated by electrophoresis. Isolates with the same electrophoretic profile are considered to be similar. Reference isolates with known identity can be included for identification of unknown strains/species (Nei and Li, 1979).

Different regions of DNA can be used for RFLP analyses. Mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) have been used extensively in fungal systematics (Bruns *et al.,* 1991; Kohn, 1992; Nicholson *et al*., 1997; Llorens *et al*., 2006; Yang *et al*., 2007). Mitochondrial DNA has been useful for evolutionary studies in fungi because of its high copy number which makes restriction fragments to be visualised clearly (Bruns *et al*., 1991). The mtDNA evolves faster than the nuclear DNA (Bruns and Szaro, 1992; Castro *et al.,* 1998) which makes mtDNA RFLP analysis ideal for use when studying closely related species (Saitou and Ueda, 1994; Vincent *et al.,* 1986). However, it is very difficult to use mtDNA when comparing distantly related species because the patterns from mtDNA RFLPs are very different (Bruns *et al*., 1991). The rDNA regions such as the internally transcribed spacer regions (ITS1 and ITS2) and intergenic spacer (IGS) regions are commonly used for RFLP analyses at species level because they evolve more slowly than the mtDNA (Bruns and Szaro, 1992; Harrington and Wingfield, 1995). These regions provide sufficient level of variation that has shown to be informative at the species level for numerous fungi (Bruns *et al*., 1991; Gardes *et al.,* 1990; Gottlieb *et al*., 2000).

Several studies have used PCR-RFLPs of the rDNA region to distinguish species of *Ganoderma.* Gottlieb *et al*. (2000) distinguished isolates of subgenera *Ganoderma* and *Elfivingia* based on restriction analysis of the ITS and 5.8 S gene regions. Latiffah *et al*. (2002) used the same technique to distinguish amongst *Ganoderma* isolates from infected oil palm and coconut stumps. They observed restriction pattern variations within and between the two groups of isolates. However, cluster analysis of the RFLP data placed both groups of isolates together. Utomo *et al*. (2005) used rDNA PCR-RFLP to determine that the causal agent of basal stem rot disease in oil palms belongs to the single species *G. boninense*.

A limitation of PCR-RFLPs stems from the fact that isolates belonging to the same species may have different profiles due to intraspecific variation (Harrington and Wingfield 1995; Bunyard *et al.*, 1996). This can lead to misidentification of species. The discriminating power of PCR-RFLP thus depends on the size and variability of the DNA segment amplified as very small digestion products may be irresolvable or could be lost during electrophoresis leading to underestimation of variation (Gottlieb *et al*., 2000). Diagnostic enzymes may also be very expensive and not commonly available in some parts of the world.

1.3.4.3 DNA sequence analyses

Nucleotide sequences from certain regions of the DNA reflect phylogeny at various taxonomic levels. Such regions need to be evolving at an appropriate rate in order to supply enough consistent differences to separate the taxa into statistically supported monophyletic groups. These regions must be present as a single copy in the genome or

evolve as a single copy region in order to avoid comparisons of different copies in different species (paralogous comparisons) if the region exists as multicopy. Also, the region should have the same function in all organisms (Bruns *et al*., 1991; Mitchell *et al*., 1995).

The ribosomal RNA (rRNA) genes, certain ribosomal elongation factors, and genes from the nuclear and the mitochondrial genomes have been useful for DNA sequence analysis in fungi (Bruns *et al*., 1991; Mitchell *et al*., 1995; Tan and Niessen, 2003; Moreau, 2006). Consequently, nucleotide sequence information from relatively conserved genes/DNA segments such as the ITS (Moncalvo *et al*., 1995a, b; Smith and Sivasithamparam, 2000a), the mitochondrial small subunit (mtSSU) (Hong and Jung, 2004), and nuclear large subunit (LSU) (Lee *et* al., 2006) rDNA have been widely used in the taxonomy and phylogeny of *Ganoderma* species. This is because the variability of these regions, which is harboured mainly in the introns, provides sufficient resolution at various taxonomic levels.

Phylogenetic analysis of the ITS sequence data was used to resolve Australian *Ganoderma* isolates into five terminal clades, and showed that a number of isolates had been misnamed (Smith and Sivasithamparam, 2000a). Based on the phylogenetic analysis of the ITS and 5.8S sequence, Latiffah *et al.* (2002) showed that *Ganoderma* isolates from infected oil palm and coconut stumps belong to the same group as classified by PCR-RFLP. Gottlieb *et al*. (2000) also used ITS-based phylogenetic analysis together with PCR-RFLPs to elucidate the taxonomy of *Ganoderma* species in South America. They reported that molecular and morphological data agree at the subgeneric level, but that it was difficult to determine relationships at the species level.

Earlier studies based on morphological identification asserted that North American *G. lucidum* and European *G. resinaceum* belong to the same biological species (Adaskaveg and Gilbertson, 1986). Based on phylogenetic relationships and nucleotide sequence variations of the ITS (Moncalvo *et al*., 1995a, b) as well as the mtSSU (Hong and Jung, 2004), these two species were shown to be different.

The gene phylogeny by Moncalvo *et al*. (1995b) has indicated that isolates that were morphologically identified as *G. lucidum* did not cluster together, neither did those identified as *G. tsugae* or *G. resinaceum*. In the phylogenetic analysis of *Ganoderma* species using mtSSU sequence data by Hong and Jung (2004), *Ganoderma* species were divided into six monophyletic groups (*G. colossus* group, *G. applanatum* group, *G. tsugae* group, Asian *G. lucidum* group, *G. meredithiae* group, and *G. resinaceum* group) that included different species that were identified based on morphological characters. Species that were identified as *G. lucidum* were scattered over three of the groups, the Asian *G. lucidum* group, the *G. resinaceum* group and the *G. tsugae* group. Also, isolates that were identified as *G. oregonense* and *G. oerstedii* did not group together. These two studies indicate that some isolates were misidentified based on morphological characters since isolates that were identified as one thing do not form a monophyletic group.

From the preceding discussion it is clear that DNA sequence analysis of the ribosomal DNA region has provided an alternative approach to elucidate the taxonomy of *Ganoderma*. These techniques have played an important role in the taxonomy of *Ganoderma*, and have proved to be more reliable than other techniques that have been used for the same purpose. Misidentification and species synonyms based on morphological identification have been reduced using the molecular techniques.

The limitations of using DNA sequence analysis arise when polymorphisms and DNA heterogeneity are observed. This implies high intraspecies diversity or the presence of more than one copy of the gene in the genome. These may predate the taxa (Bunyard *et al.,* 1996) and may cause paralogous comparisons (Bruns *et al*., 1991). Intrastrain heterogeneity in ITS sequences has been observed in some *Ganoderma* strains that were found to have more than one form of the ITS region (Wang and Yao, 2005). This also makes DNA sequence analysis more expensive and time consuming because when polymorphisms are observed in sequences, one needs to clone and then re-sequence several fragments.

1.4 SUMMARY AND CONCLUSIONS

Ganoderma species are widely distributed fungi that include economically important taxa that are pathogens of many plants such as oil palm, coconut, tea and forest trees. Species of this genus have been traditionally identified based on morphological characteristics. This morphology-based classification, however, hosts a number of problems that led to taxonomic confusion in *Ganoderma*.

Variations in morphological characteristics of *Ganoderma* have led many taxonomists to introduce biochemical and molecular methods to differentiate *Ganoderma* species. Sexual compatibility tests were also useful for distinguishing *Ganoderma* species. However, this technique has important limitations. One is that basidiospores of these species do not germinate easily *in vitro*. Isozyme analysis were also applied in distinguishing *Ganoderma* species, however, a large amount of fresh material is needed for this analysis. DNA based techniques such as PCR-RFLPs and DNA sequence

analysis of the ITS and mtSSU rDNA regions have proved to be a better alternative to elucidate the taxonomy of *Ganoderma*. However, the preferable approach is one that employs a combination of the different available techniques. In this regard, comparison of sequences for various gene regions is commonly used and it is leading to a much more lucid taxonomy for *Ganoderma*. The work presented in this dissertation will employ this taxonomic approach to identify *Ganoderma* species associated with a serious root rot disease in South Africa and Indonesia.

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Table 1: Basidiocarp characteristics of *Ganoderma* species in the *G. lucidum* complex from North America (taken from Adaskaveg and Gilbertson, 1988).

Species	*Context colour*	*Basidiospores*	Pilocystidia
G. lucidum	Tan to brown	Ellipsoid 10–12 × 6.5–8 µm	Clavate, amyloid, thick walled
G. tsugae	White to tan	Ellipsoid 11–12.5 × 6.5–8 µm	Clavate, amyloid, thick walled
G. oregonense	White to tan	Ellipsoid 13–15.5 × 7.5–9 µm	Clavate, amyloid, thick walled
G. meredithiae	Tan	Ellipsoid 9.5–11.5 × 5.5–6.5 µm	Capitate to branched, amyloid to negative, thick walled
G. zonatum	Dark brown	Narrowly ellipsoid 11–13 × 5.5–6.5 µm	Capitate to branched, amyloid to negative, thick walled
G. colossum	Tan to gray white	Broadly ellipsoid 14.5–17.5 × 9.5–11.5 µm	Clavate, negative to weakly amyloid, thin walled

Figure 1: Structure of *Ganoderma* species showing (A) laccate pilei in a member of the *G. lucidum* complex and (B) non-laccate pilei in the *G. applanatum* complex (Photos: Martin Coetzee).


A


B

Figure 2: Double-walled basidiospores of *Ganoderma* species. Bar indicates 10µm.

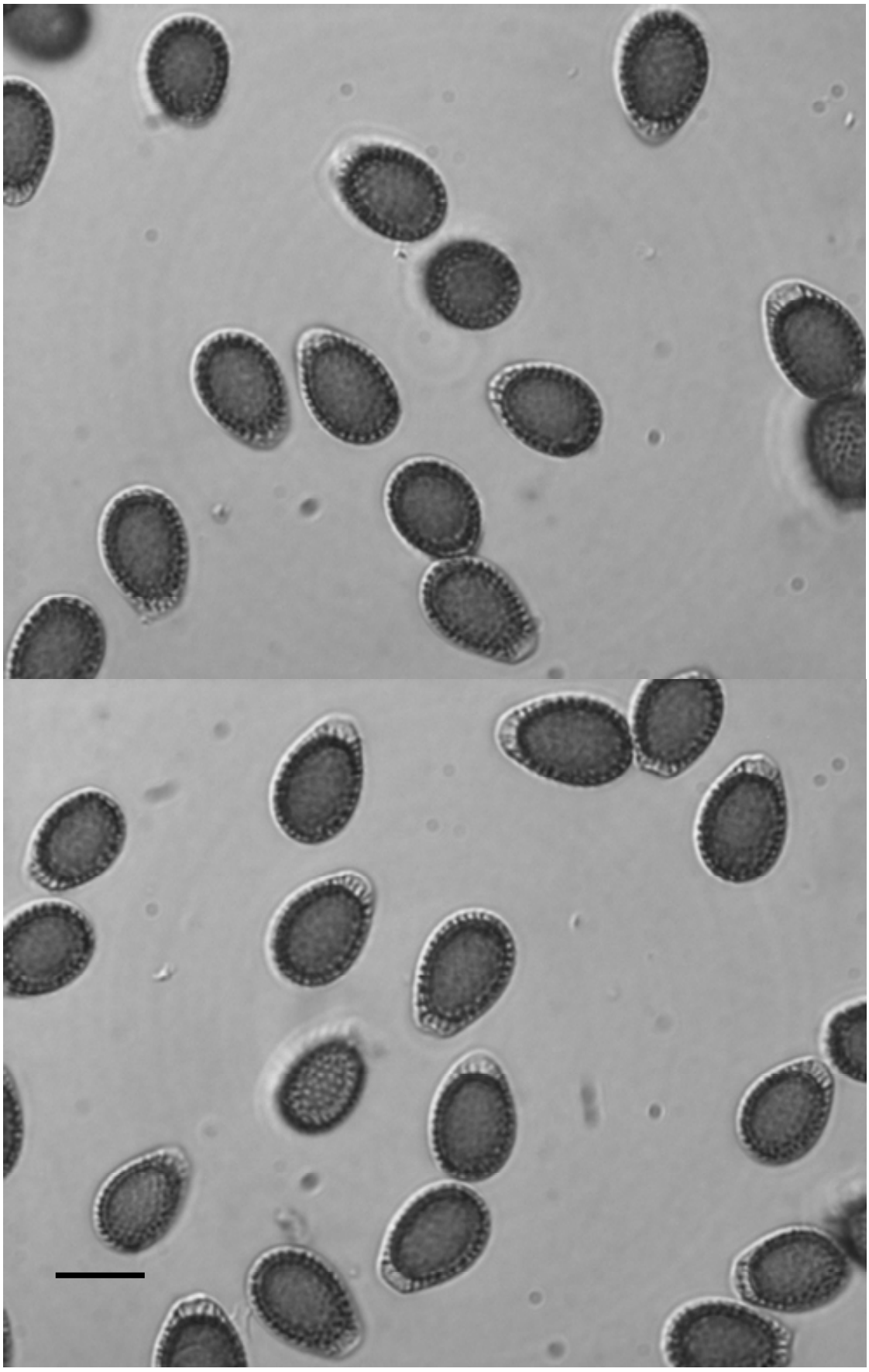


Figure 3: Microscopic cultural characters of *G. lucidum* published by Adaskaveg and Gilbertson (1986). A) Bright field microscopy of chlamydospores of *G. lucidum* (bar = 20µm). B-D) Scanning electron micrographs. B) Two adjacent chlamydospores of *G. lucidum* separated by a clamp connection (bar = 10µm). C) Branching network of staghorn hyphae produced in culture (bar = 2µm). D) Cuticular cell originating from a clamp connection produced in culture by *G. lucidum.*

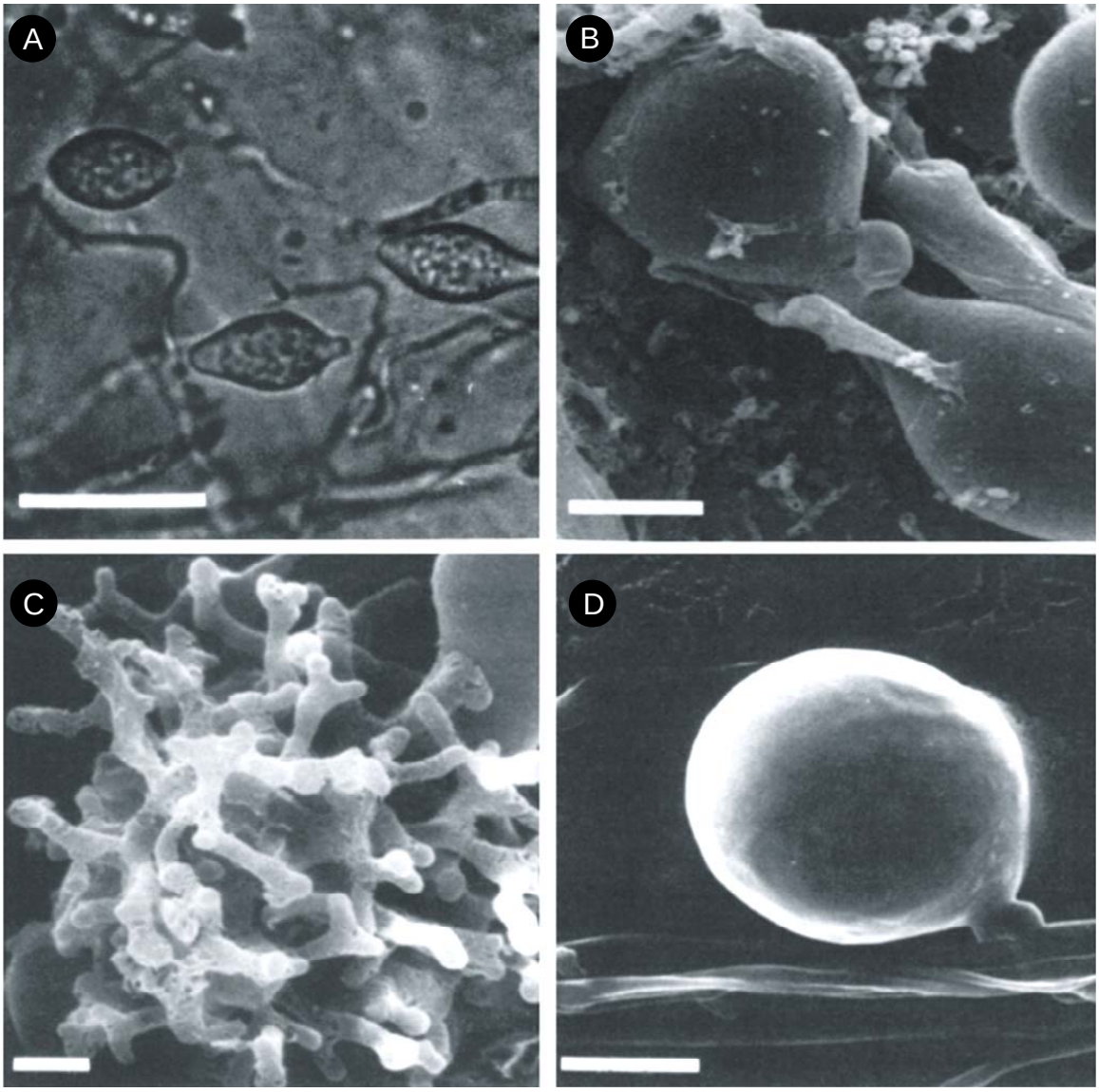
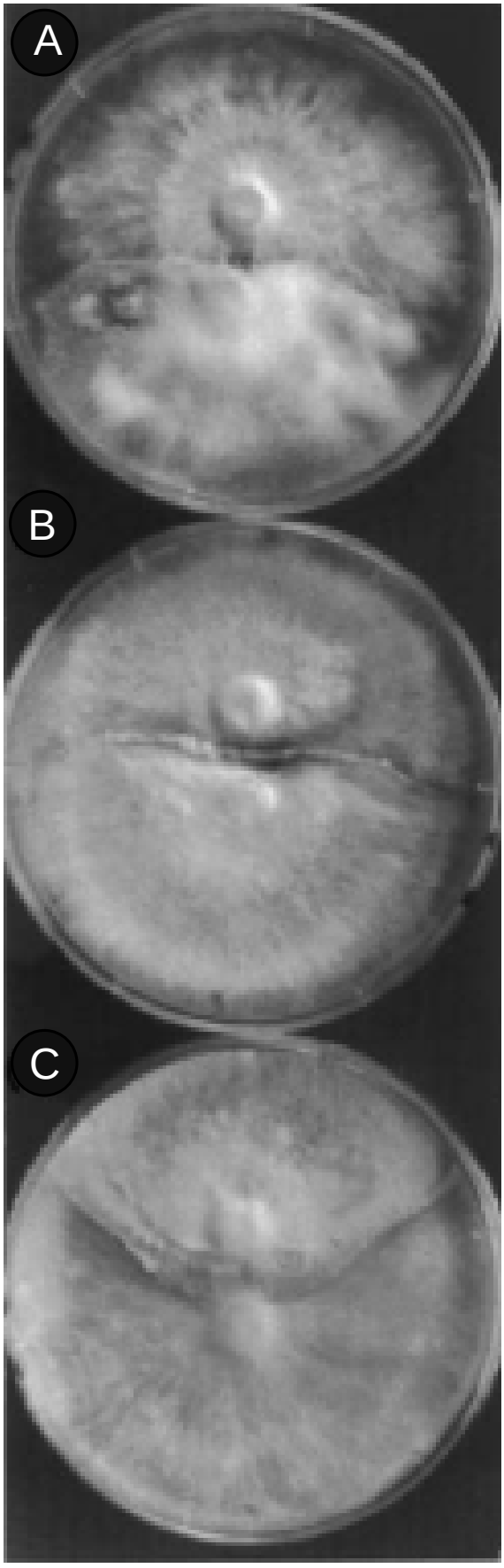
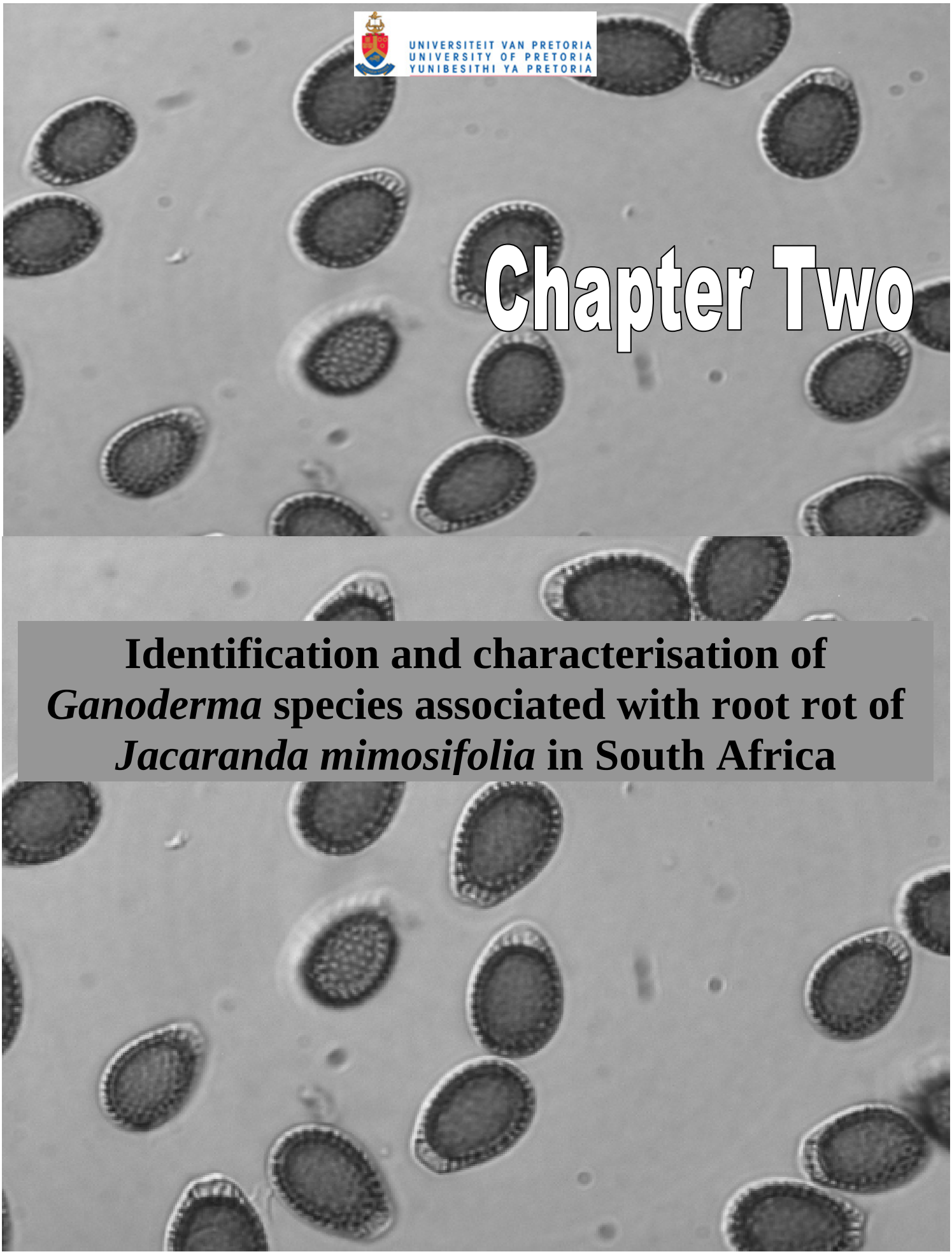

A
B
C
D

Figure 4: Interactions between haploid mycelia showing three possible antagonisms that can be observed (A) weak, (B) medium, and (C) strong (taken from Pilotti *et al*., 2002).


A
B
C



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Chapter Two

Identification and characterisation of *Ganoderma* species associated with root rot of *Jacaranda mimosifolia* in South Africa

Identification and characterisation of *Ganoderma* species associated with root rot of *Jacaranda mimosifolia* in South Africa

ABSTRACT

Pretoria is known as the "City of Jacarandas" because thousands of *Jacaranda mimosifolia* trees line its streets and adorn its gardens and parks. Large numbers of these Jacaranda trees are dying due to a root and butt rot disease apparently caused by a species of *Ganoderma.* Fruiting bodies of *Ganoderma* resembling those in the "*G. lucidum*" complex are commonly found at the bases of dying trees every year after the onset of rain in spring and early summer. The "*G. lucidum*" complex includes several paraphyletic groups that are considered to represent different species with similar morphology; the true identity of the fungus causing the disease on Jacaranda trees is therefore unknown. The aim of this study was to characterise the species of *Ganoderma* that is found on Jacaranda trees dying from this root rot disease based on morphological characteristics and DNA sequence comparisons. Basidiocarps were collected from infected trees in the suburb of Brooklyn, Pretoria and their morphology was examined. Fungal isolations were made and cultural characteristics including growth habit, colour and presence of chlamydospores were determined. DNA was extracted from the isolates and sequences obtained for the ITS regions (ITS-1, 5.8S gene and ITS-2), the IGS-1 region as well as the mitochondrial small subunit (mtSSU) gene. Morphological observations revealed that all of the isolates represented a species that was similar to those in the *G. lucidum* complex. DNA-based phylogeny showed that these isolates are closely related to *G. lucidum*. The gene phylogenies were, however, not congruent. The results of this study

therefore suggest that *G. lucidum* is a phylogenetically variable species complex and that the *Ganoderma* sp. commonly found associated with root rot disease of Jacaranda trees in Brooklyn resides in *Ganoderma lucidum sensu lato*.

INTRODUCTION

Pretoria is known as the "City of Jacarandas" as a result of the large number of *J. mimosifolia* (*Bignoniaceae*) that have been planted in its gardens and parks. This tree is native to Argentina and Bolivia in South America where it occurs mainly along rivers in warmer-temperate sub-humid areas (Gilman and Watson, 1993). Jacarandas have been planted in many parts of the world mainly for esthetic purposes due to the beautiful violet flowers that the trees produce in spring (Gilman and Watson, 1993). This tree was introduced into South Africa in 1888 when two trees were planted at a school in Arcadia, Pretoria. Seedlings were subsequently obtained from the first seeds of these trees and planted along Bosman Street, then known as Koch Street, Pretoria (Kasrils, 2001). The trees became popular and widely planted such that Pretoria becomes a haze of purple in the spring earning its name as Jacaranda city. Large numbers of these trees have, however, been observed to be dying in the Brooklyn suburb of this city due to a root rot disease apparently caused by a species of *Ganoderma.*

Ganoderma is a cosmopolitan genus that includes both saprophytic and phytopathogenic species (Seo and Kirk, 2000). Most species of *Ganoderma* are pathogenic, causing root and stem rot on a variety of monocots, dicots and gymnosperms (Ariffin *et al.*, 2000; Seo and Kirk, 2000; Sankaran *et al.*, 2005). Infection by these species mostly results in the death of affected trees. The saprophytic species cause white rot of wood by

decomposing lignin and cellulose in the wood tissue (Adaskaveg *et al.*, 1991, 1993). Some species of *Ganoderma* are also well known, especially in Asia, for their medicinal value (Jong and Birmingham, 1992).

Identification of *Ganoderma* species has traditionally been based on the morphological features of the basidiocarps (Adaskaverg and Gilbertson, 1988; Gottlieb and Wright, 1999a, b; Smith and Sivasithamparam, 2003; Pilotti *et al.*, 2004). However, identification using these characteristics is problematic because different species often share the same characteristics and therefore appear similar. Thus a high level of intra-species variation, apparently, exists in some species as a result of this morphological plasticity. Some isolates were thought to represent different species until it was later shown, based on the biological and phylogenetic species concepts, that they belong to the same species (Adaskaveg and Gilbertson, 1986; Moncalvo *et al*., 1995a, b; Hong and Jung, 2004). More than 250 *Ganoderma* species have been described though many of these were later found to be synonyms and several species complexes were also recognised. Consequently, there remains much uncertainty about many of the species described to date (Ryvarden, 1994; Adaskaveg and Gilbertson, 1986, 1988).

Problems associated with the traditional morphology-based taxonomy led to the use of alternative approaches for species recognition in *Ganoderma*. Some researchers (Adaskaveg and Gilbertson, 1986; Pilotti *et al*, 2002, 2003) have used the biological species concept and applied sexual compatibility tests, *i.e*., the ability to mate and produce fertile progeny, as the sole criterion for species recognition. These tests rely on compatible interactions between two monokaryotic isolates obtained from single basidiospores. Identifications based on sexual compatibility tests have limitations in that

basidiospores do not germinate easily *in vitro* and species-specific tester strains are needed for the purpose of species identification (Adaskaveg and Gilbertson, 1986, 1987, 1989).

Isozyme analyses have been used in the taxonomy of *Ganoderma* (Gottlieb *et al.,* 1998; Smith and Sivasithamparam, 2000b). This technique involves separating total protein extracts using gel electrophoresis, where differences in electrophoretic profiles are considered to indicate genetic variability. This method of species recognition assumes that isolates of different species produce different profiles while individuals in the same species yield similar profiles (Bonde *et al.,* 1993). The use of this method is hampered as a result of protein profiles that do not always reveal much variation at lower taxonomic levels (Gottlieb *et al*., 1998) as much of the phylogenetic signal is lost at the amino acid level due to the degeneracy in the genetic code and the fact that introns, are spliced out during transcription (Tourasse and Kolstø, 2008). In addition isozyme analysis requires availability of large amounts of fresh material (Bonde *et al.,* 1993; Smith and Sivasithamparam, 2000b). This is often difficult to achieve and impossible when no ex-type cultures are available.

DNA-based techniques such as PCR-RFLPs (Restriction Fragment Length Polymorphisms) and nucleotide sequence analysis of ribosomal genes and spacers have been successfully applied in the taxonomy of *Ganoderma* (Moncalvo *et al.,* 1995a, b; Gottlieb *et al*., 2000; Smith and Sivasithamparam, 2000a; Latiffah *et al*., 2002; Hong and Jung, 2004; Utomo *et al.,* 2005; Lee *et al.,* 2006). This method has been important in elucidating the taxonomy of the species within the genus. As a result, synonyms and misidentification of species has largely been reduced.

Little information is available on the taxonomy of *Ganoderma* in South Africa. About 20 morphological species of *Ganoderma* have previously been described from this country (Baxter and Eicker, 1994). However, DNA sequences of these species are not available and their phylogenetic relationship with other species is not known.

Fruiting bodies that are similar to those of *G. lucidum* are found at the bases of dying Jacaranda trees every year after the onset of rain in spring and early summer. This suggests that root rot disease caused by *Ganoderma* species may be responsible for the death of these trees. The identity of the *Ganoderma* species causing disease, however, has not yet been unambiguously determined since *G. lucidum* represents a species complex that includes various species with similar morphology. The aim of this study was therefore to characterise the *Ganoderma* species responsible for root rot disease on *J. mimosifolia* in Pretoria. This was achieved using morphological characteristics as well as nucleotide sequence data from a region of the ribosomal RNA operon, the internally transcribed spacer (ITS), the intergenic spacer (IGS-1) and the mitochondrial small subunit (mtSSU) gene.

MATERIALS AND METHODS

Basidiocarp morphology

Basidiocarps were collected from infected *J. mimosifolia* trees in the suburb of Brooklyn, Pretoria (Figure 1). The infected trees showed typical symptoms of root rot including crown dieback (Figure 2). The appearance (shape and colour) of the pileus for each basidiocarp collected was observed. Basidiospores were collected from spore prints of basidiocarps. These were examined under a Zeiss Axioskop light microscope by

differential inference contrast (DIC) microscopy. Spore length and width were determined from 30 spores for each isolate examined. Measurements were made with the aid of an Axiovision camera (AxioVs 40 v. 4.3.0.101, Carl Zeiss Ltd., München, Germany).

Fungal isolation and cultivation

Fungal isolations were done by aseptically placing small pieces of basidiocarp tissues on malt extract medium (MEA: 2% w/v malt extract, 1.5% w/v agar, Biolab, Midrand, South Africa) supplemented with 0.1g/litre streptomycin sulphate (Sigma-Aldrich, Missouri, USA), and incubated at 22°C in the dark for 3-5 days. Fungal colonies were then aseptically transferred to fresh MEA media without streptomycin and incubated for two weeks. Cultural characteristics including growth habit and colour were determined. The presence/absence of chlamydospores was assessed from the two-week old cultures by mounting mycelium in phloxine (1% w/v) and KOH (2% w/v) and observation under bright field microscopy. Cultures were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

DNA extraction

DNA was extracted from two-week old cultures on MEA following the method described by Murray and Thompson (1980). Mycelium was scraped from the surface of cultures and transferred into Eppendorf tubes. Pre-warmed (60-65°C) cetyltrimethylammonium bromide (CTAB) extraction buffer (5% w/v CTAB, 1.4M NaCl, 0.2% v/v 2-mercaptoethanol, 20mM EDTA pH 8, 10mM Tris-HCL pH 8, and 1% v/v

polyvinylpolyrrolidone) (700µL) was added to the mycelium, which was then pulverized using a Fast Prep (FP120 BIO 101 SAVANT) machine (Southern Cross Biotechnology, Cape Town, South Africa). The mycelium suspension was then incubated at 65°C for an hour. Cell debris was removed by centrifugation (10000 rpm, 10 minutes) and the supernatant was transferred into fresh Eppendorf tubes. Proteins were removed from the supernatant with repeated phenol/chloroform extraction. For this purpose, an equal volume of phenol/chloroform (1:1 v/v) was added to the supernatant, which was thoroughly mixed and centrifuged (10000 rpm, 10 minutes) to separate the organic and aqueous phases. The aqueous phase was transferred to a new Eppendorf and the extraction repeated until the interphase between the two layers became clear. A final chloroform extraction was done to remove the phenol residue from the aqueous layer. Nucleic acids were precipitated by adding 0.6 volume of iso-propanol and 0.1 volume of 3M sodium acetate (pH 5.2) to the clear solution and incubating the mixture at -20°C overnight. The nucleic acids were precipitated by centrifugation (13000 rpm, 30 minutes) at 4°C. The pellet was washed with 80% ethanol, dried, and re-suspended in 50µl sterile water. The RNA was removed from the nucleic acid solution by digestion with 0.01 mg/µL RNAse (Roche Diagnostics, Randburg, South Africa) at 37°C for an hour. DNA quantification was done using NANODROP (ND-1000) spectrophotometer (Nanodrop Technologies, Wilmington, USA).

PCR amplification of the ITS, IGS-1 and mtSSU region

The primers used to amplify the three gene regions and the corresponding annealing temperatures are shown in Table 2. Binding sites of the different primers used for mtSSU amplification are indicated in Figure 3. All PCR mixtures consisted of a 50ng genomic

DNA, reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_2$, 50mM KCl, Roche Diagnostics, Randburg, South Africa), 2.5 µM of each dNTP (Fermentas Life Sciences, Pretoria, South Africa), 0.4 µM of each primer, and 0.5 units of *Taq* polymerase (FABI, University of Pretoria, South Africa). PCR cycles consisted of an initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at 62°C (ITS and IGS-1) or 50°C (mtSSU) and extension at 72°C for 30 seconds (ITS and IGS-1) or 2 minutes (mtSSU). A final extension at 72°C for 7 minutes (ITS and IGS-1) or 10 minutes (mtSSU) was included to complete the reaction. PCR products were analysed by agarose (2% or 1% w/v, Whitehead Scientific, Johannesburg, South Africa) gel electrophoresis (4v/cm) and visualised by staining with ethidium bromide and UV illumination. Product size was estimated by comparison with a 100 bp-ladder DNA marker (Promega Corporation, Madison, WI, USA) or Lamda DNA (EcoRI+HindIII) marker (Fermentas Life Sciences, Pretoria, South Africa).

DNA sequencing

The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were sequenced in both directions using the same set of primers as for the respective PCR reactions. Sequence reactions were performed using an ABI Prism® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer, Warrington, UK) following the protocol supplied by the manufacturer. DNA sequences were obtained using an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA). Vector NTI version 10.3 (Invitrogen Corporation, Carlsbad, USA) was used to align and edit the DNA sequences.

Cloning and sequencing of the mtSSU rDNA

Direct sequencing of mtSSU amplicons resulted in double peaks or ambiguous site readings that could not be used for further analysis. These amplicons were thus cloned using the pGEM®-T Easy vector system (Promega Corporation, Madison, WI, USA) following the manufacturer's protocols. The inserts were recovered by colony PCRs employing vector-specific primers [T7 (5′TAATACGACTCACTATAGGG3′) and SP6 (5′TATTTAGGTGACACTATAG3′)] where cells that were picked from positively transformed (white) *Eschericia coli* colonies were used as DNA templates. The PCR conditions were the same as that used for the mtSSU region except that the extension period was limited to 30 seconds and final extension to 7 minutes. Amplified inserts were sequenced using the primers T7 and SP6.

For very large PCR products, complete sequencing of each strand could not be achieved; thus sequences from the two strands could not be assembled together. Different combinations of internal primers were therefore used in order to sequence the middle portions of such PCR products. These pairs of internal primers were BMS55 and BMS113; BMS105 and BMS153; SSUF and BMS65; SSUR and BMS103; SSUF and SSUR (Table 2). Primers SSUF and SSUR were developed in this study and designed using Primer3 (http://primer3.sourceforge.net) and they were used only in isolates that produced a 3.0Kb (kilobase pairs) fragment.

DNA sequence analysis

Sequences from the ITS, mtSSU and IGS-1 rDNA regions were compared with those in GenBank using the BLASTn (Basic Local Alignment Search Tool for nucleotides) search

algorithm. Hits from the BLASTn searches, which showed the highest similarity and coverage (from 95% to 99%) as compared to the sequences from the Pretoria isolates, were downloaded and included in this study for comparative purposes. Only 13 IGS-1 sequences are available on GenBank for *Ganoderma* isolates, sequence comparisons with other *Ganoderma* species was therefore not possible. All sequences were aligned using Multiple sequence Alignment based on Fast Fourier Transform (MAFFT, version 5, Katoh *et al.*, 2005).

Phylogenetic trees were generated based on distance analysis using PAUP* (Phylogenetic Analysis Using Parsimony and *other methods) version 4.0b10 (Swofford, 2002) and maximum likelihood (ML) with PHYML (Phylogenetic inferences using Maximum Likelihood) version 2.4.4 (Guidon and Gascuel, 2003) for the ITS and mtSSU sequence data. The HKY+I and K81uf+I+G substitution models, as determined with MODELTEST version 3.7 (Posada and Crandall, 1998) were applied to the ITS and mtSSU datasets, respectively, in the distance and maximum likelihood analyses. Trees were generated from the distance matrices using neighbour-joining (NJ) tree building algorithm (Saitou and Nei, 1987). Only distance analysis using NJ tree building algorithm was performed for the IGS-1 sequence data. Bootstrap analyses (Felsenstein, 1985) were performed with 1000 replicates.

RESULTS

Morphology of the basidiocarps

All the basidiocarps collected had similar morphological characteristics (Figure 4). Basidiomes were sessile and bracket shaped with a brown and laccate pileus. The margins of the basidiomes were wide, thick, blunt and yellowish white and the context was brown and thinner towards the margin. The tube layer was yellowish brown and hymenophore was round and cream. Basidiospores of all the isolates examined were brown and ovate with rounded apices and all the basidiospores had a double wall separated by inter-wall pillars and appeared slightly rough (Figure 5). Biometrics of basidiospores are presented in Table 3.

Fungal isolations and cultural characteristics

A total of 24 basidiocarps, one from each of the infected *J. mimosifolia* trees, were sampled. *Ganoderma* strains were successfully obtained from 22 of these basidiocarps (Table 1). Cultures from the other two samples were contaminated in spite of several re-isolations and could not be used in further studies. All isolates had white flat or fluffy mycelium which covered the Petri plates within two weeks. None of the isolates produced chlamydospores in culture.

PCR amplification

PCR amplification of the ITS and the IGS-1 regions produced a single product of about 700 base pairs (bp) (Figure 6) and 1000 bp (Figure 7), respectively, in each isolate

studied. PCR amplification of the mtSSU region using primers BMS05 and BMS173 yielded two different fragment sizes among the isolates (Figure 8). In half of the isolates (CMW29579, CMW29574, CMW29575, CMW29576, CMW29580, CMW29582, CMW29583, CMW29585, CMW29586, CMW29587, and CMW29589), the size of the amplified fragment was about 1.5 Kb (Figure 8A), whereas in the remaining isolates (CMW29571, CMW29572, CMW29573, CMW29577, CMW29578, CMW29579, CMW29581, CMW29584, CMW29588, CMW29590, and CMW29591), it was about 3.0 Kb (Figure 8B).

Sequence data and analyses

ITS sequence data

BLASTn comparisons of the ITS sequence data from the Pretoria isolates with those from GenBank revealed high similarity with various *Ganoderma* species. The highest similarity was with *G. lucidum* (AY636058.1) from China which had 99% identity, 99% coverage and 0.0 E-value with sequences from the Pretoria isolates. The aligned sequence data from Pretoria isolates and 14 *Ganoderma* strains from GenBank had a total of 747 characters including gaps (Appendix A[1]). Most of the isolates had identical sequences. Among the Pretoria isolates, sequence variation was observed in isolates CMW29586 and CMW29587, CMW29588 and CMW29589.

Phylogenetic analyses based on NJ and ML methods generated trees with similar topologies. All the Jacaranda isolates clustered together in close association with *G. lucidum* from China (AY636058.1 and AY636068.1) with a bootstrap support of 80%

[1] Appendixes are available on the CD provided with this thesis.

and 91% based on ML and NJ, respectively (Figure 9). The four isolates which had ITS sequences varying from the others formed their own subgroups within the bigger cluster of the Pretoria isolates. The CMW29586 and CMW29587 subgroup had a high bootstrap support based on both ML (87%) and NJ (86%) analyses. The subgroup including CMW29588 and CMW29589 was supported in the ML (74% bootstrap support) analysis though the support was somewhat low in the NJ (66%) analysis. Other GenBank sequences that were thought to belong to *G. lucidum* were scattered over the tree (Figure 9).

Mitochondrial SSU sequence data

BLASTn searches using the mtSSU data showed high sequence similarity with various *Ganoderma* species from GenBank. The highest similarity was with *G. oerstedii* (AF214469.1) from Argentina (97% identity and coverage with 0.0 E-value) and with *G. lucidum* (AF214475.1) from Taiwan (96% similarity, 100% coverage and 0.0 E-value).

The aligned mtSSU sequences from isolates collected in this study and a selection of 18 *Ganoderma* strains from GenBank contained a total of 3040 characters including gaps (Appendix B[1]). An indel (insertion/delition) of about 1.5 Kb was observed between position 715 and 2228 in the Pretoria isolates. An additional 1.5 Kb mtSSU sequence is thus present in certain isolates, explaining a 3.0 Kb fragment that was observed from PCR for these isolates. BLASTn search using sequences from this region revealed that this region corresponded to a Group II intron as identified by Hong and Jung (2004).

Phylogenetic analysis of the mtSSU sequence data based on NJ and ML methods generated trees with similar topologies. The Pretoria isolates grouped into two major clades (Figure 10). The one clade (group A) had a bootstrap support of 65% and 79% based on ML and NJ, respectively. This clade was placed in a cluster that included *G. oerstedii* (AF214469.1) from Argentina, *G. meridithiae* (AF248344.1), *G. oregonense* (AF248345.1) from USA, and a group of *G. lucidum* (AF248320.1, AF248342.1, AF248328.1, AF248326.1 and AF248340.1) species from Korea and Japan. This cluster formed a sister group to the second clade (group B) that included isolates from infected Jacaranda trees which also formed a sister group to *G. lucidum* (AF214475.1, AF248339.1 and AF248338.1) from Taiwan and USA (Figure 10). This clade (group B) had 99% bootstrap support based on ML and 100% support based on NJ.

Ten colonies were also sequenced for representative isolates of each of the two groups. This was done to determine if the grouping of isolates was the result of two types of mtSSU copies in the mitochondrial genome. Sequences obtained from the ten copies were the same in each isolate, indicating the presence of only one type of mtSSU copy in the genome.

IGS-1 sequence data

BLASTn searches using the IGS-1 sequence data showed similarity with few sequences for *Ganoderma* species available in GenBank. However, such sequences were not included in the analysis due to limited number of IGS-1 sequences for *Ganoderma* species in GenBank. The alignment of the IGS-1 sequence data from the Pretoria isolates resulted in a total of 892 characters including gaps (Appendix C[1]).

Two groups resulted from NJ analysis of the IGS-1 sequence data from Pretoria isolates. The IGS-1 phylogram had a tree topology that was different from those based on the mtSSU and the ITS. The ITS grouped all the Pretoria isolates together while the IGS-1 and mtSSU separated these isolates into two groups. The assemblage of the two groups in the respective trees was different; isolates that belong to one group in the mtSSU phylogram are scattered over the two groups in the IGS-1 phylogram (Figure 11).

DISCUSSION

Several species of *Ganoderma* cause root rot disease on various hosts that result in decline of productivity due to death of trees. In India, *Ganoderma* species has been reported as pathogens on different hosts including *J. mimosifolia* where *G. lucidum* was found associated with the tree (Sankaran *et al*., 2005). In this study the identity of the *Ganoderma* species that is commonly found associated with root rot of *J. mimosifolia* in the Brooklyn suburb, Pretoria was investigated and shown to represent a single species.

The trees in the sampling area showed typical symptoms of root rot and nothing else was found growing on the trees other than *Ganoderma* fruiting bodies. Also, other tree species such as *Acacia* and *Platanus* in the same area did not show any symptoms of the disease including the presence of basidiocarps. This suggested that the fungus infecting the Jacarandas prefers this host. It is assumed that a species within *Ganoderma* is the causal agent of the disease since it was the only fungus found growing on the trees.

The morphological similarity of *Ganoderma* species has caused confusion in the identification of these species. Numerous species have been described but many of them

were later found to be synonyms or represented species complexes (Bazzalo and Wright, 1982; Adaskaveg and Gilbertson, 1986, 1988). In this study morphological features including basidiocarp morphology and cultural characteristics as well as DNA based methods were used to identify the *Ganoderma* species that is commonly found associated with root rot of *J. mimosifolia* in Pretoria.

Two types of basidiocarps can be produced in *Ganoderma* depending on the particular species. These include species with laccate fruiting bodies having a shiny upper surface, or a non-laccate fruiting body with a dull upper surface. Strains with laccate pilei reside in the *G. lucidum* complex whereas those with non-laccate pilei are grouped in the *G. applanatum* complex (Moncalvo and Ryvarden, 1997). The morphological features of the basidiocarps collected in this study resembled those of the species in the *G. lucidum* complex, which have laccate pilei. Cultural characteristics of the isolates used in this study also fitted the description of cultures that were referred to as *G. lucidum* (Curtis:Fr) P. Karst (Gottlieb and Wright, 1999a) as these isolates did not produce chlamydospores.

Basidiospores of species within the Ganodermataceae have been considered to be the most characteristic taxonomic feature (Furtado, 1962) and differences in basidiospore morphology have been reported for different species within this fungal family (Adaskaveg and Gilbertson, 1986; Gottlieb and Wright, 1999a, b). All the basidiospores examined in this study shared the same characteristics having a brown colour and inter-wall pillars that are well defined. Morphology of all the isolates examined in this study as a whole appeared to be the same and similar to a number of species in the *G. lucidum* complex. However, a positive identification could not be made based on morphology alone.

Earlier work and results from the current study showed that isolates that were identified as *G. lucidum* based on morphology and deposited in GenBank do not cluster together in one monophyletic group. Using ITS and LSU sequence data, Moncalvo *et al*. (1995b) found that isolates that were morphologically identified as *G. lucidum* did not cluster together, neither did those identified as *G. tsugae* or *G. resinaceum*. The authors concluded that extensive convergence and parallelism of morphological and cultural characters must have occurred during *Ganoderma* evolution. More recently, Hong and Jung (2004) reported a phylogeny of *Ganoderma* species using mtSSU sequences. In their study, *Ganoderma* species were divided into six monophyletic groups (*G. colossus* group, *G. applanatum* group, *G. tsugae* group, Asian *G. lucidum* group, *G. meredithiae* group, and *G. resinaceum* group). Three of the groups included strains that were identified as *G. lucidum* based on morphology. Thus *G. lucidum* includes several paraphyletic groups that represent cryptic species.

The difficulty of identifying cryptic species based on morphology prompted the use of DNA sequence data to delineate the isolates from Pretoria. Nucleotide sequences from three genomic regions, the ITS and the IGS-1 regions of the ribosomal RNA operon and the mitochondrial SSU gene were analysed. There were several reasons for choosing these genes. The ITS region is commonly used in the taxonomic studies of *Ganoderma* species. The degree of variability that it encompasses has been useful to circumvent taxonomic problems within *Ganoderma* (Moncalvo *et al*., 1995a, b; Smith and Sivasithamparam, 2000a). The mtSSU, although less variable than the ITS, is believed to have the potential of complementing the ITS data in phylogenetic studies (Bruns and Szaro, 1992). The mtSSU was also found to have 3.3 times more phylogenetic information than ITS sequences among *Ganoderma* species (Hong *et al*., 2002). A large

ITS and mtSSU sequence database is also available for *Ganoderma* species, which can be used in analyses of sequences from these two regions (Moncalvo *et al*., 1995a, b; Smith and Sivasithamparam, 2000a; Hong and Jung, 2004). The IGS-1 region was included at a later stage to determine whether this region could be used to resolve the observed conflict between phylogenies based on the ITS and mtSSU.

BLASTn search of the ITS sequence data indicated a high similarity with various *Ganoderma* species that belonged to the *G. lucidum* complex. Phylogenetic trees derived from the ITS sequence data grouped all the isolates from Pretoria in one group. ITS sequences from most of the isolates were identical, however, sequence variation was observed in some isolates. The latter isolates formed two subgroups within the group from Pretoria based on the ITS sequence data. This suggested they may belong to different species although their sequence variation with other isolates was low. The isolates from Pretoria as a whole formed a sister group with what is considered to be authentic sequences of *G. lucidum* from China (GenBank database). Thus, results from the phylogenetic analysis based on ITS sequence data suggest that the Pretoria isolates belong to a species that is closely related to *G. lucidum*.

Amplification of the mtSSU yielded a single fragment for all the isolates though these were of two different sizes (1.5 Kb and 3 Kb). Sequences from the mtSSU gene revealed that the larger amplicons contained a Group II intron of 1.5 Kb, which was absent in the smaller amplicons. The presence of this intron in the mtSSU of *Ganoderma* species was not without precedent. Group II introns were found within the mtSSU of strains representing *G. lucidum, G. appalanatum, G. lobatum, G. pfeifferi,* and *G. resinaceum* by Hong and Jung (2004).

Introns are non-coding sections of a gene that are removed during transcription. Group II introns are a class of introns found in rRNA, tRNA, and mRNA of organelles in fungi and they possess very few conserved nucleotides (Tourasse and Kolstø, 2008). Consequently Group II introns may not carry information that is specific to the evolution of a lineage. For this reason, a pre-analysis was done both with and without sequence information from this intron. Phylograms generated from both analyses, with and without the intron, grouped the isolates into two clades and the grouping of the isolates in the respective clades was similar in both the analyses. Also, two isolates that lacked the intron grouped with isolates that had this intron present. Intron sequences were therefore excluded from all subsequent analyses based on the mtSSU.

Phylogenetic trees generated from the mtSSU placed the isolates from Pretoria in two relatively distant groups (groups A and B), suggesting that the isolates belong to two different species. Isolates from neither of the groups showed a strong association with sequences of *Ganoderma* species from GenBank in the phylogenetic analysis. Isolates from both groups, however, formed a cluster together with *G. oerstedii, G. meredithiae, G. oregenense* and a group of *G. lucidum* species. This indicated that the isolates from Pretoria are closely related to these species.

A phylogenetic tree generated from the IGS-1 sequence data placed the isolates from Pretoria into two groups. Although the mtSSU phylogeny also yielded a tree with two groups of Pretoria isolates, the assemblage of the groups based on the two regions were different. Isolates that belonged to one group in the mtSSU phylogram were scattered into the two groups in the IGS-1 phylogram.

The phylogenetic trees generated from the three gene regions were compared with each other in order to determine if the isolates from Pretoria represent a single or more than one species in accordance to Genealogical Concordance Species Concept (Avise and Ball, 1990). Phylogenetic species recognition based on this concept, relies on the comparison of several gene genealogies. Gene trees are concordant if they have similar topologies and the transition from concordance to conflicting topologies determines the limits of species (Taylor *et al.,* 2000). Thus, if a set of isolates group in a different assembly in several gene genealogies, there is no concordance among branches and hence incongruity. The topologies of the trees generated from the three regions in this study were not congruent. Conflict among the different trees indicated that there is not genealogical concordance and the isolates therefore belong to a single species.

The *Ganoderma* species that is commonly found in association with root rot of *J. mimosifolia* in Brooklyn, Pretoria resides in *Ganoderma lucidum sensu lato*. This is evident from the comparison of morphological and molecular characteristics done in this study. Future studies should include identifications based on biological species concept to confirm the presence of one species as well as pathogenicity tests to confirm that the species is the pathogen on *J. mimosifolia*.

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Table 1: List of *Ganoderma* isolates used in this study collected from *J. mimosifolia* in Pretoria.

Isolate numbers[a]	Identity	Culture number[b]	GPS coordinates
PTA1	*Ganoderma* sp.	CMW29570	S:25º45'76.1", E:28º13'76.1"
PTA2	*Ganoderma* sp.	CMW29571	S:25º45'49.7", E:28º14'06.7"
PTA3	*Ganoderma* sp.	CMW29572	S:25º45'48.1", E:28º14'00.5"
PTA4	*Ganoderma* sp.	CMW29573	S:25º45'40.9", E:28º14'38.5"
PTA5	*Ganoderma* sp.	CMW29574	S:25º45'41.4",E:28°13'55.6"
PTA6	*Ganoderma* sp.	CMW29575	S:25°45'45.2", E:28°14'11.7"
PTA7	*Ganoderma* sp.	CMW29576	S:25°45'53.7", E:28°14'46.5"
PTA8	*Ganoderma* sp.	CMW29577	S:25°45'53.6", E:28°14'19.6"
PTA9	*Ganoderma* sp.	CMW29578	S:25°45'52.3", E:28°14'18.9"
PTA10	*Ganoderma* sp.	CMW29579	S:25°45'48.1", E:28°14'00.9"
PTA11	*Ganoderma* sp.	CMW29580	S:25°46'04.8", E:28°14'46.0"
PTA12	*Ganoderma* sp.	CMW29581	S:25°45'51.1", E:28°14'40.7"
PTA13	*Ganoderma* sp.	CMW29582	S: 25°45'32.2", E: 28°14'04.3"
PTA14	*Ganoderma* sp.	CMW29583	S: 25°45'44.2", E: 28°14'05.6"
PTA15	*Ganoderma* sp.	CMW29584	S: 25°45'46.5", E: 28°13'53.9"
PTA16	*Ganoderma* sp.	CMW29585	S: 25°45'50.3", E: 28°14'11.2"
PTA17	*Ganoderma* sp.	CMW29586	S: 25°45'45.6", E: 28°13'45.7"
PTA18	*Ganoderma* sp.	CMW29587	S: 25°45'59.6", E: 28°14'27.2"
PTA19	*Ganoderma* sp.	CMW29588	S: 25°46'02.9", E: 28°14'27.2"

Table 1 (continued)

Isolate numbers[a]	Identity	Culture number[b]	GPS coordinates
PTA20	*Ganoderma* sp.	CMW29589	S: 25°45'51.0", E: 28°14'14.2"
PTA22	*Ganoderma* sp.	CMW29590	S: 25°45'56.4", E: 28°14'47.6"
PTA24	*Ganoderma* sp.	CMW29591	S: 25°45'50.4", E: 28°14'33.0"

[a]Isolate numbers refer to the names given by the collectors

[b]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Table 2: List of primers used in this study for the amplification and sequencing the ITS (White *et al.,* 1990), IGS-1 (Coetzee *et al*., 2003) and mt SSU rDNA (Hong and Jung, 2004) regions.

Primer	5′ to 3′ sequence	Region amplified	Annealing temperature
ITS1	TCCGTAGGTGAACCTGCGG	ITS	62°C
ITS4	GCTGCGTTCTTCATCGATGC	ITS	62°C
P-1	TTGCAGACGACTTGAG	IGS-1	60°C
O-1	AGTCCTATGGCCGTGGAT	IGS-1	60°C
BMS05	TTAATTTTGGTTCNGATTGAACG	mtSSU	50°C
BMS55	GAGTGAACTAGCTAACTGAAATC	mtSSU	50°C
BMS65	CTGGTGCCAGAAGACTCGGTAA	mtSSU	50°C
BMS105	ATTAGTCGGTCTCGAAGCAAACG	mtSSU	50°C
BMS103	CACTTCGTTTGCTTCGAGACCGAC	mtSSU	50°C
BMS113	GACAGCCATGCAACACCTGTA	mtSSU	50°C
BMS153	CGACAGACAGTTAGTTCATCACG	mtSSU	50°C
BMS173	TGCTATGACTTTTGAGATGTTAC	mtSSU	50°C
SSUF*	GATAGGGTAACGGCCTACCAAG	mtSSU	50°C
SSUR*	CACCAGATTTGGACGACACTC	mtSSU	50°C

*Primers that were designed in this study.

Table 3: Basidiospore biometrics of representative *Ganoderma* isolates from Pretoria.

Isolate	Average length (µm)	Average width (µm)	Size (µm)	Colour
PTA1	12.4	8.2	(11–)12–13 × (7–)8–9	Brown
PTA3	12.5	8.3	12–13(–14) × (7–)8–9	Brown
PTA4	12.6	8.4	(11–)12–13(–14) × (7–)8–9	Brown
PTA5	12.3	8.0	(11–)12–13 × (7–)8–9	Brown
PTA6	12.4	7.9	(12–)12–13 × (7–)8–8(–9)	Brown
PTA7	12.3	8.0	(11–)12–13(–14) × (7–)8–9	Brown
PTA8	12.1	8.0	(11–)12–13 × (7–)8–8(–9)	Brown
PTA9	12.3	8.2	(11–)12–13 × (7–)8–9	Brown
PTA10	12.5	8.3	(11–)12–13(–14) × (7–)8–9	Brown
PTA11	12.4	8.3	12–13 × (7–)8–9	Brown
PTA22	12.4	8.2	(11–)12–13(–14) × (7–)8–9	Brown

Figure 1: Sampling position of isolates in Brooklyn. Dots indicate trees that were sampled.

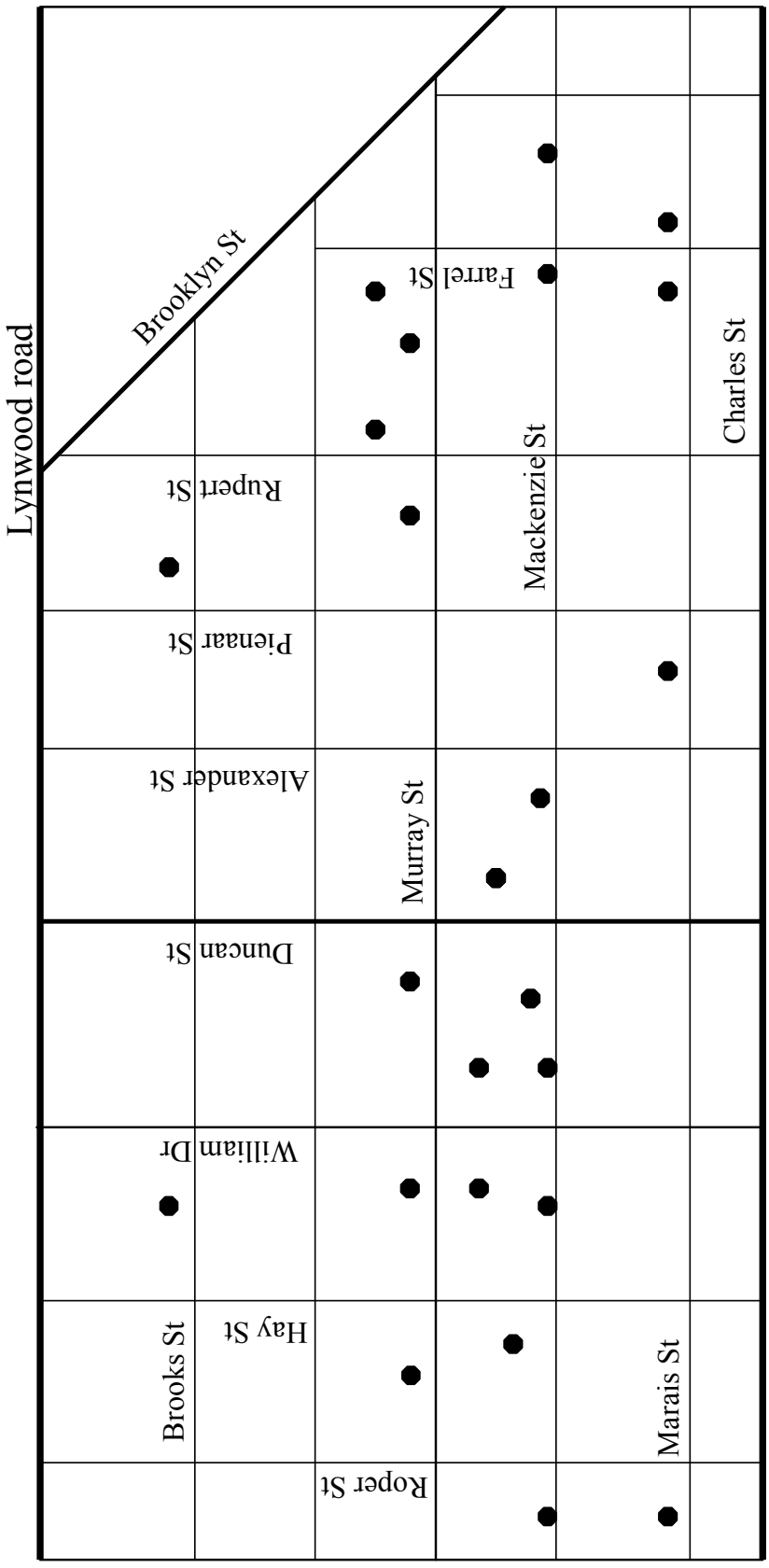

Lynwood road
Brooklyn St
Ruper St
Pienaar St
Alexander St
Duncan St
William Dr
Hay St
Brooks St
Roper St
Murray St
Mackenzie St
Farrel St
Marais St
Charles St

Figure 2: Die-back of *J. mimosifolia* as a symptom of root rot caused by *Ganoderma* species (Photos: Martin Coetzee).

Figure 3: Location of the primer binding sites used in PCR amplification of mt SSU rDNA (Hong and Jung, 2004 and also *primers designed in this study).

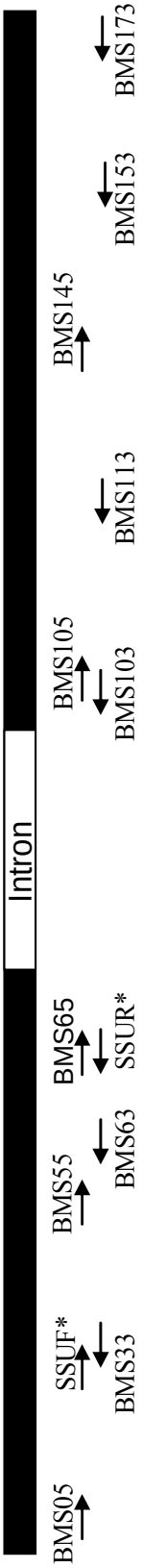

BMS05
SSUF*
BMS33
BMS55
BMS63
BMS65
SSUR*
Intron
BMS105
BMS103
BMS145
BMS113
BMS153
BMS173

Figure 4: Basidiocarps of *Ganoderma* isolates obtained from *Jacaranda mimosifolia* in Pretoria (Photos: Martin Coetzee).

Figure 5: Morphology of basidiospores of *Ganoderma* isolates from Pretoria. Inter-wall pillars (arrow) are well defined. Bars represent 10µm.

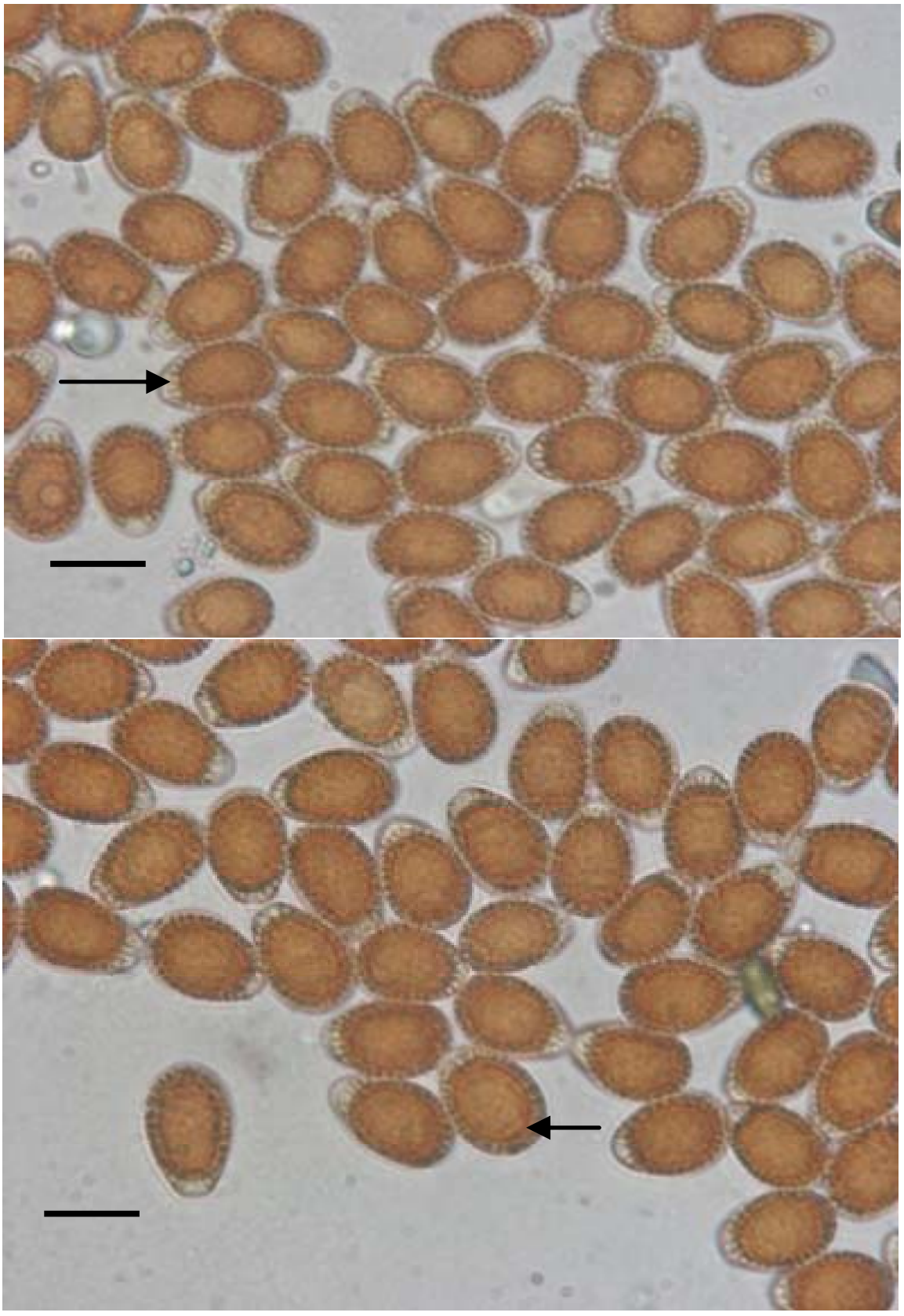


Figure 6: Picture of a 2% agarose gel stained with ethidium bromide to showing ITS PCR fragments for *Ganoderma* isolates from Pretoria amplified using primers ITS1 and ITS4 (Lanes 1-22). A 100 bp high molecular marker was loaded in lanes labeled M. The band sizes are indicated at the sides of the gel in base pairs.

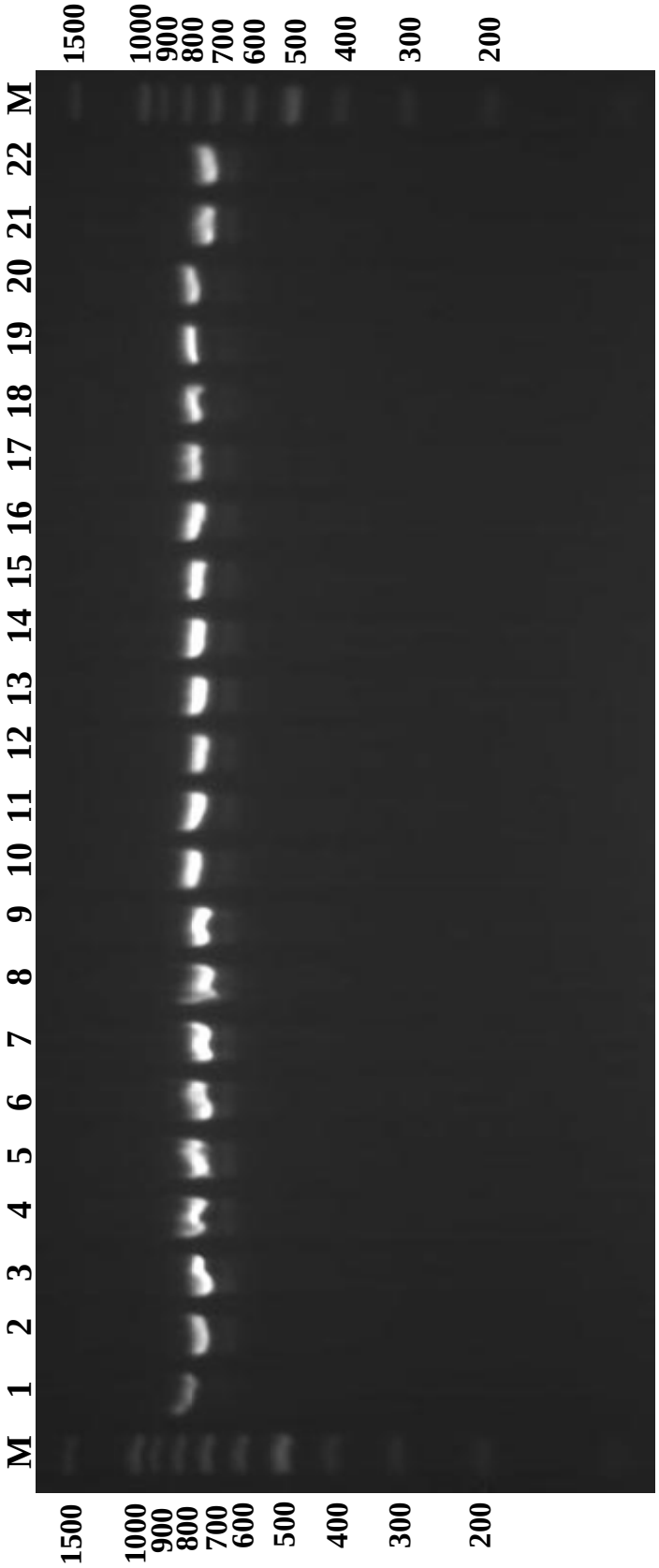

1500
1000
900
800
700
600
500
400
300
200
M
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
M
1500
1000
900
800
700
600
500
400
300
200

Figure 7: Picture of a 2% ethidium bromide stained agarose gel showing IGS-1 PCR fragments for the isolates used in this study. Lanes 1-22 show the PCR fragments for isolates from Pretoria. A 100 bp high molecular marker was loaded in lanes labeled M. The band sizes are indicated in base pairs at the sides of the gel.

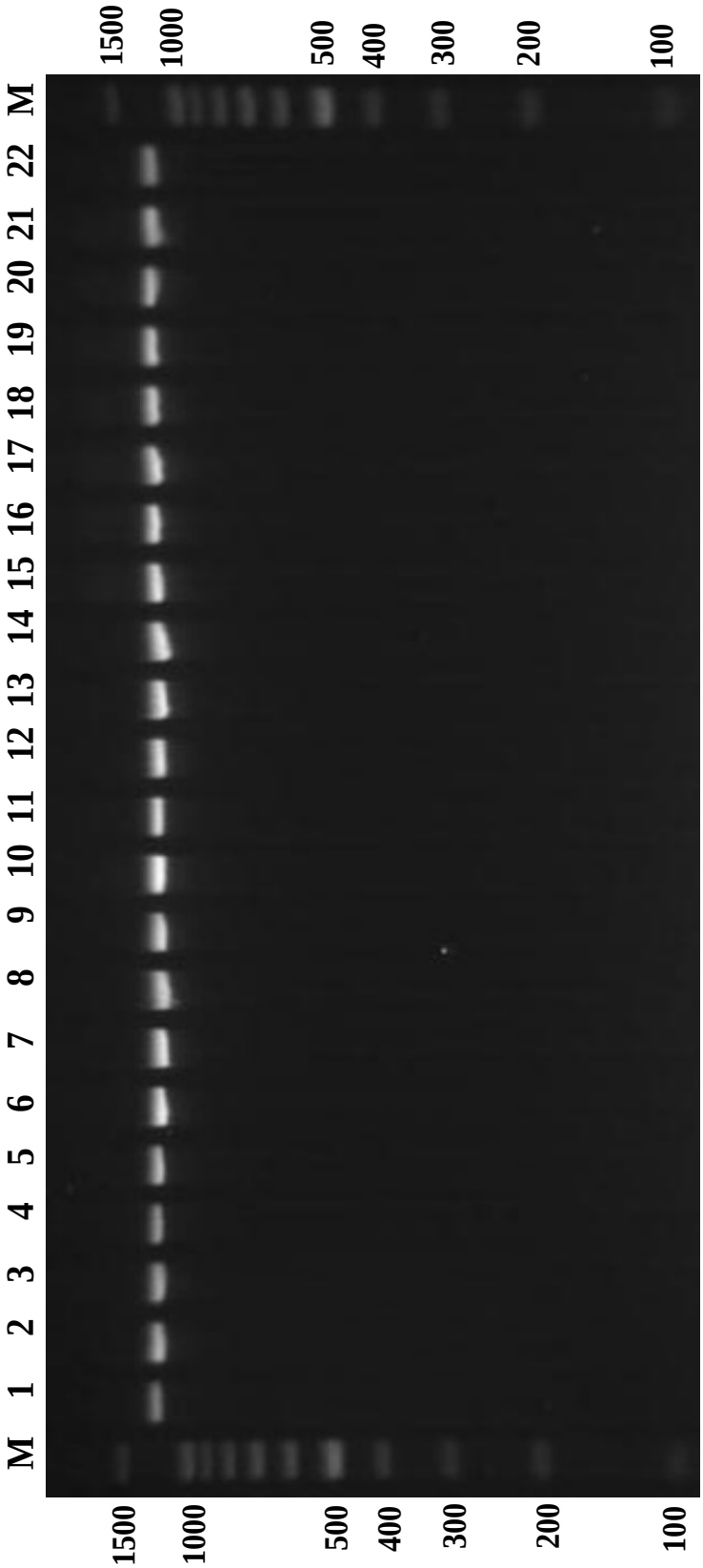
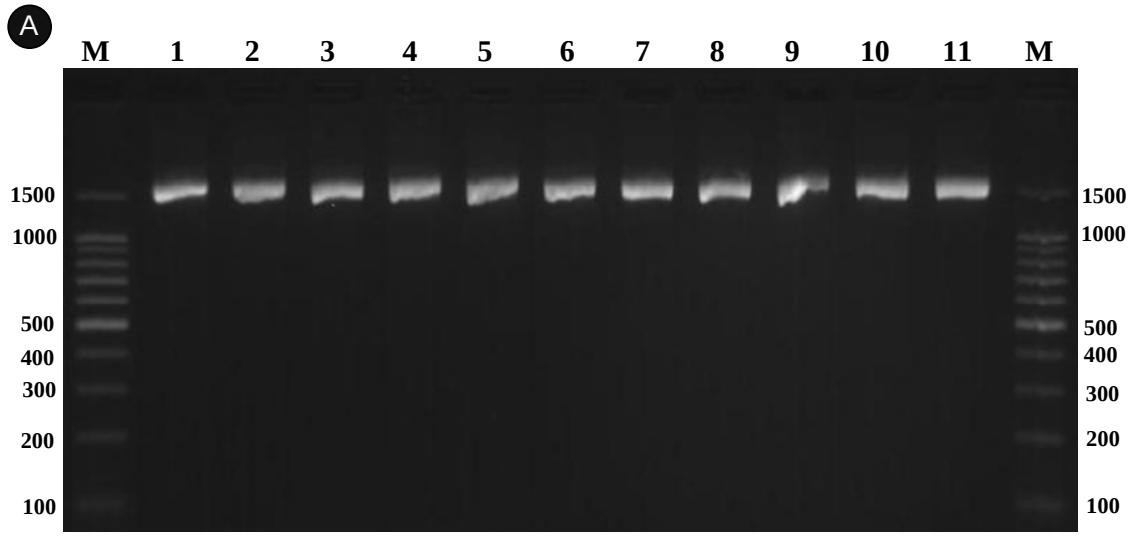

1500
1000
500
400
300
200
100
M
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
M
1500
1000
500
400
300
200
100

Figure 8: Pictures of an ethidium bromide stained agarose gels showing the mtSSU PCR fragments amplified with primers BMS05 and BMS173. A) A 2% agarose gel showing fragments for half of the isolates that produced 1.5Kb fragment size (Lanes 1-11). Lanes labeled M contains a 100 base pair molecular weight marker. The fragment sizes are indicated in base pairs at the sides of the gel. B) Agarose gel (1%) showing a 3.0Kb fragment produced for the other half of *Ganoderma* isolates from Pretoria. A Lamda DNA (EcoRI+HindIII) marker was loaded in lanes labeled M and its fragment sizes are indicated in base pairs.


A
M 1 2 3 4 5 6 7 8 9 10 11 M
1500
1000
500
400
300
200
100

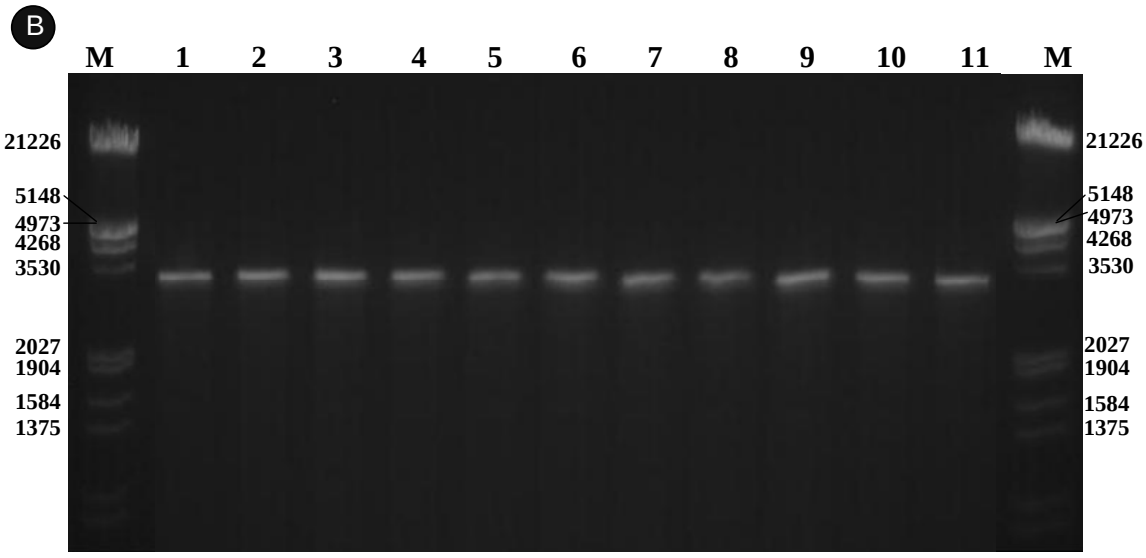

B
M 1 2 3 4 5 6 7 8 9 10 11 M
21226
5148
4973
4268
3530
2027
1904
1584
1375

Figure 9: Phylogram generated after distance and maximum likelihood analyses of the ITS sequence data for isolates from Pretoria and other *Ganoderma* species from GenBank. Bootstrap support values are indicated above the branches for maximum likelihood, below the branches for neighbor-joining analysis. The scale bar indicates the number of substitution per site. The tree was rooted with *G. lipsiense* and *G. applanatum.*

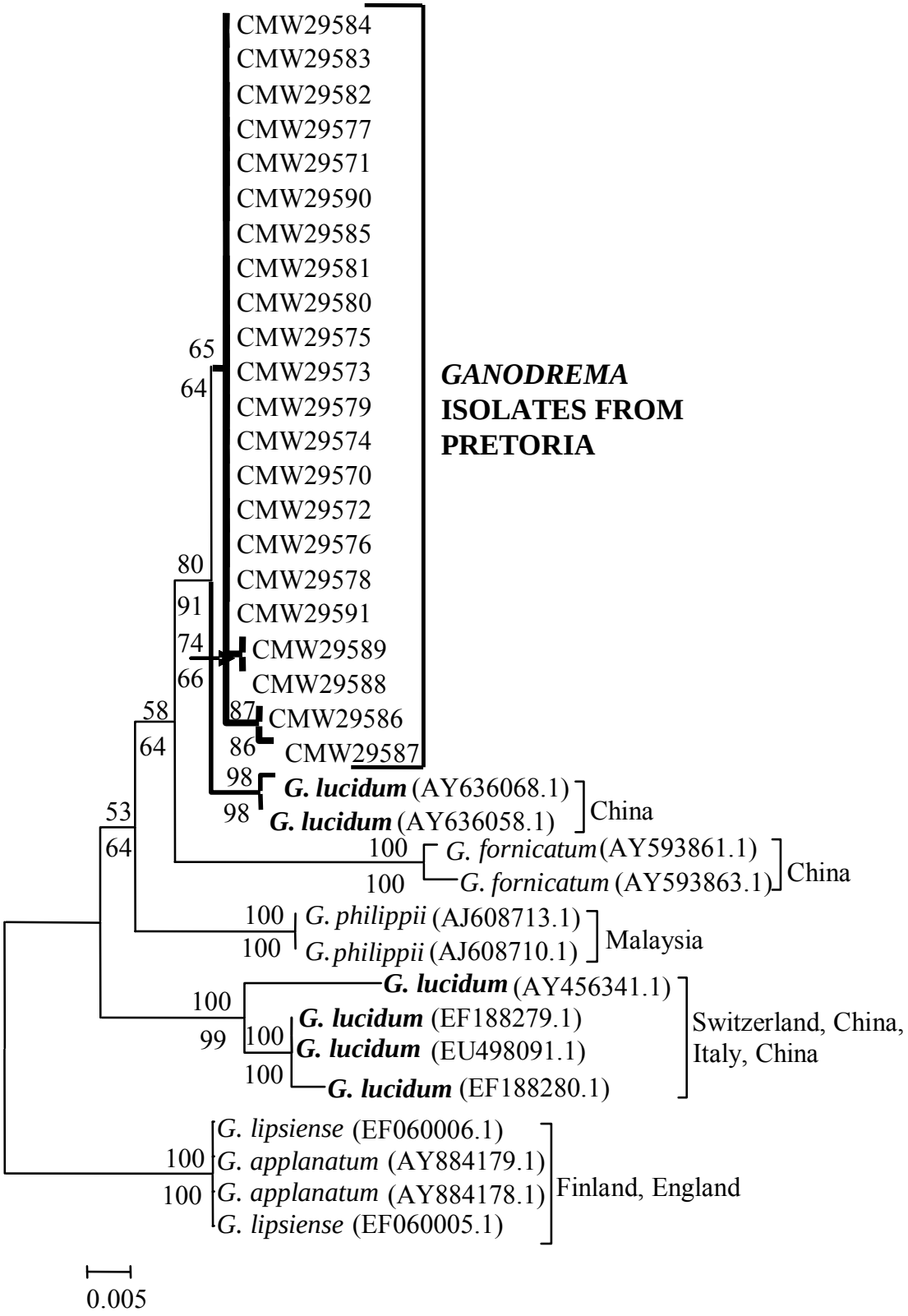

CMW29584
CMW29583
CMW29582
CMW29577
CMW29571
CMW29590
CMW29585
CMW29581
CMW29580
CMW29575
CMW29573
CMW29579
CMW29574
CMW29570
CMW29572
CMW29576
CMW29578
CMW29591
CMW29589
CMW29588
CMW29586
CMW29587
GANODREMA ISOLATES FROM PRETORIA
G. lucidum (AY636068.1)
G. lucidum (AY636058.1)
China
G. fornicatum (AY593861.1)
G. fornicatum (AY593863.1)
China
G. philippii (AJ608713.1)
G. philippii (AJ608710.1)
Malaysia
G. lucidum (AY456341.1)
G. lucidum (EF188279.1)
G. lucidum (EU498091.1)
G. lucidum (EF188280.1)
Switzerland, China, Italy, China
G. lipsiense (EF060006.1)
G. applanatum (AY884179.1)
G. applanatum (AY884178.1)
G. lipsiense (EF060005.1)
Finland, England
0.005

Figure 10: Phylogram generated after distance and maximum analyses of the mtSSU sequence data. Bootstrap support values are indicated above the branches for maximum likelihood, below the branches for neighbor-joining analysis. Isolates in Group A contained an intron except for CMW29574 and CMW29589. The scale bar indicates the number of substitutions per site. The tree is rooted to *G. applanatum.*

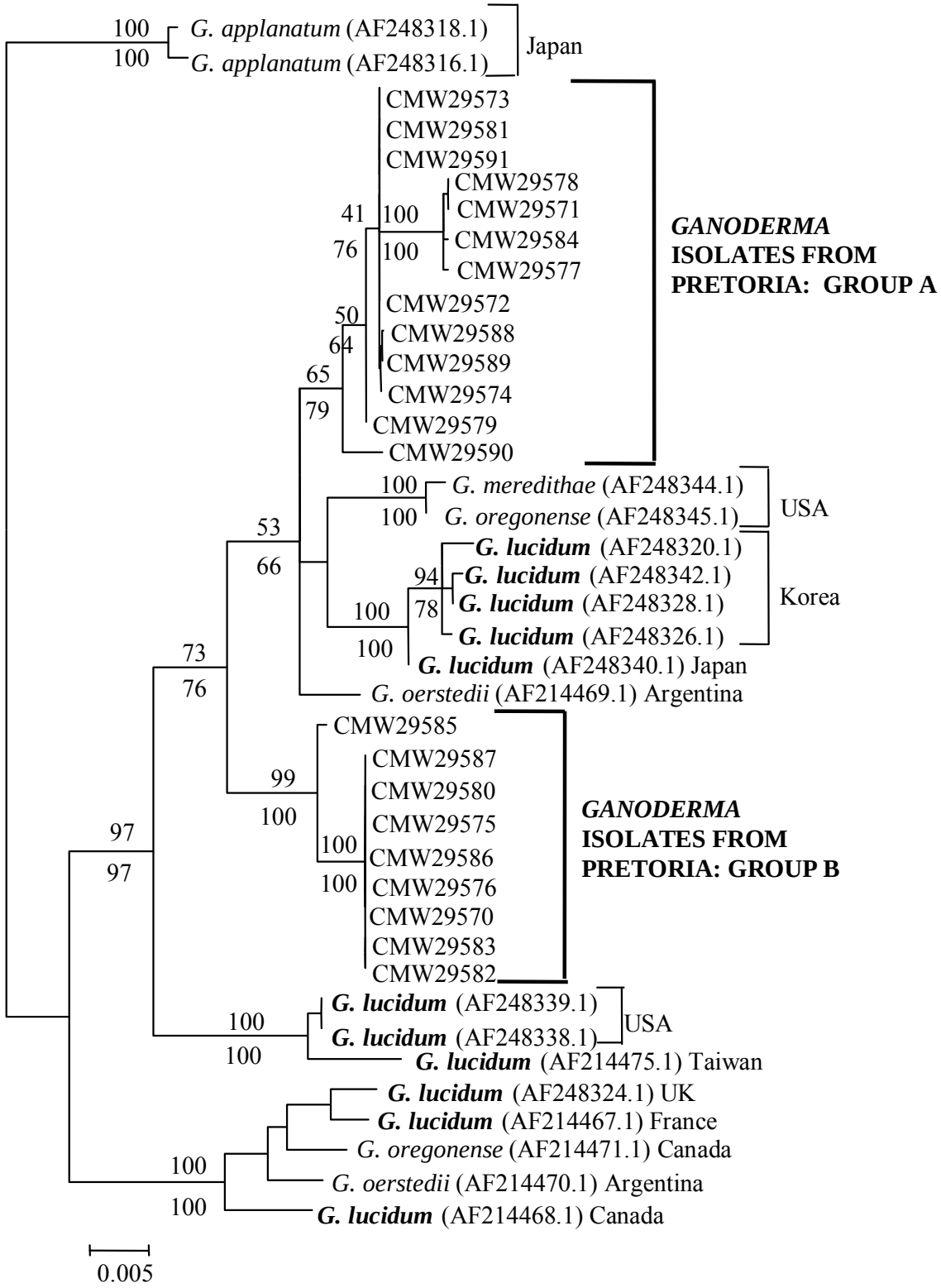
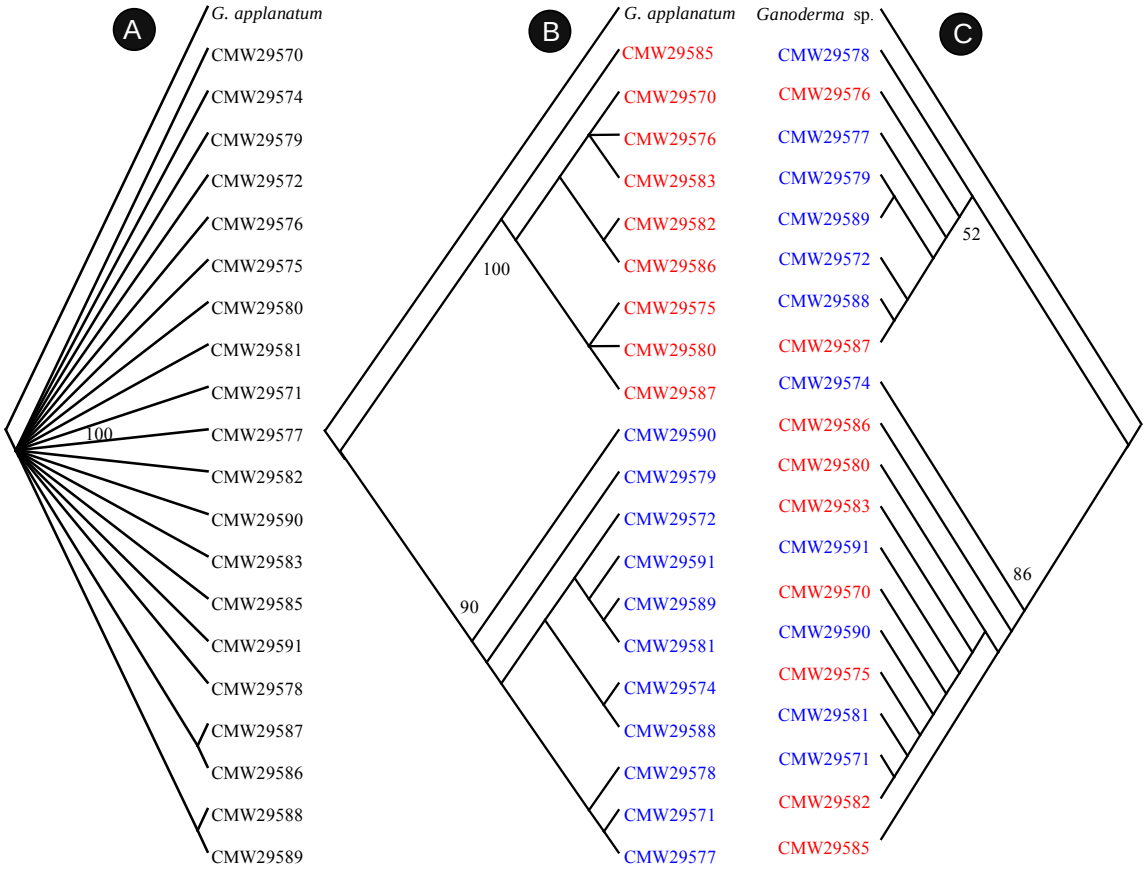

100
100
G. applanatum (AF248318.1)
G. applanatum (AF248316.1)
Japan
CMW29573
CMW29581
CMW29591
CMW29578
41
100
CMW29571
76
100
CMW29584
CMW29577
GANODERMA ISOLATES FROM PRETORIA: GROUP A
50
CMW29572
64
CMW29588
65
CMW29589
CMW29574
79
CMW29579
CMW29590
100
G. meredithae (AF248344.1)
100
G. oregonense (AF248345.1)
USA
53
66
G. lucidum (AF248320.1)
94
G. lucidum (AF248342.1)
100
78
G. lucidum (AF248328.1)
Korea
100
G. lucidum (AF248326.1)
G. lucidum (AF248340.1) Japan
73
76
G. oerstedii (AF214469.1) Argentina
CMW29585
CMW29587
99
CMW29580
100
CMW29575
97
100
CMW29586
97
100
CMW29576
CMW29570
CMW29583
CMW29582
GANODERMA ISOLATES FROM PRETORIA: GROUP B
G. lucidum (AF248339.1)
100
G. lucidum (AF248338.1)
USA
100
G. lucidum (AF214475.1) Taiwan
G. lucidum (AF248324.1) UK
G. lucidum (AF214467.1) France
G. oregonense (AF214471.1) Canada
100
G. oerstedii (AF214470.1) Argentina
100
G. lucidum (AF214468.1) Canada
0.005

Figure 11: Cladograms based on the distance analysis of the A) ITS sequence data, B) mtSSU sequence data, and C) IGS-1 sequence data indicating the incongruity of the three genes used. Isolates belonging to one group in the mtSSU tree are indicated by either blue or red font colour and in the IGS-1 tree, blue or red font colour indicate isolates that belong to the same group in the mtSSU tree being scattered over the tree. The trees are rooted with *G. applanatum* (A and B) and *Ganoderma* sp. (C).


A
G. applanatum
CMW29570
CMW29574
CMW29579
CMW29572
CMW29576
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Ganoderma sp.
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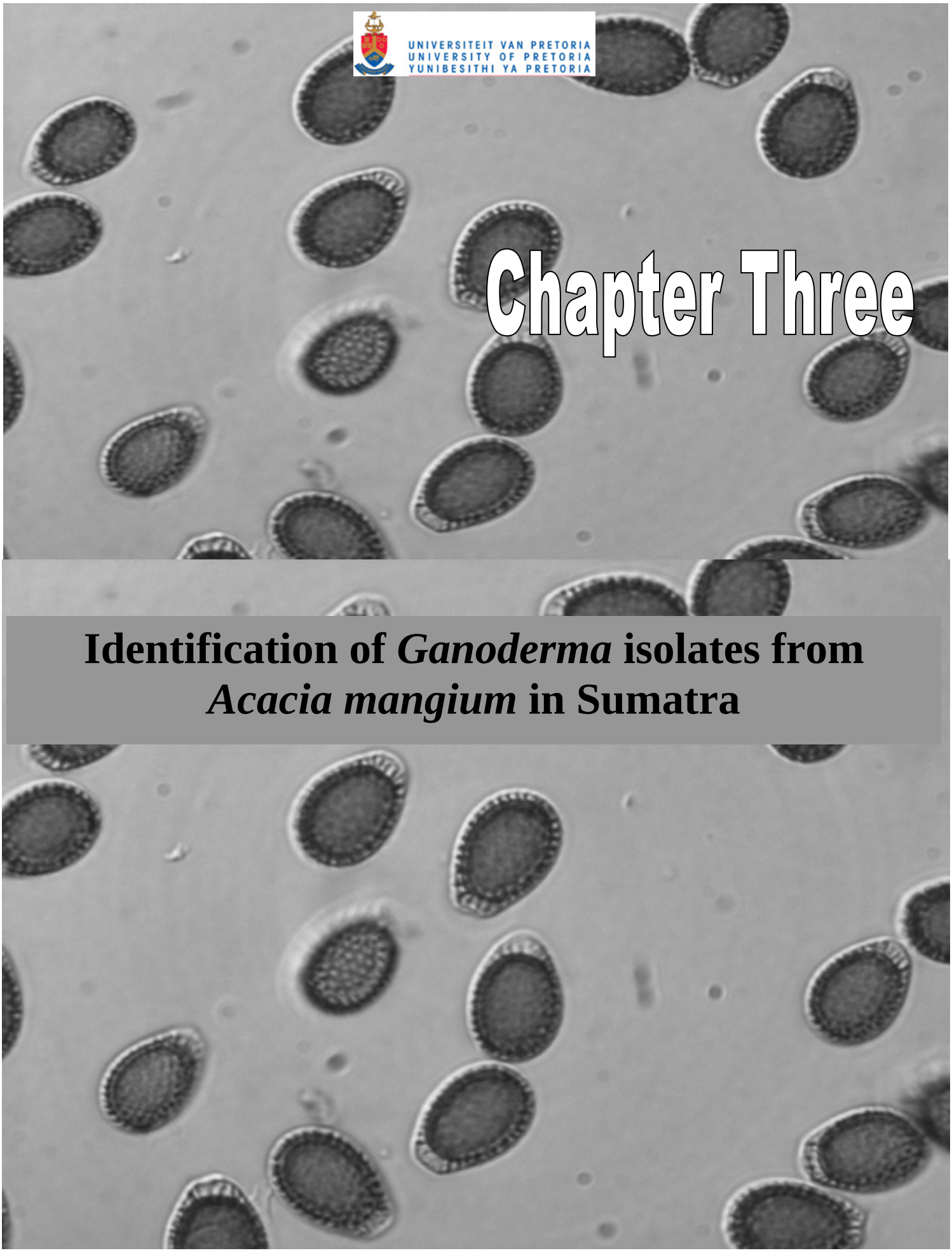

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Chapter Three

Identification of *Ganoderma* isolates from *Acacia mangium* in Sumatra

Identification of *Ganoderma* isolates from *Acacia mangium* in Sumatra

ABSTRACT

Ganoderma root rot is a significant threat to commercially planted *Acacia mangium* and *A. crassicarpa* trees in plantations in Indonesia. Several *Ganoderma* species are known to cause root rot disease on various plant hosts, but the identity of the *Ganoderma* species causing disease in *Acacia* plantations in Sumatra is uncertain. The aim of this study was to characterise *Ganoderma* isolates associated with root rot of *A. mangium* in Sumatra. Root samples were collected from infected trees in Sumatra plantations and isolations were made from these on a basidiomycete-selective medium. DNA was isolated from each of these cultures and nucleotide sequences obtained for the ITS region (ITS-1, 5.8S and ITS-2) of the ribosomal RNA operon and used in DNA sequence comparisons and phylogenetic analyses. Phylogenetic comparisons using ITS sequences showed that there is a single *Ganoderma* sp. associated with Ganoderma root rot disease on *A. mangium* and this is *G. philippii.*

INTRODUCTION

There are approximately 1000 *Acacia* species native to Australia, Indonesia, Papua New Guinea (PNG) and some Pacific island nations (Potter *et al.,* 2006). Several species, including *A. mangium* Willd, are planted in South East Asia mainly for pulp production. The total area of tropical *Acacia* plantations established in China, Indonesia, Malaysia, PNG, the Philippines, Thailand, Vietnam, and on Melville Island in Australia is about 2 million hectares. Of these, approximately 1.2 million hectares are in Indonesia with the major species planted being *A. mangium* (Potter *et al.,* 2006).

Acacia mangium has been grown as a plantation species in Indonesia for a considerable time, where it was first used in fire breaks, land rehabilitation and reforestation of grasslands. The wood of this tree is used for the production of paper pulp, sawlogs and timber, which is utilised in furniture production and other higher-value products (Old *et al.,* 2000; Potter *et al*., 2006). Establishment of large-scale *A. mangium* plantations in Indonesia began in the early 1990's due to the rapid growth rate of the tree (Arisman and Hardiyanto, 2006).

Acacia plantations in Indonesia have been reported to be threatened by root rot diseases. These are considered to be the most economically damaging diseases affecting *A. mangium* (Almonicar, 1992; Lee, 2000; Old *et al.,* 2000; Potter *et al.,* 2006). Red, brown, black and white root rot diseases have been reported from *A. mangium* plantations. These diseases are associated with various basidiomycete fungi including *Amauroderma* cf. *parasiticum* (black rot), *Ganoderma* species (red rot), *Phellinus* species (brown rot), *Rigidoporus lignosus* (white rot) and *Tinctoporellus epimiltinus* (brown rot)

(Almonicar, 1992; Lee, 2000; Old *et al.,* 2000; Potter *et al.,* 2006). *Ganoderma* species have been reported as the most frequent causal agents of root rot of *A. mangium* in Malaysia and Indonesia (Lee 2000).

Ganoderma root rot is spread primarily through contact between diseased roots or infested woody debris and healthy roots (Turner, 1965; Ariffin *et al.,* 2000). When basidiocarps are formed, these fungi can disperse by means of basidiospores (Pilotti *et al*., 2003; Pilotti, 2005; Sanderson, 2005). The spores become incorporated into the soil and germinate from where the hyphae grow into the roots of the trees causing decay on the wood and bark (Schwarze and Ferner, 2003).

Ganoderma root rot can be recognised by a number of symptoms on infected trees that are usually in discrete patches due to spread by root contact (Figure 1). A mild to severe wilting of leaves, crown dieback and reduced growth are typically observed. Due to reduced water and mineral uptake, the foliage becomes chlorotic (pale green), sparse and reduced in size (Figure 1A). Depending on the environmental conditions, infected trees become very susceptible to wind throw and die gradually (Ariffin *et al.,* 2000; Old *et al*., 2000). Bracket shaped basidiocarps may be produced on the stems of infected trees (Figure 1B). In some cases, on fast growing plantation trees characteristic basidiocarps may be absent on diseased trees (Lee 2000, Old *et al*., 2000). Roots of infected trees are commonly covered with a reddish-brown rhizomorphic skin that becomes visible after the roots are cleaned (Figure 1C); and mycelium forming white mottled patterns is found on the underside of the bark (Figure 1D) (Almonicar, 1992; Lee 2000; Mohammed *et al*., 2006).

Surveys on disease incidence and mortality levels of *A. mangium* have previously been conducted in South Sumatran plantations in the Riau areas. Results from these surveys suggested that *G. philippii* is causing root rot on *A. mangium* (Mohammed *et al*., 2006). Given that the fruiting structures of numerous basidiomycetes including *Ganoderma* species are seen on the stems or exposed roots of dying trees, it is unknown whether the major root disease problem is caused by one or more than one *Ganoderma* species. Moreover, there is uncertainty as to the identity of the *Ganoderma* sp. involved. This study arose from the opportunity to isolate specifically from the roots of dying trees in South Sumatra *A. mangium* plantations in an attempt to determine whether one or more species of *Ganoderma* are involved in the disease problem and to identify the resulting isolates. This was achieved using sequence data from the internally transcribed spacer (ITS) of the ribosomal RNA operon.

MATERIALS AND METHODS

Sampling sites and fungal isolations

Root samples were collected from infected trees at five localities in Sumatra. These included compartments at Baserah, Logas North, Logas South, Teso East and Teso West (Figure 2). Fungal isolations were done by transferring small pieces of mycelium from infected roots to malt extract medium (MEA: 2% w/v malt extract, 1.5% w/v agar, Biolab, Midrand, South Africa) containing streptomycin sulphate (100mg/L, Sigma-Aldrich, Missouri, USA). The samples were incubated at room temperature until white aerial mycelia appeared. Cultures were purified by transferring small plugs of medium with hyphae to fresh MEA containing streptomycin. Cultures were subsequently grown

on potato dextrose agar medium (PDA: 0.4% w/v potato extract, 2% w/v dextrose, and 1.5% w/v agar, Biolab, Midrand, South Africa) at 24°C for 2 weeks for the purpose of DNA extraction. Isolates that were obtained during this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa (Table 1).

DNA extraction

DNA was extracted from 2-week-old cultures grown on PDA. Mycelial mats were transferred to Eppendorf tubes to which preheated (60-65°C) DNA extraction buffer [5% w/v cetyltrimethylammonium bromide (CTAB), 1.4M NaCl, 0.2% v/v 2-mercaptoethanol, 20mM EDTA pH 8, 10mM Tris-HCL pH 8, and 1% v/v polyvinylpolyrrolidone)] (700µL) was added. The mycelium was then homogenized using a Fast Prep (FP120 BIO 101 SAVANT) machine (Southern Cross Biotechnology, Cape Town, South Africa). The mycelium-suspension was subsequently frozen in liquid nitrogen and then incubated at 60°C for 1hour. This procedure was repeated twice after which the suspension was transferred to 1.5 mL Eppendorf tubes. Cell debris was removed by centrifugation (10000 rpm, 10 minutes), followed by several phenol: chloroform (1:1 v/v) extractions through centrifugation (10000 rpm, 10 minutes) to remove proteins. This was repeated until the interphase between the aqueous layer and the organic layer became clear. A final chloroform extraction was done to remove the phenol residue from the aqueous layer. Nucleic acids were precipitated from the aqueous layer using 96% ice-cold iso-propanol (0.6 v/v) and 3M sodium acetate (0.1 v/v, pH 5.2). The precipitate was collected by centrifugation (13000 rpm, 30 minutes) at 4°C, washed with ice cold 70% ethanol, dried and re-suspended in sterile distilled water. The DNA

obtained was quantified using a NANODROP (ND-1000) spectrophotometer (Nanodrop Technologies, Wilmington, USA).

PCR amplification

The ITS region (ITS-1, 5.8S, and ITS-2) of the ribosomal RNA operon was amplified using primer pair ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′GCTGCGTTCTTCATCGATGC3′) (White *et al.*, 1990). The PCR mixtures consisted of a final concentration of 5ng/µL genomic DNA, reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_2$, 50mM KCl, Roche Diagnostics, Randburg, South Africa), 2.5 µM of each dNTP (Fermentas Life Sciences, Pretoria, South Africa), 0.4 µM of each primer, and 0.5 units of *Taq* polymerase (FABI, University of Pretoria, South Africa). PCR reaction conditions were as follows: initial denaturation and activation of the enzyme at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, elongation at 72°C for 30 seconds, and completed by a final elongation at 72°C for 7 minutes. PCR products were analysed by 2% (w/v) agarose (Whitehead Scientific, Johannesburg, South Africa) gel electrophoresis and visualised by staining with ethidium bromide and UV illumination. Product size was estimated by comparison with a 100 bp-ladder DNA marker (Promega Corporation, Madison, WI, USA). Amplicons were purified from unincorporated nucleotides, excess primer and salts using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

DNA sequencing

DNA sequences were obtained from the purified PCR products in both directions using primers ITS1 and ITS4. Sequence reactions were carried out with the ABI Prism® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer, Warrington, UK) following the manufacturer's protocol. DNA sequences were obtained using an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA). Vector NTI version 10.3 (Invitrogen Corporation, Carlsbad, USA) was used for editing and assembling of sequences.

Cloning of ITS amplicons

ITS PCR products from isolates that yielded poor sequencing results were cloned into pGEM®-T vector. Cloning and transformation reactions utilised the pGEM®-T cloning kit (Promega Corporation, Madison, WI, USA) with *Escherichia coli* JM109 High Efficiency Competent Cells following the manufacturer's instructions. Positive insertion of the ITS fragments were verified by direct amplification from the transformed *E. coli* colonies using vector specific primers T7 (5′TAATACGACTCACTATAGGG3′) and SP6 (5′TATTTAGGTGACACTATAG3′). The PCR reaction mixture included reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_2$, 50mM KCl, Roche Diagnostics, Randburg, South Africa), 2.5 µM of each dNTP (Fermentas Life Sciences, Pretoria, South Africa), 0.4 µM of each primer, and 0.5 units of *Taq* polymerase (FABI, University of Pretoria, South Africa), and *E. coli* cells from positively transformed colonies (white) were used as a template. The PCR conditions included an initial denaturation at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds. A final elongation at 72°C

for 7 minutes was included to complete the reaction. PCR products were visualized and purified as described above. Cloned fragments from five to ten positively transformed colonies were selected for each isolate to be sequenced as described above but using the primers T7 and SP6.

Sequence analysis

The ITS sequences obtained were compared with other *Ganoderma* ITS sequences in GenBank using BLASTn (Basic Local Alignment Search Tool for nucleotides) searches. GenBank sequences that showed high similarity and coverage (from 95% upwards) with the sequences from Sumatra isolates were downloaded and included in this analysis for comparative purposes. DNA sequences were aligned using Multiple sequence Alignment based on Fast Fourier Transform (MAFFT) version 5 (Katoh *et al.*, 2005).

Phylogenetic analysis of the ITS sequence data was conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Tamura *et al*., 2007). The Kimura 2-parameter nucleotide substitution model was applied. A phylogenetic tree was generated using neighbour-joining tree building algorithm (Saitou and Nei, 1987). Confidence levels of the nodes were determined by bootstrap with 1000 replicates (Felsenstein, 1985) using the same substitution model.

RESULTS

Fungal isolates

A total of 183 isolates were obtained from infected *A. mangium* roots collected from different plantation sites in Sumatra (Table 1, Figure 2). The majority of isolates appeared as white with either flat or fluffy mycelium in culture. Few isolates had a brown fluffy morphology in culture.

PCR amplification and sequence analyses

The ITS region was successfully amplified for all 183 isolates and yielded PCR fragments of approximately 700 base pairs (bp) in length. Sequences were obtained for all 183 isolates. PCR products from some isolates were cloned due to polymorphisms that were detected from electropherograms with double peaks after sequencing with primers ITS1 and ITS4. Two types of the ITS sequences were obtained after sequencing the cloned fragments, indicating heterogeneity (Figure 3) of the ITS region in individual isolates. BLASTn searches revealed a high similarity (95% to 99%) between the ITS sequence for the majority of isolates used in this study with those for various *Ganoderma* species in GenBank. The highest similarity was with *G. philippii* (99%). Few isolates showed similarity with other basidiomycete species associated with root rot disease but with low similarity to *Ganoderma* species.

A phylogenetic tree generated from the ITS sequence data grouped the isolates from Sumatra in a strongly supported cluster (95% bootstrap support) with sequences representing *G. philippii* (AJ627584, AJ608710, and AJ608713) that originated from *A.*

mangium in Malaysia and Indonesia (Bougher unpublished, GenBank). Five isolates from Sumatra (CMW30066, CMW29744, CMW29998, CMW29844 and CMW29740) grouped outside this cluster. Isolate (CMW30066) grouped together with *G. mastoporum* (AJ627585.1) with a 99% bootstrap support while the rest grouped together with a sequence labelled as *Ganoderma* sp. but without bootstrap support (Figure 4).

DISCUSSION

In this study, a large number of isolates were collected specifically from the roots of dying *A. mangium* trees in South Sumatra. The collections were specifically focussed on determining whether there is one dominant pathogen associated with the disease and also to identify the fungus. The results showed clearly that a single species of *Ganoderma* is the major pathogen involved in wide-spread death of trees and that this is *G. philippii.*

Identification of *Ganoderma* species has traditionally been based on basidiocarp morphology. However, the employment of characters from the basidiocarps has led to many synonyms, species complexes and possible misidentification of species in the genus (Bazzalo and Wright, 1982; Adaskaveg and Gilbertson, 1986, 1988, 1989). This is due to the morphological plasticity of the basidiocarps and the few differences that are often displayed between species. In general, the taxonomy of *Ganoderma* is considered uncertain (Steyaert, 1972; Ryvarden, 1994; Gottlieb *et al.,* 2000). This makes morphological identification of the species that cause root rot disease difficult. Also, basidiocarps are often not available during field collection and identification using morphology is thus not an option. This has certainly led to the very large number of pathogen names associated with root rot of *Acacia* species in Indonesia. In this study,

and due to the availability of DNA sequencing to identify isolates, we were specifically able to identify the fungus most closely associated with freshly diseased roots of *A. mangium*. It thus seems certain that *G. philippii* is the primary pathogen of concern and future studies should focus primarily on it.

Various *Ganoderma* species have been associated with root rot disease on various hosts. Species reported to be the causal agent of root rot of *Acacia* species include *G. applanatum, G. australe, G. boninense, G. lucidum, G. multiplicatum, G. philippii, G. rotundatum* and *G. steyaertanum* (Lee, 2000; Sankaran *et al*., 2005; Irianto *et al.,* 2006). *Ganoderma philippii* and *G. lucidum* were identified as the causal agents of root rot of *A. mangium* in Southeast Asia (Lee 2000; Irianto *et al.,* 2006). The latter *Ganoderma* species were however, later shown to belong to *G. steyaertanum* (Smith and Sivasithamparam, 2003)

BLASTn results of the ITS sequence data from Sumatra isolates showed that they were similar to various *Ganodema* species, with the highest similarity being with *G. philippii*. A few isolates, however, were found to be highly similar to other basidiomycete species that are associated with various root rot diseases but with a low similarity to various *Ganoderma* species. In addition, some of *Ganoderma* isolates from Sumatra were found to have heterogeneous ITS sequences. Intrastrain ITS heterogeneity has not been frequently encountered but has been reported previously for *G. applanatum* var. *gibbosum, G. fornicatum, G. neojaponicum,* and *G. japonicum* (Wang and Yao, 2005).

The phylogram generated from neighbour-joining analysis of the ITS sequence data confirmed the results obtained from BLASTn searches. The isolates from Sumatra

grouped in one major clade together with sequences labelled as *G. philippii* reported from *A. mangium* in Malaysia and Indonesia. The isolates that showed low similarity to *Ganoderma* species in GenBank grouped outside the major group with one isolate grouping together with *G. mastoporum* while the rest closely related to a sequence labelled as *Ganoderma* sp.

Identification of *G. philippii* as the major cause of *A. mangium* root disease in Sumatra is not necessarily surprising. This fungus causes the disease known as red rot disease in a variety of perennial crops worldwide such as *Camellia sinensis* (tea), *Hevea brasiliensis* (rubber) and *Acacia* species (Chee, 1990; Lee, 2000). It is distributed throughout Southeast Asia and has been identified as one of the two major root rot fungi causing significant economic losses in *A. mangium* and rubber plantations in Indonesia and Malaysia (Chee, 1990; Lee, 2000). It is, therefore, not unexpected that this fungus would also be shown to be the causal agent of Acacia root rot disease in Indonesia.

The results of this study showed that *G. philippii* is the major species that is found associated with *A. mangium* trees affected by root rot diseases in the South Sumatran plantations investigated. However, there might be other root rot related basidiomycetes such as *Phellinus* species, but their effect is not currently as severe as that of *G. philippii*. Although pathogenicity tests were not conducted in this study, the trees in the sampling areas showed typical symptoms of root rot including red rhizomorphic skin on the roots which is caused by *Ganoderma* species and some of the trees were already dead. We are, therefore, confident that *G. philippii*, as the main species found associated with the trees, is the primary pathogen since it is a well-known pathogen of *A. mangium* in the Southeast Asia.

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Table1: List of *Ganoderma* isolates collected from *A. mangium* in different estates that were used in this study and their identity based on ITS sequence data.

Isolate number [a]	Culture number [b]	Estate	Species I.D based on ITS phylogeny and BLASTn
GSB-1.1	CMW29998	Baserah	*Ganoderma* sp.
GSB-1.2	CMW30060	Baserah	*G. philippii*
GSB-1.3	CMW29740	Baserah	*Ganoderma* sp.
GSB-1.4	CMW30061	Baserah	*G. philippii*
GSB-1.5*	CMW29843	Baserah	*G. philippii*
GSB-1.6*	CMW29907	Baserah	*G. philippii*
GSB-1.7	CMW29844	Baserah	*Ganoderma* sp.
GSB-2-1.3	CMW29745	Baserah	*G. philippii*
GSB-2-4.11*	CMW30062	Baserah	*G. philippii*
GSB-2-9.4	CMW29908	Baserah	*G. philippii*
GSB-2-9.5	CMW30006	Baserah	*G. philippii*
GSB-3-1.1	CMW29999	Baserah	*G. philippii*
GSB-3-1.6	CMW30000	Baserah	*G. philippii*
GSB-3-2.6	CMW30001	Baserah	*G. philippii*
GSB-3-3.2	CMW29748	Baserah	*G. philippii*
GSB-3-3.4	CMW29746	Baserah	*G. philippii*
GSB-3-3.8	CMW29943	Baserah	*G. philippii*
GSB-3-4.3	CMW29944	Baserah	*G. philippii*
GSB-4-10.7	CMW29751	Baserah	*G. philippii*
GSB-4-3.1	CMW29750	Baserah	*G. philippii*
GSB-4-3.7	CMW30063	Baserah	*G. philippii*
GSB-4-6.1	CMW30002	Baserah	*G. philippii*
GSB-4-6.3	CMW29743	Baserah	*G. philippii*
GSB-4-7.1	CMW30003	Baserah	*G. philippii*
GSB-4-9.3	CMW29744	Baserah	*Ganoderma* sp.
GSB-5-(-1.10)	CMW29847	Baserah	*G. philippii*
GSB-5-(-1.11)	CMW29848	Baserah	*G. philippii*
GSB-5-(-1.9)	CMW30064	Baserah	*G. philippii*
GSB-5-(-2.13)	CMW29845	Baserah	*G. philippii*
GSB-5-2.4	CMW30065	Baserah	*G. philippii*
GSB-5-2.7	CMW30004	Baserah	*G. philippii*
GSB-5-9.5	CMW30005	Baserah	*G. philippii*
GSL-1-10.9	CMW29856	Logas	*G. philippii*
GSL-1-12.4	CMW29857	Logas	*G. philippii*
GSL-1-3.11	CMW29950	Logas	*G. philippii*
GSL-1-3.9	CMW29910	Logas	*G. philippii*
GSL-1-4.12	CMW29788	Logas	*G. philippii*
GSL-1-5.13	CMW29792	Logas	*G. philippii*
GSL-1-7.2	CMW29789	Logas	*G. philippii*
GSL-1-7.3	CMW29790	Logas	*G. philippii*
GSL-1-8.4*	CMW29791	Logas	*G. philippii*
GSL-1-9.5	CMW29951	Logas	*G. philippii*
GSL-2-1.3	CMW29858	Logas	*G. philippii*
GSL-2-1.5	CMW30010	Logas	*G. philippii*
GSL-2-10.3	CMW29793	Logas	*G. philippii*
GSL-2-2.4	CMW29794	Logas	*G. philippii*
GSL-2-2.5	CMW29796	Logas	*G. philippii*
GSL-2-2.6	CMW29911	Logas	*G. philippii*
GSL-2-5.2	CMW29795	Logas	*G. philippii*
GSL-3-1.2	CMW29797	Logas	*G. philippii*
GSLM3	CMW30066	Logas	*G. mastoporum*

Table 1 (continued)

Isolate number [a]	Culture number [b]	Estate	Species I.D based on ITS phylogeny and BLASTn
GSL-4-10.1	CMW30067	Logas	*G. philippii*
GSL-4-10.5	CMW29804	Logas	*G. philippii*
GSL-4-11.4	CMW30024	Logas	*G. philippii*
GSL-4-12.3	CMW30011	Logas	*G. philippii*
GSL-4-13.2	CMW30068	Logas	*G. philippii*
GSL-4-16.4	CMW29805	Logas	*G. philippii*
GSL-4-16.5	CMW29952	Logas	*G. philippii*
GSL-4-17.6	CMW29860	Logas	*G. philippii*
GSL-4-3.3	CMW29803	Logas	*G. philippii*
GSL-4-4.6	CMW29912	Logas	*G. philippii*
GSL-4-7.1	CMW29802	Logas	*G. philippii*
GSL-5-11.3	CMW30069	Logas	*G. philippii*
GSL-5-11.4	CMW30012	Logas	*G. philippii*
GSL-5-13.4	CMW30070	Logas	*G. philippii*
GSL-5-13.5	CMW29955	Logas	*G. philippii*
GSL-5-14.3	CMW29861	Logas	*G. philippii*
GSL-5-14.5	CMW29913	Logas	*G. philippii*
GSL-5-15.4	CMW30071	Logas	*G. philippii*
GSL-5-15.7	CMW29815	Logas	*G. philippii*
GSL-5-2.5	CMW29953	Logas	*G. philippii*
GSL-5-2.6	CMW29954	Logas	*G. philippii*
GSL-5-4.3	CMW29806	Logas	*G. philippii*
GSL-5-4.4	CMW30013	Logas	*G. philippii*
GSL-5-4.6	CMW30014	Logas	*G. philippii*
GSL-5-5.3	CMW30015	Logas	*G. philippii*
GSL-5-5.5	CMW30016	Logas	*G. philippii*
GSL-5-7.3	CMW30017	Logas	*G. philippii*
GSL-5-8.3	CMW30018	Logas	*G. philippii*
GSL-5-8.4	CMW30072	Logas	*G. philippii*
GSL-5-9.3	CMW30074	Logas	*G. philippii*
GSL-6-(-3.6)	CMW30019	Logas	*G. philippii*
GSL-6-(-4.6)	CMW29807	Logas	*G. philippii*
GSL-6-(-7.7)	CMW30020	Logas	*G. philippii*
GSL-6-2.3	CMW30075	Logas	*G. philippii*
GSL-6-2.4	CMW30076	Logas	*G. philippii*
GSL-6-4.4	CMW30078	Logas	*G. philippii*
GSL-6-5.10	CMW29813	Logas	*G. philippii*
GSL-6-5.5	CMW30021	Logas	*G. philippii*
GSL-6-5.9	CMW30022	Logas	*G. philippii*
GSL-6-6.10	CMW29814	Logas	*G. philippii*
GSL-6-6.3	CMW30079	Logas	*G. philippii*
GSL-6-6.7	CMW30080	Logas	*G. philippii*
GSL-6-6.9	CMW30081	Logas	*G. philippii*
GSL-7-1.2	CMW30023	Logas	*G. philippii*
GSL-7-1.6	CMW29863	Logas	*G. philippii*
GSL-7-10.11	CMW29811	Logas	*G. philippii*
GSL-7-10.3	CMW29808	Logas	*G. philippii*
GSL-7-10.4	CMW30082	Logas	*G. philippii*
GSL-7-2.3	CMW30025	Logas	*G. philippii*
GSL-7-5.2	CMW29812	Logas	*G. philippii*
GSL-7-5.3	CMW29864	Logas	*G. philippii*
GSL-7-5.4	CMW29914	Logas	*G. philippii*
GSL-7-6.3	CMW29810	Logas	*G. philippii*
GSL-7-6.8	CMW30026	Logas	*G. philippii*
GSL-7-7.7	CMW30027	Logas	*G. philippii*

Table 1 (continued)

Isolate number [a]	Culture number [b]	Estate	Species I.D based on ITS phylogeny and BLASTn
GSL-7-7.9	CMW29865	Logas	*G. philippii*
GSL-7-8.8	CMW30083	Logas	*G. philippii*
GSL-8-1.4	CMW29816	Logas	*G. philippii*
GSL-8-2.1	CMW29915	Logas	*G. philippii*
GSL-8-2.9	CMW29866	Logas	*G. philippii*
GSL-8-3.2	CMW29817	Logas	*G. philippii*
GSL-8-3.3	CMW30084	Logas	*G. philippii*
GSL-8-3.8	CMW29867	Logas	*G. philippii*
GSL-8-4.2	CMW29820	Logas	*G. philippii*
GSL-8-4.8	CMW30029	Logas	*G. philippii*
GSL-8-5.3	CMW29868	Logas	*G. philippii*
GSL-8-5.7	CMW30030	Logas	*G. philippii*
GSL-8-5.9	CMW29818	Logas	*G. philippii*
GSL-8-6.2	CMW30085	Logas	*G. philippii*
GSTE-1-2.3*	CMW29784	Teso East	*G. philippii*
GSTE-1-4.12	CMW29788	Teso East	*G. philippii*
GSTE-1-4.6	CMW29782	Teso East	*G. philippii*
GSTE-2-2.1	CMW29945	Teso East	*G. philippii*
GSTE-2-4.2	CMW29916	Teso East	*G. philippii*
GSTE-2-4.4	CMW29859	Teso East	*G. philippii*
GSTE-2-5.3	CMW30086	Teso East	*G. philippii*
GSTE-3-1.3	CMW30087	Teso East	*G. philippii*
GSTE-3-3.10	CMW30088	Teso East	*G. philippii*
GSTE-3-6.8	CMW29783	Teso East	*G. philippii*
GSTE-3-9.3	CMW29786	Teso East	*G. philippii*
GSTE-4-(-1.9)	CMW29781	Teso East	*G. philippii*
GSTE-4-2.9	CMW30089	Teso East	*G. philippii*
GSTE-4-4.1	CMW29785	Teso East	*G. philippii*
GSTE-4-4.7	CMW29946	Teso East	*G. philippii*
GSTE-4-4.8	CMW30009	Teso East	*G. philippii*
GSTE-4-5.6	CMW30090	Teso East	*G. philippii*
GSTE-4-7.12	CMW29780	Teso East	*G. philippii*
GSTW-6-1.2	CMW29947	Teso West	*G. philippii*
GSTW-6-1.3	CMW29763	Teso West	*G. philippii*
GSTW-6-1.5	CMW29755	Teso West	*G. philippii*
GSTW-6-3.3	CMW29762	Teso West	*G. philippii*
GSTW-6-4.10	CMW30007	Teso West	*G. philippii*
GSTW-6-4.3	CMW29917	Teso West	*G. philippii*
GSTW-6-5.10	CMW29948	Teso West	*G. philippii*
GSTW-6-7.6	CMW29756	Teso West	*G. philippii*
GSTW-6-9.8	CMW29761	Teso West	*G. philippii*
GSTW-7-(-1.20)	CMW29765	Teso West	*G. philippii*
GSTW-7-(-2.4)	CMW29766	Teso West	*G. philippii*
GSTW-7-1.1	CMW29764	Teso West	*G. philippii*
GSTW-7-1.2*	CMW29918	Teso West	*G. philippii*
GSTW-7-1.4	CMW29919	Teso West	*G. philippii*
GSTW-7-2.9	CMW29769	Teso West	*G. philippii*
GSTW-7-3.14	CMW29768	Teso West	*G. philippii*
GSTW-7-3.4	CMW29767	Teso West	*G. philippii*
GSTW-7-3.9*	CMW29754	Teso West	*G. philippii*
GSTW-7-4.19	CMW29770	Teso West	*G. philippii*
GSTW-7-4.22	CMW29771	Teso West	*G. philippii*
GSTW-8-(-1.13)	CMW29922	Teso West	*G. philippii*
GSTW-8-(-1.3)	CMW29851	Teso West	*G. philippii*
GSTW-8-(-10.3)	CMW29921	Teso West	*G. philippii*

Table 1 (continued)

Isolate number [a]	Culture number [b]	Estate	Species I.D based on ITS phylogeny and BLASTn
GSTW-8-(-2.2)	CMW29850	Teso West	*G. philippii*
GSTW-8-(-3.2)	CMW30091	Teso West	*G. philippii*
GSTW-8-(-9.3)	CMW29852	Teso West	*G. philippii*
GSTW-8-1.1*	CMW29753	Teso West	*G. philippii*
GSTW-8-1.2	CMW29849	Teso West	*G. philippii*
GSTW-8-2.1*	CMW29757	Teso West	*G. philippii*
GSTW-8-2.5	CMW29920	Teso West	*G. philippii*
GSTW-8-4.1	CMW29773	Teso West	*G. philippii*
GSTW-8-5.2	CMW29772	Teso West	*G. philippii*
GSTW-8-5.7*	CMW29774	Teso West	*G. philippii*
GSTW-8-6.6	CMW29758	Teso West	*G. philippii*
GSTW-9-1.10	CMW29776	Teso West	*G. philippii*
GSTW-9-1.1	CMW29775	Teso West	*G. philippii*
GSTW-9-1.5	CMW29854	Teso West	*G. philippii*
GSTW-9-1.7	CMW29759	Teso West	*G. philippii*
GSTW-9-2.2	CMW29778	Teso West	*G. philippii*
GSTW-9-2.6	CMW29853	Teso West	*G. philippii*
GSTW-9-2.8	CMW29777	Teso West	*G. philippii*
GSTW-9-4.1	CMW29949	Teso West	*G. philippii*
GSTW-9-4.2	CMW30008	Teso West	*G. philippii*
GSTW-9-4.3	CMW29855	Teso West	*G. philippii*
GSTW-9-4.5	CMW29779	Teso West	*G. philippii*

[a] The isolate numbers refers to the name given during collection of isolates

[b] CMW refers to the culture collection of the FABI, University of Pretoria.

* These isolates were found to contain heterogeneity in the ITS region.

Figure 1: Symptoms of Ganoderma root rot. A) Reduction of foliage growth and density with leaves becoming pale green or yellow. B) Fruiting bodies of *Ganoderma*. C) Red rhizomorphs on the roots (indicated by arrow). D) White mottled mycelium under the bark (indicated by arrow).

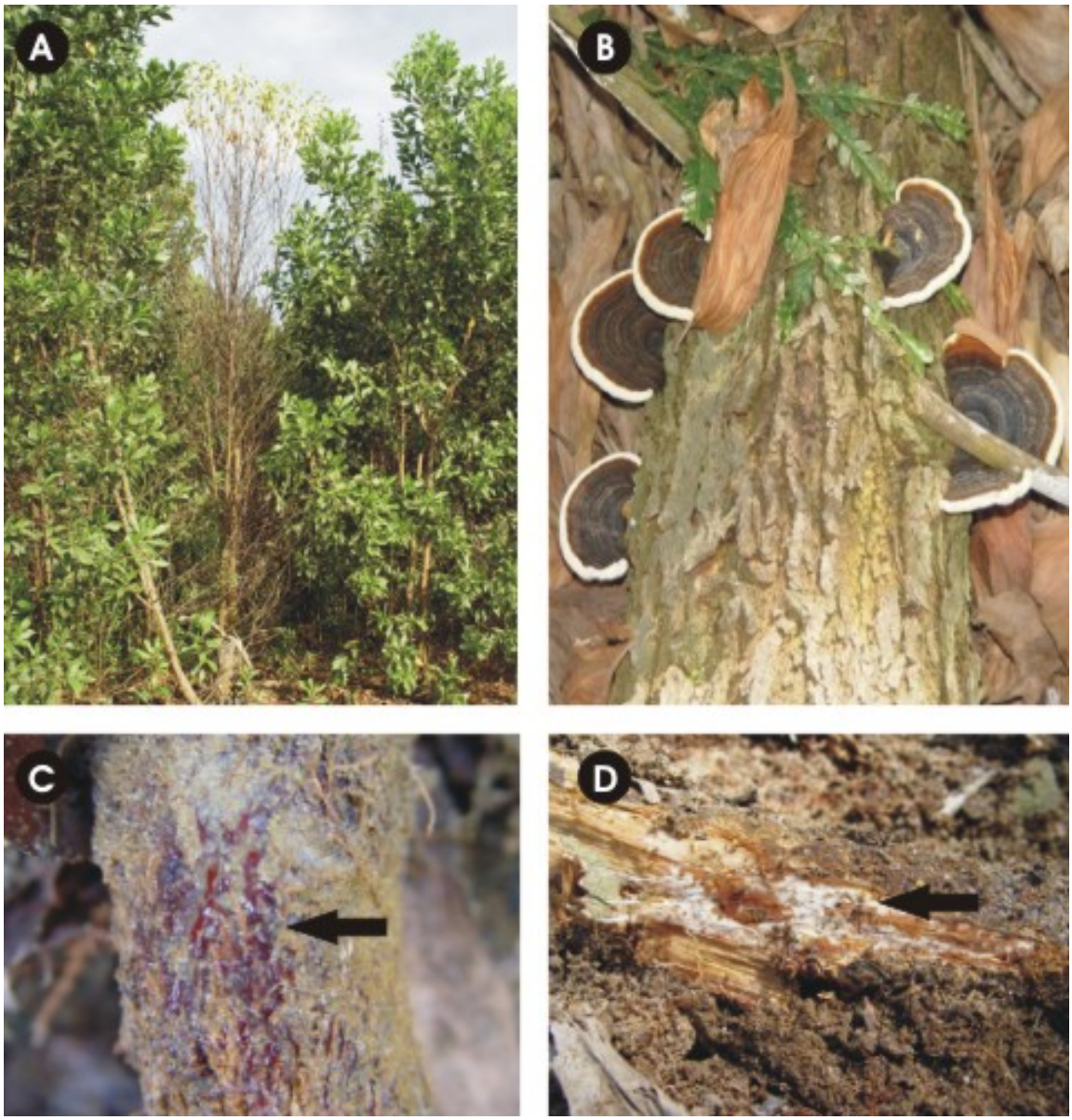

A
B
C
D

Figure 2: Relative geographic position of estates from which root samples were collected in Sumatra RAPP plantations. Filled circles indicate compartments that were sampled.

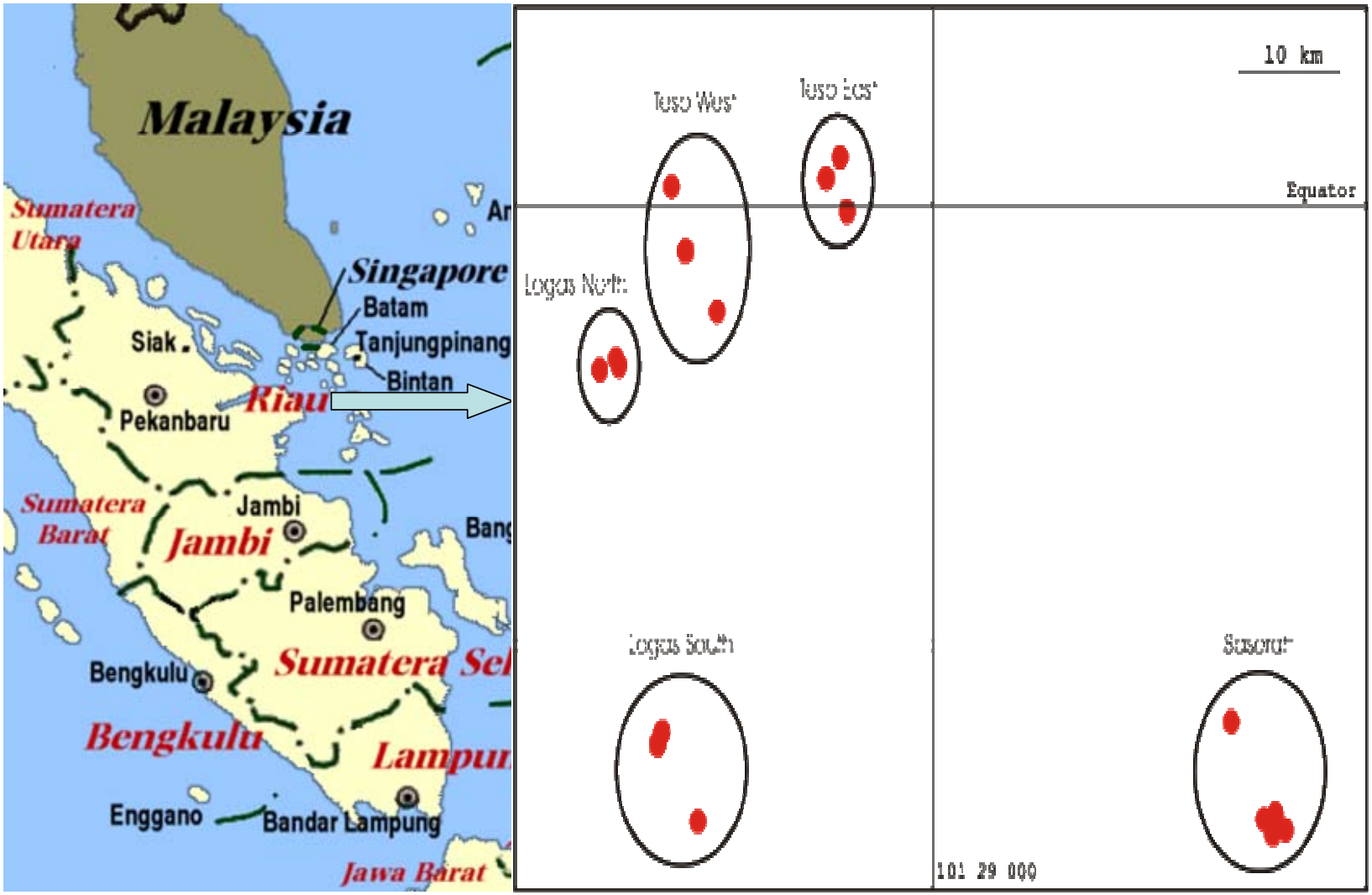


Figure 3: Aligned DNA sequences for a portion of the ITS region generated from cloned fragments of one of the Sumatran isolates. Dots (.) indicate nucleotides that are identical to those for the first sequences. Nucleotide base substitution (highlighted in grey) indicate heterogeneity in the ITS region.

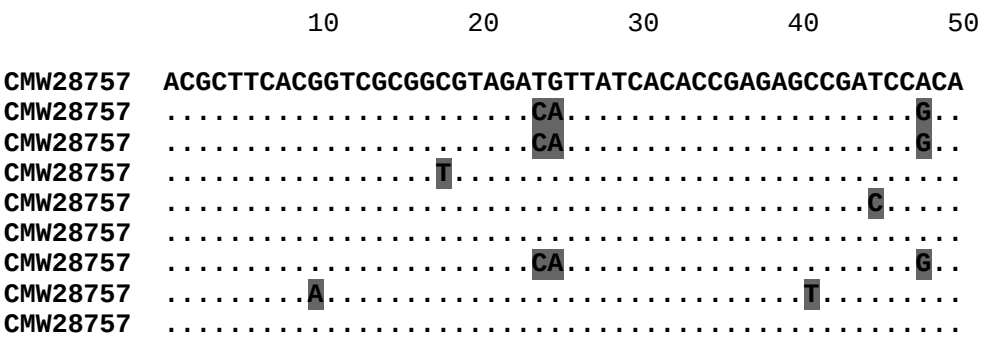
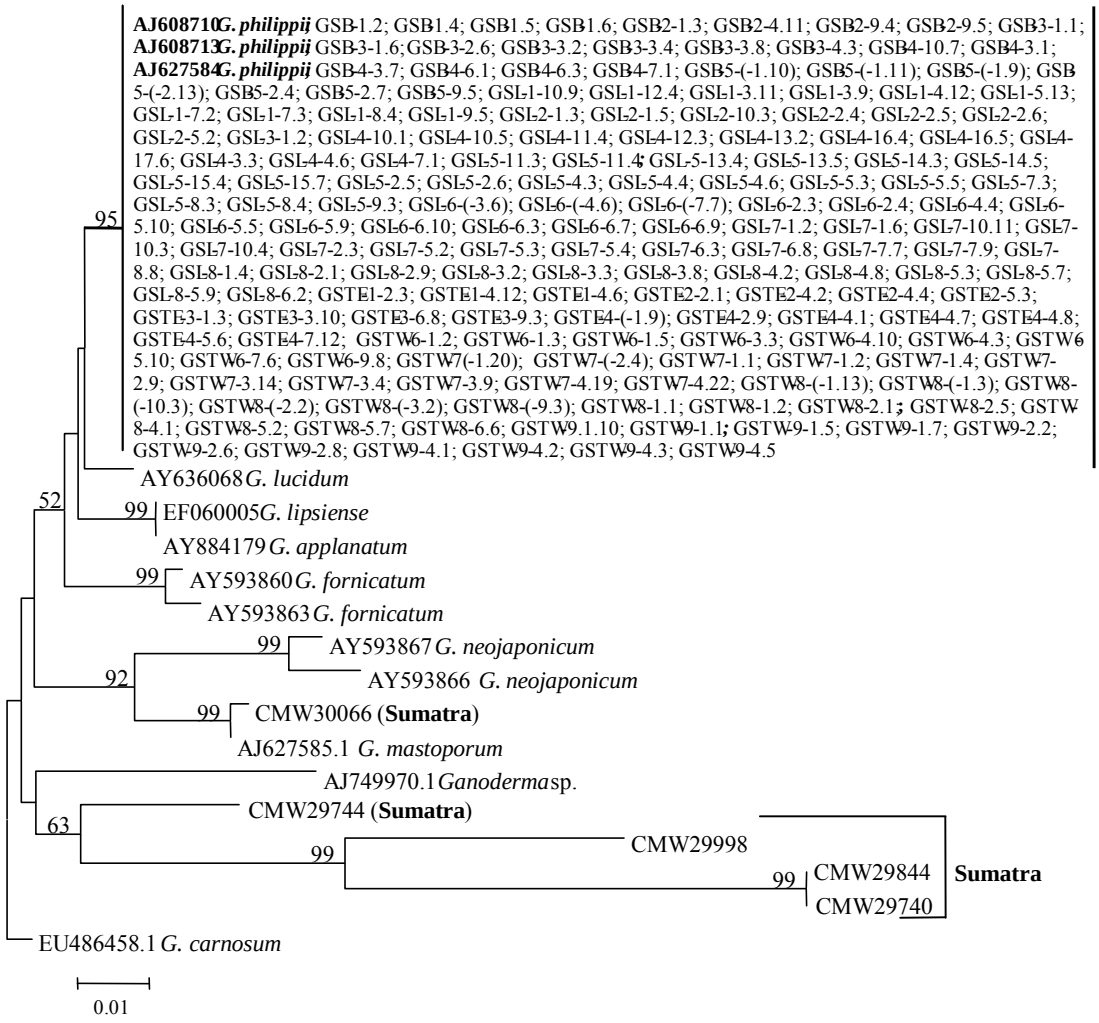

10
20
30
40
50
CMW28757 ACGCTTCACGGTCGCGGCGTAGATGTTATCACACCGAGAGCCGATCCACA
CMW28757 ..........CA..........G..
CMW28757 ..........CA..........G..
CMW28757 ....T..............
CMW28757 ....C.....
CMW28757 ....
CMW28757 ..........CA..........G..
CMW28757 .........A..............T.........
CMW28757 ....

Figure 4: Phylogenetic tree generated from the ITS sequence data of *Ganoderma* isolates from Sumatra and *Ganoderma* species obtained from GenBank using neighbour-joining tree building algorithm. Only bootstrap values greater than 50% are indicated above the branches. The tree is rooted to *G. carnosum.* The scale bar indicates the number of substitutions per site.


AJ608710*G. philippii* GSB-1.2; GSB1.4; GSB1.5; GSB1.6; GSB2-1.3; GSB2-4.11; GSB2-9.4; GSB2-9.5; GSB3-1.1;
AJ608713*G. philippii* GSB-3-1.6;GSB-3-2.6; GSB3-3.2; GSB3-3.4; GSB3-3.8; GSB3-4.3; GSB4-10.7; GSB4-3.1;
AJ627584*G. philippii* GSB-4-3.7; GSB4-6.1; GSB4-6.3; GSB4-7.1; GSB5-(-1.10); GSB5-(-1.11); GSB5-(-1.9); GSB
5-(-2.13); GSB5-2.4; GSB5-2.7; GSB5-9.5; GSL1-10.9; GSL1-12.4; GSL1-3.11; GSL1-3.9; GSL1-4.12; GSL1-5.13;
GSL1-7.2; GSL1-7.3; GSL1-8.4; GSL1-9.5; GSL2-1.3; GSL2-1.5; GSL2-10.3; GSL2-2.4; GSL2-2.5; GSL2-2.6;
GSL2-5.2; GSL3-1.2; GSL4-10.1; GSL4-10.5; GSL4-11.4; GSL4-12.3; GSL4-13.2; GSL4-16.4; GSL4-16.5; GSL4-
17.6; GSL4-3.3; GSL4-4.6; GSL4-7.1; GSL5-11.3; GSL5-11.4; GSL5-13.4; GSL5-13.5; GSL5-14.3; GSL5-14.5;
GSL5-15.4; GSL5-15.7; GSL5-2.5; GSL5-2.6; GSL5-4.3; GSL5-4.4; GSL5-4.6; GSL5-5.3; GSL5-5.5; GSL5-7.3;
GSL5-8.3; GSL5-8.4; GSL5-9.3; GSL6-(-3.6); GSL6-(-4.6); GSL6-(-7.7); GSL6-2.3; GSL6-2.4; GSL6-4.4; GSL6-
5.10; GSL6-5.5; GSL6-5.9; GSL6-6.10; GSL6-6.3; GSL6-6.7; GSL6-6.9; GSL7-1.2; GSL7-1.6; GSL7-10.11; GSL7-
10.3; GSL7-10.4; GSL7-2.3; GSL7-5.2; GSL7-5.3; GSL7-5.4; GSL7-6.3; GSL7-6.8; GSL7-7.7; GSL7-7.9; GSL7-
8.8; GSL8-1.4; GSL8-2.1; GSL8-2.9; GSL8-3.2; GSL8-3.3; GSL8-3.8; GSL8-4.2; GSL8-4.8; GSL8-5.3; GSL8-5.7;
GSL8-5.9; GSL8-6.2; GSTE1-2.3; GSTE1-4.12; GSTE1-4.6; GSTE2-2.1; GSTE2-4.2; GSTE2-4.4; GSTE2-5.3;
GSTE3-1.3; GSTE3-3.10; GSTE3-6.8; GSTE3-9.3; GSTE4-(-1.9); GSTE4-2.9; GSTE4-4.1; GSTE4-4.7; GSTE4-4.8;
GSTE4-5.6; GSTE4-7.12; GSTW6-1.2; GSTW6-1.3; GSTW6-1.5; GSTW6-3.3; GSTW6-4.10; GSTW6-4.3; GSTW6
5.10; GSTW6-7.6; GSTW6-9.8; GSTW7(-1.20); GSTW7-(-2.4); GSTW7-1.1; GSTW7-1.2; GSTW7-1.4; GSTW7-
2.9; GSTW7-3.14; GSTW7-3.4; GSTW7-3.9; GSTW7-4.19; GSTW7-4.22; GSTW8-(-1.13); GSTW8-(-1.3); GSTW8-
(-10.3); GSTW8-(-2.2); GSTW8-(-3.2); GSTW8-(-9.3); GSTW8-1.1; GSTW8-1.2; GSTW8-2.1; GSTW-8-2.5; GSTW
8-4.1; GSTW8-5.2; GSTW8-5.7; GSTW8-6.6; GSTW9.1.10; GSTW9-1.1; GSTW9-1.5; GSTW9-1.7; GSTW9-2.2;
GSTW9-2.6; GSTW9-2.8; GSTW9-4.1; GSTW9-4.2; GSTW9-4.3; GSTW9-4.5
Ganoderma isolates from Sumatra
95
AY636068 *G. lucidum*
52
99
EF060005 *G. lipsiense*
AY884179 *G. applanatum*
99
AY593860 *G. fornicatum*
AY593863 *G. fornicatum*
99
AY593867 *G. neojaponicum*
92
AY593866 *G. neojaponicum*
99
CMW30066 (**Sumatra**)
AJ627585.1 *G. mastoporum*
AJ749970.1 *Ganoderma* sp.
CMW29744 (**Sumatra**)
63
99
CMW29998
99
CMW29844
CMW29740
Sumatra
EU486458.1 *G. carnosum*
0.01

SUMMARY

Ganoderma root rot disease has been reported world wide causing the death of affected hosts. The taxonomy of the genus *Ganoderma* is considered to be in disarray due to the use of basidiocarp morphological characters to differentiate the species which resulted in many synonyms, species complexes and possible misidentifications of species within the genus. The use of sexual compatibility tests and molecular techniques became powerful diagnostic tools to elucidate the taxonomy of *Ganoderma* species. Application of these techniques has resolved some of the taxonomic problems but the use of certain species names in the genus is still causing contention among taxonomists.

The literature surrounding the taxonomy and techniques used in the taxonomy of the root rot fungus *Ganoderma* are considered in this thesis. It is clear that the taxonomy of *Ganoderma* is very difficult and it is still largely obscured by species complexes and incorrect species identifications. It is also evident that a single species concept will not aid in the identification of *Ganoderma* species. Rather, a combination of concepts based on morphology, mating tests and DNA sequence data should be used in elucidating the taxonomy of *Ganoderma*.

Morphological characteristics as well as nucleotide sequence analysis of three gene regions; the internally transcribed spacer (ITS), the mitochondrial small subunit (mtSSU) and the intergenic spacer (IGS-1), were used to identify the causal agent of Ganoderma root rot of *J. mimosifolia* in the suburb of Brooklyn, Pretoria, South Africa. Morphological observations and DNA-based phylogenies revealed that all isolates

collected from infected trees belong to a single species that reside in the *G. lucidum sensu lato* complex.

Acacia mangium is a leguminous tree that is grown as an exotic plantation species in Indonesia. These economically important trees are threatened by Ganoderma root rot disease. This disease is considered to be the most important cause of losses in *A. mangium* plantations. Phylogenetic analysis of ITS sequence data showed that *G. philippii* is the primary agent of Ganoderma root rot in *A. mangium* in Sumatra, Indonesia.