Molecular and Biochemical Characterisation of *Armillaria*

Submitted 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 as hitherto not been submitted for any degree at any other university or faculty.

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PREFACE

Species of the fungal genus *Armillaria* cause the disease known as Armillaria root rot. This disease occurs mainly on woody plants and it occurs worldwide. Although species of *Armillaria* are reasonably well defined, there still remain a number of isolates that have not been identified and which probably represent species new to science.

This thesis is presented in a series of chapters, of which chapters 2, 3 and 4 are in manuscript format. This has resulted in some duplication of references and details with regards to the details of the isolates studied and the techniques used for some of the studies. This style is the one that is preferred as it provides experience in scientific writing.

Chapter one of this thesis focuses on the published literature on the genus *Armillaria*. The chapter starts with the history of *Armillaria*. The focus then shifts to identification techniques that have been applied in *Armillaria* classification. Identification techniques are subdivided into morphological identification, identification based on biological characteristics, isozyme and protein pattern identification, and DNA based methods of identification. The chapter concludes with a discussion on the features that are particular to *Armillaria*.

Chapter two deals with the characterisation of Zimbabwean *Armillaria* isolates. Three different techniques are applied in this chapter; RFLP profiles, DNA sequences of the IGS- 1 region of the rRNA operon and AFLP studies. Armillaria root rot disease has been

reported to cause considerable damage in Zimbabwean pine plantations and fruit orchards, but the identity of the species causing the disease remains unclear. At least three taxonomic groups of isolates of *Armillaria* have been reported to occur in Zimbabwean plantations. *Armillaria fuscipes* has recently been confirmed to be present in Zimbabwe but the identity of the remaining groups remains unresolved.

Chapter three investigates with the phylogenetic relationships of a wide range of *Armillaria* spp. from different hosts and geographic areas in Zimbabwe. The relationships between isolates has been investigated using DNA sequence data of Elongation Factor 1 alpha (EF 1-α). This is the first study using a single copy, protein coding gene to examine the phylogeny of *Armillaria.* Results obtained in this study confirm the phylogeny proposed previously based on the ribosomal RNA genic regions.

Chapter four deals with the use of pectic isozyme profiles in determining the relationships among a wide range of isolates of *Armillaria* from different hosts and regions. Pectic enzymes have been used previously to study a limited number of *Armillaria* species. This study is the first to use a comprehensive set of isolates representing the majority of the known species in this genus. The results of this study show that pectic enzymes can provide a cheap and effective method of distinguishing between the majority of *Armillaria* species

Armillaria root rot is an economically important pathogen in the plantations and orchards of Zimbabwe. In order to effectively manage this disease, accurate identification of the

species responsible for the disease is essential. The research questions treated in this thesis were conceived to provide some useful answers with regard to the identity of the fungi causing disease as well as some solutions for accurate and cheap identification of these fungi.

Chapter 1:

Literature review: Armillaria root disease, distinct characters and identification techniques.

1.0 INTRODUCTION

Armillaria (Fr.:Fr.) Staude (Basidiomycetes, Agaricales, Tricholomataceae) is a basidiomycete genus including many species that are the causal agents of root and butt rot diseases. *Armillaria* resides in the fungal sub-class Homobasidiomycetes because the basidia do not have walls and it produces mushrooms in the sexual stage. The genus resides in the order Agaricales based on morphological features such as presence of a cap and stipe. In relation to other homobasidiomycete taxa, *Armillaria* spp. are unusual in having diploid rather than dikaryotic vegetative mycelia (Watling et al., 1982). Most *Armillaria* spp. are pathogens of woody plants. Some species have evolved as successful secondary or facultative pathogens while others are primary pathogens (Gregory et al., 1991).

At least 38 *Armillaria* spp. have been described (Volk and Burdsall, 1995) and these are listed in Table 1. These species are distributed worldwide (Hood et al., 1991). In Africa, before research on *Armillaria* intensified, it was assumed that only two species were present; *A. heimii* Pegler and *A. mellea* (Vahl: Fr.) P. Kumm. (Mohammed et al., 1994). Recent reports suggest that at least four unique species of *Armillaria* occur in Africa (Chillali et al., 1997; Coetzee et al., 2001b; 2003b; Mwenje et al., 2003; Otieno et al., 2003). Mwenje and Ride (1996; 1997) showed that three of these species occur in Zimbabwe. Coetzee et al. (2000b) reported that *A. fuscipes* Petch was present in South Africa and Zimbabwe. Mwenje et al. (2003) more recently examined a larger collection of isolates from Zimbabwe and confirmed that *A. fuscipes* was present in the country.

Armillaria spp. reported in Australia and New Zealand include *A. luteobubalina* Watling and Kile, *A. novae-zelandiae* (G. Stev.) Herink, *A. fumosa* Kile and Watling, *A. limonea* (G. Stev.) Boesew., *A. fella* (Hongo) Kile and Watling, *A. hinnulea* Kile and Watling and *A. pallidula* Kile and Watling (Kile and Watling, 1983, 1988; Watling et al., 1991). Morphological species known to occur in Europe include *A. mellea, A. ostoyae* (Romagn.) Herink*, A. cepistipes* Velen. [= *A. bulbosa*], *A. borealis* Marxmüller and Korhonen*, A. tabescens* (Scop.) Emel*, A. cepistipes* Velen. and *A. ectypa* (Fr.) Emel (Rishbert, 1982; Gregory and Watling, 1985; Morrison et al., 1985a; Roll-Hansen, 1985; Termorshuizen and Arnolds, 1987; Chillali et al., 1998; Zolciak et al., 1997). Species that have been reported in North America are *A. calvescens* Bérubé and Dessur*., A. gallica* Marxmüller and Romagn. [= *A. lutea*]*, A. sinapina* Bérubé and Dessur*., A. mellea, A. gemina* Bérubé and Dessur*., A. ostoyae, A. tabescens, A. nabsnona* Volk and Burdsall and *A .cepistipes* (Bérubé and Dessureault, 1988; Miller et al., 1994; Volk et al., 1996). Asian species include *A. mellea, A. gallica, A. cepistipes, A. sinapina, A. tabescens, A. singula* Cha and Igarashi*, A. jezoensis* Cha and Igarashi and *A. nabsnona* (Terashita and Chuman, 1989; Cha et al., 1994; Mohammed et al., 1994).

For many years, the name *A. mellea* was arbitarily assigned to new collections of *Armillaria* (Watling et al., 1982; Mohammed et al., 1994). *Armillaria mellea* is a single but polymorphic species. Thus, the name *A. mellea sensu lato* is common in the literature although the taxa most likely do not represent this fungus. Watling et al. (1982) suggested complete abandonment of the term *A. mellea sensu lato* in reference to *Armillaria* spp. because it caused unnecessary confusion.

A major advance in *Armillaria* taxonomy arose from the application of the biological species concept for the genus (Korhonen, 1978; Anderson and Ullrich, 1979). Korhonen (1978) described five European Biological Species (EBS) of *Armillaria* and Anderson and Ullrich (1979) ten North American Biological Species (NABS) that were previously included in the *A. mellea* complex. The delineation of *A. mellea* into more than one biological species based on incompatibility reactions introduced a more precise delimitation of species (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979; Guillaumin and Berthelay, 1981; Volk and Burdsall, 1995). Various interfertility groups (biological species) are currently recognised from various parts of the world (Korhonen, 1978; Ullrich and Anderson, 1978; Mohammed et al., 1994; Cha et al., 1994).

Identification using DNA-based techniques is currently employed to characterise *Armillaria* species. The most commonly used techniques are RFLPs (Smith and Anderson, 1989; Harrington and Wingfield, 1995; Buscot et al., 1996), DNA sequence comparisons (Anderson and Stasovski, 1992; Chillali et al., 1997; 1998; Coetzee et al., 2000a/b; 2001a/b; 2003a/b) and AFLPs (Pérez-Sierra et al., 2004). These methods are now widely used in identifying new isolates. These techniques complement established morphological and biological methods.

Many studies have been conducted on the geographic distribution and ecological preferences of *Armillaria* spp. (Raabe, 1962; Guillaumin and Berthelay, 1981; Hood et

al., 1991; Watling et al., 1991; Blodgett and Worrall, 1992). The genus tends to be mostly confined to areas with moderate temperatures, high rainfall and high altitude (Ivory, 1987). There are however, extreme cases, for example in the Congo where Armillaria root disease has been reported in areas of low altitude (less than 500m) but with high rainfall (Mwangi et al., 1994). The disease is prominent in temperate regions and in tropics where both conifers and broadleaved trees are infected (Morrison et al., 1985a; Proffer et al., 1987; Harrington and Rizzo, 1993).

The aim of this literature review is to consider key literature pertaining to Armillaria root disease, and more specifically techniques that are currently being applied to identify species of *Armillaira*.

2.0 Taxonomic history

Taxonomic and nomenclatural confusion has surrounded *Armillaria* (basionym: *Armillariella*) since the introduction of the genus into the taxonomic literature (Watling et al., 1982; 1991). At one time the genus was referred to as *Armillariella* (Singer, 1975; Watling et al., 1982; 1991; Volk and Burdsall, 1995). Karsten (1881) studied three Finish species of *Armillariella* namely *Arm. mellea* (Vahl: Fr.) P.Karst.; *Armillariella dryina* (Pers.) P. Karst. and *Arm. corticata* (Fr.) P. Karst. The correct generic name for the genus was disputed by several authors (Singer, 1975; Watling et al., 1982; 1991; Termorshuizen and Arnolds, 1987; Burdsall and Volk, 1993; Volk and Burdsall, 1995). This caused confusion for taxonomists and plant pathologists and has consequently impacted

negatively on research on this widely distributed and economically important genus of fungi (Watling et al., 1982).

A fungus belonging to the genus *Armillaria* might have been observed in 1704 by John Ray but was only fully described twenty-five years later by Micheli (cited in Watling et al., 1982). Fries (1821) accepted *Armillaria* as a tribe within *Agaricus* and he included twelve species, the last of which was *Ag. melleus* Vahl: Fr. In 1825, Fries combined the tribes *Armillaria* and *Lepiota*, discarding the former and retaining the latter name (Watling et al., 1982). Later in 1838, he re-established the tribe and included double the number of species presented in 1821 (Watling et al., 1982). At this stage Fries split the tribe into three groups: *Tricholomata subannulata* (Fries), *Clitocybae annulatae* (Fries), and *Collybiae annulatae* (Fries). Many of these species are placed in different sub-families of *Tricholomataceae* in modern taxonomic classifications (Watling et al., 1982).

Staude (1857), following the Friesian arrangement presented in 1821, raised *Armillaria* as a tribe to generic rank. Staude included only four German species (*Ag. mucidus, Ag. melleus, Ag. aurantius* and *Ag. robustus*). Many taxonomists believe Staude to be the authority, and that his description was sufficient for the description of a genus (Termorshuizen and Arnolds, 1987; Watling et al., 1982; Burdsall and Volk, 1993; Volk and Burdsall, 1995). Singer (1955; 1986), however, considered Kummer as the authority of the genus. Quélet (1872) is also sporadically cited as the authority of *Armillari*a but this is because the authors were not aware of the work done by Staude (1857) and Kummer (1871) (cited in Watling et al., 1982; Pegler, 2000). Staude is, however, the

accepted authority for the genus *Armillaria* (Watling et al., 1982; 1991; Burdsall and Volk, 1993; Pegler, 2000) and *Armillariella* is regarded as an obligate synonym of the earlier *Armillaria* (Pegler, 2000).

Herink (1973) (cited in Watling et al., 1982) and Pegler (2000) divided the genus *Armillaria* into two sub-genera, *Armillaria* and *Desarmillaria.* In this classification, *Armillaria* includes annulate taxa with a veil whereas *Desarmillaria* those without an annulus and veil. *Armillaria mellea* (Vahl: Fr.) P. Kumm. was considered to be the type species for *Armillaria* and *A. socialis* (DC: Fr) Herink for *Desarmillaria.* However, the majority of species previously accepted in *Desarmillaria* are still incorporated into the genus *Armillaria* (Volk and Burdsall, 1995).

3.0 Identification methods employed in *Armillaria* taxonomy

In order to understand the biogeography and pathology of *Armillaria* spp., it is essential that the species are correctly identified. Several identification techniques are available to delineate *Armillaria* species. Identification methods include morphological characterisation (Watling et al., 1982; Bérubé and Dessureault, 1988; 1989), mating tests (Motta and Korhonen, 1986; Proffer et al., 1987; Dumas, 1988; Blodgett and Worrall, 1992; Banik et al., 1996), isozyme and protein analysis (Morrison et al., 1982b; 1985b; Lin et al., 1989; Wahlström et al., 1991; Mwenje and Ride, 1996) and DNA based molecular characterisation (Jahnnke et al., 1987; Smith and Anderson, 1989; Anderson and Stasovski, 1992; Harrington and Wingfield, 1995; Banik et al., 1996; Volk et al., 1996; Terashima et al., 1998).

3.1 Identification based on morphology

Armillaria shares similar morphological features with other members of the Agaricales such as presence of a stipe and caps with gills. Agaricales bear their spores on gills and a spore print is often obtainable (Arona, 1986). Three characteristics, however, distinguish *Armillaria* spp. from other agarics. These features include rhizomorphs (root like structures), production of mushroom-like basidiocarps, and mycelial fans which occur between the bark and wood of infected plants (Rishbeth, 1972; Garraway et al., 1991). *Armillaria* uses these attributes to infect and colonize a wide range of hosts and substrates. These features enable pathologists and taxonomists to establish *Armillaria* as the causal agent of death of plants in infection centres.

3.1.1 Rhizomorphs

Rhizomorphs are discrete, filamentous aggregations and highly differentiated fungal structures that grow out from a food source into a substrate that may not support its growth (Garraway et al., 1991). Early mycologists viewed these structures as representing a separate fungal species named *Rhizomorpha fragilis.* The species was further divided into two sub-forms; *R. subterranean* and *R. subcorticalis* (Garraway et al., 1991). It was, however, later shown that rhizomorphs are not distinct species but are structures of *Armillaria* species. These structures are considered the main means of plant infection through their extension from one tree to another (Gregory et al., 1991).

Although almost all species of *Armillaria* produce rhizomorphs in culture (Redfern and Filip, 1991), variation in the absence or presence of rhizomorphs in the field is also a

well-recognised phenomenon. Swift (1972), for example, found that rhizomorphs were very rare in species of *Armillaria* occurring in Zimbabwe. Some species, for example, *A. limonea,* produce rhizomorphs frequently whilst in others, such as *A. tabescens*, they apparently do not produce rhizomorphs in the field (Rishbeth, 1982). *Armillaria luteobubalina* readily produces rhizomorphs in culture (Morrison, 1982b) but very rarely in natural forests (Podger et al., 1978; Kile and Watling, 1981).

Armillaria spp. produce two kinds of rhizomorphs. In one type they are dichotomously branched, while in the other they are monopodial (Morrison, 1982b) as shown in Figure 1. Morrison (1982b) divided *Armillaria* isolates from England into three types based on the type and frequency of rhizomorph branching. Type I has a monopodial branching system where the main axis is formed by the continued growth of the rhizomorph tip and lateral branches arise perpendicular to the main axis at a distance behind the primary tip. Sometimes dichotomous branching of the primary tip is observed. Type II has a dichotomous branching pattern with both branches being of the same size. Type II is further divided into type IIa and IIb, with the former branching less frequently than the latter with lateral branches being rarely observed. The branching types of rhizomorphs are shown in Figure 1. Type IIa rhizomorphs are fragile and break easily while Type IIb rhizomorphs are more robust.

Rhizomorph branching pattern depends on the *Armillaria* species. *Armillaria gallica*, has rhizomorphs with a monopodial branching pattern whereas those of *A. mellea, A. ostoyae* and *A. cepistipes* have a dichotomous branching pattern (Rishbeth, 1982; Redfern and

Filip, 1991). Morrison (1989) suggested that species with dichotomously branched rhizomorphs tended to be more pathogenic than species with monopodial rhizomorphs.

Rhizomorph formation depends on the interaction between the *Armillaria* spp. and environmental conditions. Furthermore, the type and distribution of rhizomorphs is influenced by various abiotic factors in addition to the species type. Factors that influence rhizomorph production include soil composition (Swift, 1972; Redfern, 1973; Morrison, 1982a; Redfern and Filip, 1991; Termorshuizen, 2000), aeration (Smith and Griffin, 1971; Morrison, 1976; Rishbeth, 1978), nutrition (Morrison, 1982a) and temperature (Pegler, 1977; Redfern and Filip, 1991).

Pure sand can partially inhibit rhizomorph production, growth rate, number and branching of rhizomorphs (Redfern and Filip, 1991). Redfern (1973) also noticed that peat stimulates rhizomorph formation. Swift (1972), after several experiments using sterilized soil extracts, attributed absence of rhizomorphs from forest soils in Zimbabwe to a water-soluble inhibitor. Available evidence suggests that rhizomorph growth depends on soil nutrition (Morrison, 1975; 1982a). Morrison (1982a) observed that monopodially branching isolates change their growth habit to dichotomous with higher branching frequency and an increase in diameter and growth rate when humus is added to sand. Humus contains larger amounts of organic carbon, total nitrogen and exchangeable cations, as sources of nutrients, in comparison to sand (Morrison, 1982a).

Aeration has an effect on the total yield and distribution of rhizomorphs (Smith and Griffin, 1971; Morrison, 1976; Rishbeth, 1978). Low carbon dioxide and high oxygen concentrations result in higher rhizomorph yield (Rishbeth, 1978). It was suggested that oxygen and carbon dioxide levels may play a role in the chemical reactions resulting in rhizomorph formation (Smith and Griffin, 1971).

Garrett (1956) found that soil moisture and water holding capacity had no effect on rhizomorph growth within the 40-80% and 25-75% ranges, respectively. Rhizomorphs are rarely present in permanently wet soils. *Armillaria luteobubalina* growth has been found to be suppressed at levels less than 25% moisture holding capacity (Redfern and Filip, 1991). Water logging has been reported to be able to indirectly, through the soil atmosphere, restrict growth or prevent rhizomorph formation of isolates in pot experiments (Rishbeth, 1978).

Temperature may also influence the production, branching and growth of rhizomorphs (Rishbeth, 1968; Redfern, 1973; Termorshuizen, 2000). The optimum temperature for rhizomorph growth is about 22 °C. Limited growth can occur at 5 °C and 28 °C. No growth was observed at 30 °C (Rishbeth, 1968). Rishbeth (1978) suggested that absence of rhizomorphs in forest soils at low elevations in tropical Africa can be attributed to high soil temperature. Low temperature can be a limiting factor in many forest soils in the north temperate zones (Rishbeth, 1978). Temperature might also affect the number and branching pattern of rhizomorphs initiated from woody inoculum (Redfern, 1973).

3.1.2 Basidiocarps

Basidiocarps represent the sexual fruiting structures of basidiomycetes. The basidiomycetes are divided into the Homobasidiomycetes and Heterobasidiomycetes based on basidium morphology and mode of basidiospore germination (Patouillard, 1900 cited in Swann and Taylor, 1993). In homobasidiomycetes, such as *Armillaria*, basidiospores germinate via germ tubes only and have aseptate basidia. Heterobasidiomycetes, in contrast, are characterised by septate or aseptate basidia and basidiospores are capable of two or more modes of germination.

Armillaria spp. have traditionally been identified based on differences in their basidiocarp morphology. Some problems are, however, associated with classifications using morphological characteristics of basidicarps as identification criteria. Basidiocarps are seasonal and their morphology is influenced by environmental conditions (Kile and Watling, 1981). In some species they are also very rare (Swift, 1972). Wet conditions favour basidiocarp formation while severe winter frosts cause basidiocarp deterioration (Kile and Watling, 1981). A combination of macro-morphological features such as colour, size, shape of the cap and stipe, and colour of the gills, as well as micro-morphological structures such as basidiospore shape and size, presence or absence of clamp connections, are needed for unambiguous identification (Bérubé and Dessureault, 1988). Other features such as growth studies must therefore be included to distinguish related species. Certain species, for example *A. gallica* [=*A. lutea*] and *A. clavescens,* are similar in their basidiocarp morphology (Bérubé and Dessureault, 1989). *Armillaria*

ostoyae and *A. gemina* are morphologically identical and were only differentiated using vegetative features (Bérubé and Dessureault, 1989).

It is possible to produce basidiocarps *in vitro* (Kile and Watling, 1981; Shaw et al., 1981; Abomo-Ndongo et al., 1997). However, artificial basidiocarps obtained in this way do not always resemble those found in nature, limiting their use in identification procedures (Kile and Watling, 1981). Furthermore, *in vitro* production of basidiocarps can be time consuming and is in many instances unsuccessful.

3.1.3 Mycelial fans and vegetative culture morphology

Armillaria spp. can be differentiated from other fungi by the presence of mycelial fans produced under the bark of infected plants (Rishbeth, 1986; Watling et al., 1991). Mycelial fans can aid in disease spread by direct contact between roots from diseased trees with those of healthy trees. Despite differences observed between species, it is difficult to differentiate *Armillaria* spp. primarily on their mycelial characteristics. It is thus recommended to consider other additional tests such as growth studies, response to light and chemicals when identifying species (Watling et al., 1991).

Morphology of vegetative isolates has been used to differentiate *Armillaria* species. Rishbeth (1986) described differences in vegetative culture morphology between *A. mellea*, *A. ostoyae*, *A. bulbosa* and *A. tabescens*. He observed that colonies of *A. mellea* were pale, with a woolly margin and a buffy, woolly centre, and sometimes dark and sclerotic. Colonies of *A. ostoyae* had a pale, translucent appearance with concentric

zones, also a translucent margin and a very sclerotic centre was observed in some instances. Two different colony forms were observed for *A. cepistipes* [=*A. bulbosa*]; one was thin and translucent that produced a brown pigment while the other had a translucent margin and a sclerotic centre with an umber or rust colour, and a woolly surface (Rishbeth, 1986). In most cases colonies of *A. tabescens* are not pigmented and the colony centres are buff, woolly and at times sclerotic (Rishbeth, 1986). Kile and Watling (1983) observed differences in vegetative culture morphology of the Australian species *A. hinnulea*, *A. novae-zealandiae*, *A. fumosa* and *A. luteobubalina*. They concluded that vegetative culture morphology can be effectively used to differentiate *Armillaria* species.

3.2 Identification based on biological species concept

Mating tests were introduced into *Armillaria* taxonomy as a means to overcome the problems associated with basidiocarp and vegetative culture morphology. These tests are based on the interchange of genetic material between isolates of the same biological species (Korhonen, 1978; Anderson et al., 1980). This technique has proved to be useful and reliable and has played a significant role in *Armillaria* classification, especially for species from Europe and North America (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979; Anderson et al., 1980).

3.2.1 Mating tests

Fungi have two kinds of mating system which are referred to as unifactorial and bifactorial, allowing non-self compatibility between strains of the same species. *Armillaria* has a bifactorial sexual incompatibility system where monospore isolates from a fruit body segregate as four mating type loci (Hintikka, 1973; Korhonen, 1978). Hyphal confrontation between two haploid monosporous isolates can be used to identify biological species and to distinguish incompatible mating types (Hintikka, 1973; Korhonen, 1978).

In *A. mellea,* each basidiocarp produces basidiospores with four different incompatibility genotypes. Confronting single spore isolates give the following interactions A=B=; A≠B=; A=B≠; A≠B≠ (Hintikka, 1973; Guillaumin et al., 1991) which are interpreted as follows:

- ► Incompatible mating (A=B=): In this situation the mycelium of paired isolates grows side by side without any interaction.
- ► Hemi-compatible common-B (A≠B=): In most instances it is similar to incompatible mating.
- ► Hemi-compatible common-A mating (A=B≠): The aerial mycelium is either completely lacking or if present is very sparse. Microscopically, partially disintegrated septa can be observed in the submerged hyphae. This is an indication that there has been nuclear migration.

► Compatible mating (A≠B≠): There is no boundary between the mating mycelium which changes to a crustose morphology. Hyphae have migrating nuclei and disintegrated septa. Compatible reactions show that the isolates belong to the same biological species (Hintikka, 1973; Guillaumin et al., 1991).

Mating tests are performed between haploid heterothallic tester strains with known identity and the isolate that needs to be identified. The unknown isolate is paired with all the tester strains, and the result of the mating reactions scored according to the appearance of the mycelium. Haploid cultures are generally fluffy while diploid cultures are crustose and flat (Hintikka, 1973; Korhonen, 1978; Anderson et al., 1980; Anderson et al., 1987). When hyphal confrontation results in a crustose or more appressed colony, the isolates are taken to be compatible and of the same biological species. If the colonies remain white and fluffy, then they are considered to be incompatible and of different biological species (Korhonen, 1978; Anderson et al., 1980; Guillaumin et al., 1991).

A phenomenon resembling the Buller phenomenon (Buller, 1931) is also operational in *Armillaria* (Korhonen, 1978). When a cottony haploid mycelium is mated with a crustose diploid isolate it has been observed that the cottony nature of the haploid slowly turns into the crustose type (Korhonen, 1978). This indicates that the diploid isolate has donated a nucleus to the haploid isolate thereby converting it to a diploid. Darmono and Burdsall (1992), however, observed that not all compatible interactions result in crustose colonies. Some colonies only became appressed, while others maintained a fluffy

appearance but displayed other morphological changes such as short aerial mycelium, hyphal discolouration, or rhizomorph proliferation (Darmano and Burdsall, 1992).

There are problems associated with using mating type incompatibility to distinguish species of *Armillaria.* These include the fact that it can be difficult to interpret results and familiarity with mating type incompatibility reactions is necessary. The technique is also time consuming (Anderson et al., 1989; Mohammed et al., 1994). It is, furthermore, advisable to use fresh monosporous isolates and in certain cases these are not available. Fluffy monosporous isolates assume an appressed morphology after an extended time in storage (Darmono and Burdsall, 1992). Basidiomes associated with a biological species are often difficult to obtain, consequently it hampers production of monospore haploid cultures (Burdsall et al., 1990).

A major limitation of mating compatibility tests is its dependence on heterothallic mating systems for a sexual interaction. In heterothallic species the haploid monokaryon is self sterile and it becomes diploid only when two haploids carrying different alleles at the mating locus come into contact and mate. With homothallic species the haploid monokaryon is self fertile and it becomes diploid without mating with another haploid. Most African *Armillaria* spp. have been reported to be homothallic (Mohammed et al., 1994; Abomo-Ndongo et al., 1997). The European species *A. ectypa* was also reported to be homothalic (Zolciak et al., 1997). It is impossible to use sexual compatibility tests on homothallic isolates.

In pairing tests, the appearance of a black line between two isolates is regarded as evidence that they belong to different species. If they belong to the same species, sexual incompatibility and intersterility regulate mating in *Armillaria* (Anderson et al., 1987). Intersterility barriers appear to be absolute among sympatric species, while in allopatric species, intersterility barriers are in some cases not obvious. Anderson et al. (1980) showed that a species from one continent might show partial interfertility with more than one species from another continent.

3.2.2 Biological species in *Armillaria*

The biological species concept has been used in identification of *Armillaria* isolates (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979; Anderson et al., 1980; Guillaumin and Berthelay, 1981; Anderson, 1986; Mohammed et al., 1994; Volk et al., 1996). Anderson and Ullrich (1979) used the concept to define ten biological species in North America. However, Anderson (1986) reported that biological species IV and V, and species VI and VIII, were interfertile and hence equivalent. This resulted in the reduction in the number of NABS to eight. Korhonen (1978) described five European biological species and Morrison et al. (1985a) further defined an additional biological species in Europe. The list of NABS and EBS is given in Table 2 and the mating interactions in Table 3. The concept has been used to identify new field isolates in Africa (Mohammed et al., 1994; Abomo-Ndongo et al., 1997), New Zealand and Australia (Kile and Watling, 1983; 1988) and Asia (Cha et al., 1994; Ota et al., 1998). Overall, the concept has played a significant role in *Armillaria* characterisation.

3.3 Isozyme and protein pattern analysis

Isozymes are enzymes that catalyse the same reaction but have different molecular weight and hence electrophoretic mobilities (D'Ovidio et al., 2004). The most common mechanism for the formation of isozymes involves the arrangement of subunits arising from different genetic loci in different combinations to form the active polymeric enzyme (D'Ovidio et al., 2004). Genetically distinct species will have isozymes of different sizes. The possession of many and different forms of an enzyme is advantageous to the fungus as it confers some flexibility to the species with regards to pathogenicity.

Isozyme and protein pattern analysis has been used to characterise fungal species (Cruinkshank and Pitt, 1987; Karlsson and Stenlid, 1991; Chang and Mills, 1992; Mwenje and Mguni, 2001). Isozymes have been widely used in *Armillaria* characterisation. Lin et al. (1989) used esterase and total protein patterns to study four *Armillaria* spp. of North American biological species (NABS). They found that NABS I had two esterase banding patterns that seemed to correspond to geographic origin. Biological species VII produced esterase bands with a different colour on staining. The species showed distinct protein patterns and could be differentiated from each other. They, however, shared one common protein pattern band.

Pectic esterases and polygalacturonases have been used to separate European *Armillaria* isolates (Whalström et al., 1991). *Armillaria mellea* was found to possess additional bands absent in other species. They also found that isozyme production was substrate induced. *Armillaria ostoyae* and *A. borealis* had closely related isozyme patterns.

Overall, the technique separated isolates into five species. Morrison (1982b) used isozymes to show the existence of three biological species within the *A. mellea* complex, and the results were supported by rhizomorph growth patterns. Morrison et al. (1985b) used esterase and polyphenol oxidase to study intersterility groups occurring in British Columbia. Banding pattern separated the isolates into *A. bulbosa*, biological species group IX, group X was placed within *A. ostoyae*, and group F was placed within group V.

Agustian et al. (1994), using isozyme banding patterns, separated African *Armillaria* isolates into five groups. These groups were an unknown species, *A. mellea* sub sp. *africana, A. heimii* (heterothallic), and two subgroups of *A. heimii* (homothallic), one comprising isolates from Congo and Zimbabwe and the other comprising isolates isolated from *Pinus elliottii* in Malawi and Tanzania. Mwenje and Ride (1996) used pectic enzymes to group *Armillaria* isolates from Zimbabwe into three different taxonomic groups. The authors were able to determine that different groups have varying levels of enzymatic activity with group II having the highest activity for most of the isozymes except for pectin-lyase and beta-glucosidase, which were prominent in group III isolates. Protein patterns were in total agreement with isozyme analysis. Mwenje et al. (1998) showed that different Zimbabwean groups have different pathogenicities on cassava with group III being the most pathogenic and group II being the least pathogenic.

3.4 DNA based identification

DNA based techniques have become increasingly useful in *Armillaria* taxonomy during the past two decades. Earlier methods relied on the quantity of mitochondrial and nuclear

DNA to differentiate species (Motta et al., 1986; Peabody and Peabody, 1986). Differences in Restriction Fragment Length Polymorphisms (RFLPs), DNA sequences and Amplified Fragment Length Polymorphisms (AFLPs) are now widely used to separate *Armillaria* spp. (Anderson et al., 1989; Smith and Anderson, 1989; Anderson and Stasovski, 1992; Harrington and Wingfield, 1995; Banik et al., 1996; Chillali et al., 1997; 1998; Volk et al., 1996; Terashima et al., 1998; Coetzee et al., 2000a/b; 2001a/b; Gezahgne et al., 2004; Pérez-Sierra et al., 2004).

A region of the ribosomal gene repeat with adequate but not excessive variation is often chosen for identification of species (Bruns et al., 1991). Two such regions are the Internally Transcribed Spacer (ITS1 and ITS2) regions and the Intergenic Spacer (IGS-1) region of the ribosomal RNA operon. The IGS-1 and ITS regions are highly polymorphic gene spacer regions (Molnar and Fedak, 1989; Kambhampati and Rai, 1991). These regions are often employed in identification of *Armillaria* spp. but they offer little variation within closely related species (Anderson and Stasovski, 1992; Coetzee et al., 2001a). Ribosomal genes are non-orthologous and can be involved in recombination and might behave as pseudogenes thereby compromising phylogenetic studies (Buckler et al., 1997; O'Donnell and Cigelnik, 1997).

Identifying organisms on the basis of their DNA requires knowledge of the correct location of the target DNA (Buscot et al., 1996). The ITS and IGS-1 spacer regions are located within the rDNA operon (Veldman et al., 1981; Kurtzman and Liu, 1990; Collins et al., 1991). The 5S rRNA gene is located within the IGS region of the rRNA operon in

Armillaria (Duschesne and Anderson, 1990). Coetzee et al. (2000b) showed that the orientation of the 5S gene of the ribosomal rRNA operon is inverted in African *Armillaria* isolates. The effect of this anomaly is that the primers employed for amplifying this region of the gene for isolates from other geographical regions cannot be used in African *Armillaria*. Different *Armillaria* spp. give IGS-1 and ITS PCR fragments of varying sizes, highlighting the variability of these regions between species. The IGS-1 is more variable than the ITS region hence, nucleotide sequences are more difficult to align and consequently it becomes difficult to use this DNA region to determine relationships between distantly related *Armillaria* species.

3.4.1 Restriction fragment length polymorphisms

Restriction fragment length polymorphisms have been successfully applied in characterising *Armillaria* spp. (Smith and Anderson, 1989; Harrington and Wingfield, 1995; Chillali et al., 1997; 1998; Coetzee et al., 2000a/b; 2001a/b; 2003a/b; Mwenje et al., 2003; Gezahgne et al., 2004). *Armillaria* spp. were initially characterised based on differences in RFLPs using mitochondrial DNA (Jahnke et al., 1987; Smith and Anderson, 1989) and rDNA (Anderson et al., 1989). These were more recently superseded by RFLPs using amplified products of the IGS-1 (Harrington and Wingfield, 1995) and ITS regions (Chillali et al., 1997; 1998). This method provides a quick and relatively reliable means to identify *Armillaria* species. The RFLP profiles of the IGS-1 region are currently known for the majority of *Armillaria* spp. and are provided in Table 4. It must, however, be noted that some closely related species can have similar restriction patterns when PCR amplicons are digested with a single enzyme. In these

cases, it is necessary to use additional restriction enzymes to distinguish between these species.

3.4.2 DNA sequencing

DNA sequences for *Armillaria* spp. identification have largely been obtained from the ribosomal RNA operon (Anderson and Stasovski, 1992; Chillali et al., 1997; 1998; Coetzee et al., 2000a; 2001b; 2003a; Gezahgne et al., 2004). This gene region has certain characteristics that make it attractive for phylogenetic studies. Ribosomal RNA genes from nuclear genomes have the same function in all taxa, have a high copy number and they evolve at approximately the same rate (Harrington and Rizzo, 1999; Mitchell et al., 1995). The rRNA operon consists of highly conserved and variable regions. The conserved regions include the large subunit (LSU), small subunit (SSU), 5.8 S and the 5S rRNA genes (Kurtzman and Liu, 1990; Collins et al., 1991). Variable regions are found in the Internal Transcribed Spacers (ITS1 and ITS2) and the Intergenic spacer region (IGS-1). The genic and intergenic regions evolve at different rates thus allowing differentiation of taxa at different levels (Mitchell et al., 1995)

Sequencing of PCR products has been very useful in identification of *Armillaria* species. Coetzee et al. (2000a), for example, showed through ITS and IGS-1 DNA sequence analyses that *A. mellea* isolates from different geographic areas are genetically isolated and might be undergoing speciation. DNA sequence results are considered to be unambiguous and reliable because they reflect the exact composition of the genetic makeup of the organism. A considerable number of *Armillaria* DNA sequences are

available in GenBank and these can now be used for comparative purposes. In total there are 282 ITS and 148 IGS-1 in GenBank as of (12/05/2005). Polymorphic genes have some alleles that are shared by different individuals and this limits their use in species deliniation (Taylor et al., 2000). It is necessary to look at more loci because different individuals will have differing allele combinations. A single gene region will mostly likely group isolates into monophyletic groups rather than into phylogenetic species (Taylor et al., 2000). It is therefore necessary to generate information from many data sets to confidently define species.

The need for more information to increase resolution of fungal taxonomy has lead to a number of gene regions being suggested for use in fungi. The Assembling the Fungal Tree Of Life (AFTOL: http://ocid.nacse.org/research/aftol/links.php) project aims to understand the evolution and phylogeny of the fungal Kingdom. It proposes to sample nuc-ssu rDNA, nuc-lsu rDNA, RPB1, RPB2, EF-1α, ATP6 and ITS regions from all major groups of fungi and generate datasets of molecular and subcellular characters that can be used for phylogenetic analysis. A comprehensive phylogenetic study of fungi, in addition to taxonomic studies will enable the description of the many undescribed fungal species.

No protein-coding gene has yet been used in *Armillaria* identification but this is true also for many of the basidiomycetous fungi. Piercey-Normore et al. (1998) used anonymous sequences to study the phylogeny of North American Biological Species (NABS) of *Armillaria* but not protein coding genes. Currently the β-tubulin (Landvik et al., 2001)

and glyceralderhyde3-phosphate dehydrogenase (Berbee et al., 1999) are being used for the AFTOL project while elongation factor 1-α sequences have been used successfully in some basidiomycetes (Thornewell et al., 1995; Wendland and Kothe, 1997; Kauserud and Schumacher, 2001; 2003).

3.4.3 Amplified Fragment Length Polymorphism (AFLP) fingerprinting technique

Vos et al. (1995) described the AFLP method as a DNA fingerprinting technique. The technique is reliable because it employs robust reaction conditions and targets the whole genome. The method involves restriction digestion of genomic DNA and ligation of oligonucleotide adaptors, followed by preamplification and selective amplification and finally gel analysis. The method has been employed in plant population studies (Sanchez et al., 1998; Singh et al., 2002), insects (Parson and Shaw, 2001) and in fungal identification (Majer et al., 1996; Marasas et al., 2001; Abdel-Satar et al., 2003; Jurgenson et al., 2002a/b).

Very little work has been done using AFLPs in *Armillaria* taxonomy. Pérez-Sierra et al. (2004) used the technique in an attempt to group African isolates thought to represent *A. heimii*. Their results divided the isolates into two groups generally based on geographic origin. Isolates from West Africa group together but also included an isolate from East Africa. The second group comprised isolates from East and Southern Africa. They concluded that these groups represent two distinct species namely *A. heimii* and an unknown *Armillaria* species.

4.0. CONCLUSIONS

► *Armillaria* is an economically important root rot fungus. Its damage in forest trees and in high value crops cannot be underestimated. Armillaria root rot is most serious in newly established plantations. Resistance to infection tends to increase with increase in plant age. Attempts to eradicate the disease from some defined areas have not been entirely successful.

► Available evidence indicates that the genus *Armillaria* is large and includes at least 38 well studied and documented species. These species have a worldwide distribution in tropical and temperate regions. The genus has a broad host range with some species being host specific and others overlapping between hosts. Species differ greatly in their ability to cause disease.

► Three features are known to be particular to species within the genus *Armillaria*. These are rhizomorphs, basidiocarps and mycelial fans. These features play a role in disease spread. They are useful in *Armillaria* identification. Occurrence of these features is, however, dependent on the environment.

► Fungal identification techniques differ in their degrees of resolution. Identification of *Armillaria* spp. based on morphology is frustrated by the seasonal occurrence of basidiocarps. Interfertility tests are time consuming and require familiarity with the technique. DNA based methods and isozyme pattern analysis are reasonably fast and they are now commonly used in *Armillaria* spp. identification.

► The advent of molecular techniques, in addition to morphological and mating tests has been very useful in clearly defining *Armillaria* spp., which for a long time have been grouped together as *A. mellea.* The genus *Armillaria* now consists of a number of biological species that can be clearly defined by use of interfertility tests. However, more research needs to done to fully understand *Armillaria* spp. and their relationships with each other. There are for example still some isolates for which no species description is currently available.

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Table 1: List of known *Armillaria* species

Species	Reported in
A. affinis (Singer) Volk & Burdsall	Carribean, Central America
A. borealis Marxmüller & Korhonen	Britain, Russia
A. calvescens Bérubé & Dessur.	Canada, USA
A. camerunensis (Henn.) Volk & Burdsall	Africa
A. cepistipes Velenovsky	Germany, Japan, USA
A. duplicata (Berk.) Sacc.	India
A. ectypa (Fr.) Lamoure	France
A. fella (Hongo) Kile & Watling	New Guinea
A. fumosa Kile & Watling	Australia
A. fuscipes Petch	East and West Africa, Madagascar, Sri-lanka
A. gallica Marxmüller & Romagn.	Europe, Japan, USA
A. gemina Bérubé & Dessur.	Canada, USA
A griseomellea (Singer) Kile & Watling	South America
A. heimii Pegler	Cameroon
A. hinnulea Kile & Watling	South-eastern Australia
A. jezoensis Cha & Igarashi	Japan
A. limonea (Stevenson) Boesewinkel	New Zealand
A. luteobubalina Watling & Kile	Australia
A. mellea (Vahl: Fr.) P.Kumm.	Kenya, England, Japan, Canada, North Asia

Species	Reported in
A. montagnei (Singer) Herink	South America, Denmark
A. nabsnona Volk & Burdsall	Washington
A. nigtitula Orton	Great Britain
A. novae-zealandiae (Stevenson) Herink	Eastern Australia, New Guinea, New Zealand, South America
A. omnituens (Berk) Sacc.	India
A ostoyae (Romagn) Herink	France, Japan, Canada, USA
A. pallidula Kile & Watling	Australia
A. pelliculata Beeli	Africa
A. procera Speg.	South America
A puiggarri Speg.	South America
A. sinapina Bérubé & Dessur.	Canada, USA
A. singula Cha & Igarashi	Japan
A.solidipes Peck	North America
A sparrei (Singer) Herink	South America
A tabescens (Scop.: Fr.) Emel	England, USA
A. tigrensis (Singer) Raith	South America
A. viridiflava (Singer) Volk & Burdsall	South America. Europe
A. yunensis (Singer) Herink	South America

Data from Guillaumin and Berthelay, 1981; Gregory and Watling, 1985; Rishbeth, 1986; Bérubé and Dessureault 1988; 1989; Mohammed et al., 1989; Gregory et al., 1991; Watling et al., 1991; Whalström et al., 1991; Blodgett and Worrall, 1992; Agustian et al., 1994; Volk and Burdsall, 1995; Banik et al., 1996; Zolciak et al., 1997; Otieno et al., 2003.

Table 2: List of North America and European biological species of *Armillaria*

North American Biological Species (NABS)	
NABS I	*A. ostoyae* (Romagn.) Herink
NABS II	*A. gemina* (Bérubé & Dessur.)
NABS III	*A. calvescens* (Bérubé & Dessur.)
NABS V (IV)	*A. sinapina* (Bérubé & Dessur.), partially infertile with *A. cepistipes*
NABS VI	*A. mellea* (Vahl: Fr.) P. Kumm.
NABS VII	*A. gallica* (Marxmüller & Romagn.)
NABS IX	*A. nabsnona* (Volk & Burdsall)
NABS X	unnamed
NABS XI	Interfertile with *A. cepistipes* (Velenovsky) = Biological species F (Morrison et al., 1985a)

Anderson and Ullrich, 1979; Anderson et al., 1986; Volk et al., 1996

European Biological Species (EBS)	
EBS A	*A. borealis* (Marxmüller & Korhonen)
EBS B	*A. cepistipes* (Velenovsky) = Biological species F (Morrison et al 1985a)
EBS C	*A. ostoyae* (Romagn.) Herink
EBS D	*A. mellea* (Vahl: Fr.) P. Kumm.
EBS E	*A. gallica* (Marxmüller & Romagn.)

Korhonen, 1978; Morrison et al., 1985a

Table 3: Mating interactions between European and North American Biological Species of *Armillaria*.

EBS	**NABS**	I	II	III	IV	V	VI	VII	VIII	IX	X
A. mellea		-	-	-	-	-	+	-	x	-	-
A. bulbosa		-	-	x	-	-	-	+	-	-	-
Species A		-	-	-	-	-	-	-	-	-	-
Species B		-	-	x	+	-	-	x	-	-	+
Species C		+	-	-	-	-	-	x	-	-	-

- complete intersterility; + pairings compatible; x reduced growth

Adapted from Anderson et al., 1980.

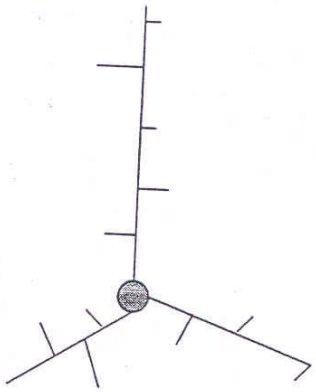
Table 4: *Alu*I restiction fragment sizes for *Armillaria* species for the IGS-1 region.

Species	Fragment Sizes (in base pairs)
A. borealis (A)	310, 200, 135
A. borealis (B)	310, 200, 104
A. calvescens	582, 240
A. cepestipes (A)	399, 200, 183
A. cepestipes (B)	310, 200, 135
A. gallica (America)	582, 240
A. gallica (Europe)	399, 240, 183
A. gemina	310, 200, 135
A. mellea (A)	490, 180
A. mellea (B)	320, 155
A. nabsnona (A)	534, 200
A. nabsnona (B)	306, 230, 196
A. nabsnona (C)	560, 321, 237, 203
A. ostoyae	310, 200, 135
A. sinapina	399, 200, 135
A. tabescens (A)	430, 240
A. tabescens (B)	320, 240, 100
NABS X	399, 183, 142
NABS XI	413, 203, 185
A. fuscipes	380, 255, 130
Armillaria sp.	485, 255, 170
A. heimii	480, 230, 175

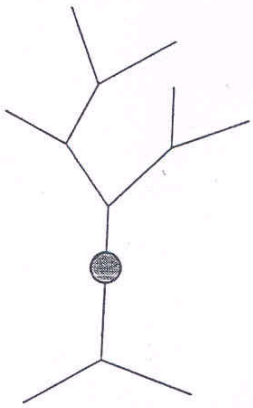
Data from Harrington and Wingfield, 1995; Banik et al., 1996; Volk et al., 1996; Coetzee et al., 2000b.

Figure 1: Schematic diagram showing rhizomorph branching pattern. Type I is monopodial branching, Type IIa shows dichotomous branching with less branching frequency and Type IIb shows high frequency dichotomous branching.

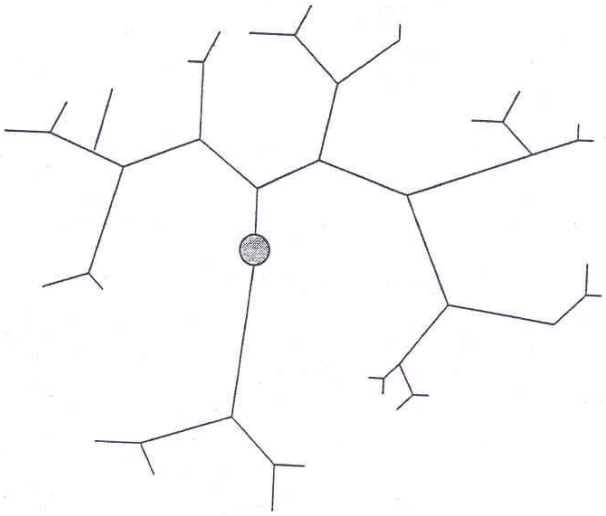
Type I



Type IIa



Type IIb



Chapter 2:

Characterisation of Zimbabwean *Armillaria* using IGS-1 gene sequences and AFLPs.

Characterisation of Zimbabwean Armillaria using IGS-1 gene sequences and AFLPs.

Abstract

Armillaria root and butt rot disease is a common problem in peach orchards, tea and pine plantations in the Eastern Highlands of Zimbabwe. The species of *Armillaria* causing this disease have not been accurately identified but it is believed that at least three species are involved. These included *A. fuscipes* (previously referred to as RFLP Group I) and two unnamed species known as RFLP Group II and RFLP Group III. The aim of the study was to use PCR-RFLP, sequences of the IGS-1 region of the rDNA operon and AFLP fingerprinting to characterise 27 Zimbabwean *Armillaria* isolates. PCR-RFLP tests showed that the isolates resided in five groups. Analysis of sequence data elucidated four groups, which were also supported by AFLP data. Eleven isolates belonged to RFLP Group I which is considered to represent *A. fuscipes*, four isolates were most similar to those previously referred to as Zimbabwean RFLP Group II and two isolates clustered most closely with RFLP Group III. The remainder of the isolates appear to represent *Armillaria* taxa not previously found in Zimbabwe.

INTRODUCTION

Armillaria root rot disease is well known in the eastern highlands and northern parts of Zimbabwe (Swift, 1972; Mwenje and Ride, 1996). These areas are characterised by high altitudes that ranges from 1000-2000 m above sea level and the annual rainfall exceeds 1000 mm (http://www.krref.krefeld.schulen.net). Armillaria root rot has been a problem in Zimbabwean plantations for many years with the first studies of the disease dating back to 1972 (Swift, 1972). Since that time, substantial losses due to this disease have been reported in pine plantations and fruit orchards in the eastern highlands of Zimbabwe (Mwenje et al., 1998).

The identity of the *Armillaria* spp. causing root rot in Zimbabwe has been the subject of a number of studies (Mwenje and Ride, 1996; Mwenje et al., 2003). These have utilised various collections of isolates and have applied various techniques. Yet, the identity of these fungi remains to be fully resolved. At the present time, it is believed that at least three taxa occur in the country and these have been referred to as RFLP Groups I -III. Of these three groups, one (RFLP Group I) is thought to represent *A. fuscipes* (Mwenje et al., 2003).

Several techniques have been applied in identifying *Armillaria* species in various parts of the world. These include interfertility tests (Hintikka, 1973), DNA based molecular techniques (Smith and Anderson, 1989; Anderson and Stasovski, 1992; Harrington and Wingfield, 1995; Volk et al., 1996; Coetzee et al., 2000a/b; Mwenje et al., 2003),

isozyme and protein analysis (Morrison et al., 1985; Wahlström et al., 1991; Mwenje and Ride, 1996), immunological assays (Burdsall et al., 1990) and morphological characterisation (Watling et al., 1982; Bérubé and Dessureault, 1988). Morphological characterisation is easy to perform but its major draw back in Zimbabwe is the rare occurrence of basidiocarps (Swift, 1972) or rhizomorphs (Mwenje and Ride, 1996) in the field. Interfertility tests are time consuming and can give ambiguous results. The technique is also only applicable to heterothallic species and most African isolates are reported to be homothallic (Abomo-Ndongo et al., 1997). Isozyme patterns, protein patterns and immunological assays give reproducible results but their disadvantage is that they can be time consuming.

Of the available techniques for the identification of *Armillaria* spp, DNA based methods appear to give the most rapid and consistent results. Currently, sequences of the IGS-1 and ITS are most commonly used (Anderson and Stasovski, 1992; Chillali et al., 1998; Coetzee et al., 2000b). However, for rapid identification, RFLPs arising from restriction digests of IGS-1 can give relatively accurate and rapid results. This approach has been used for North American species of *Armillaria* (Harrington and Wingfield, 1995). It has also been used in preliminary studies to characterise species of *Armillaria* from Southern Africa including Zimbabwe (Coetzee et al., 2000b; Mwenje et al., 2003).

It has been assumed for a long time that African *Armillaria* species include the two species *A. heimii* and *A. mellea* (Mohammed et al., 1989). Mwenje and Ride (1996), using isozymes, showed that Zimbabwean *Armillaria* consists of three groups. Coetzee et

al. (2000b) using basidiocarp morphology and DNA based methods showed that *A. fuscipes* is present in Southern Africa including Zimbabwe and this was later confirmed by Mwenje et al. (2003) using RFLP profiles and sequence data for the IGS-1 region of the RNA operon. None of the isolates studied by Mwenje et al. or Coetzee et al. were closely related to *A. mellea,* even though it has been suggested that this species occurs in Africa (Mohammed et al., 1989).

Amplified Fragment Length Polymorphisms (AFLP) represent a DNA fingerprinting method that is commonly used in population genetic studies. The method described by Vos et al. (1995) has thus been used to generate DNA fingerprints for a range of organisms. The technique provides a powerful tool for studying genetic variability and relatedness in many groups of organisms. Recently Pérez-Sierra et al. (2004) used the technique to consider the identity of African *Armillaria* isolates that they treated as *A. heimii*. These authors showed that their isolates could be separated into two distinct groups, one of which they referred to as *A. heimii* and the other as an unknown species. In their study, isolates from West Africa grouped with an isolate from east Africa while southern and eastern Africa isolates grouped together. This was consistent with the findings of Coetzee et al. (2000b) who showed that isolates from South Africa represented a single taxon (*A. fuscipes*) that differed from isolates from Cameroon, Zambia and Zimbabwe.

The primary aim of this study was to characterise a collection of Zimbabwean *Armillaria* isolates from different hosts. This was achieved using different techniques previously

applied and also to compare these with the AFLP technique. Isolates previously characterised by Mwenje et al. (2003) were included in this study to provide a framework for the analyses.

MATERIALS AND METHODS

Origin of Isolates

All samples were collected from the eastern highlands and northern parts of Zimbabwe (Table 1). These isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) University of Pretoria, South Africa.

DNA extraction and amplification

Isolates were grown on malt yeast extract agar (MYA; 1.5% Biolab Malt extract; 0.2% Biolab Yeast extract and 1.5% Biolab Agar) at 22 ºC in the dark for two weeks. The isolates were then transferred to liquid MY (1.5% Biolab Malt Extract and 0.2% Biolab Yeast Extract) and grown at 22 ºC for three weeks. Mycelium was harvested by filtration, freeze-dried and ground to a fine powder in liquid nitrogen. One thousand µL of extraction buffer (200 mM Tris-HCl pH 8; 25 mM EDTA; 250 mM NaCl and 0.5% SDS) was added to approximately 0.9 g of ground mycelium and incubated at 57 ºC for one hour. Cell debris was precipitated by centrifugation (15300 *g,* 30 minutes). Proteins were removed using a phenol: chloroform (1:1) extraction. This was repeated until a clean interphase was obtained. Traces of phenol were removed using a final chloroform extraction. Nucleic acids were precipitated from the aqueous layer overnight at –20 °C

using cold Ethanol (2:1 v/v). The DNA precipitate was collected by centrifugation (15300 *g*, 15 minutes). The resulting pellet was washed in 70% Ethanol, dried at 56 ºC and resuspended in 50 µl sterile distilled water. DNA concentration was quantified by UV Spectroscopy using a Beckman Du Series 7500 UV Spectrophotometer. The DNA samples were stored at –20 °C until needed.

DNA from the isolates was used as a template for PCR amplification of the IGS-1 region. The IGS-1 region between the 3' end of the large subunit (LSU) and the 5' end of the 5S gene of the RNA operon was amplified using the primer pair P-1 (5' TTG CAG ACG ACT TGA ATG G 3') (Hsiau, 1996) and 5S-2B (5' CAC CGC ATC CCG TCT GAT CTG CG 3') (Coetzee et al., 2000b). The PCR reaction mixture included 1.75 U of *Taq* Polymerase (Roche Diagnostics), 1 mM dNTPs, 0.01 µM of each primer, PCR buffer with $MgCl_2$, 0.25 mM of additional $MgCl_2$ and approximately 90 ng of template DNA. PCR conditions were an initial denaturation at 94 ºC for one minute, followed by 35 cycles of denaturation (94 ºC, 30 seconds), primer annealing (60 ºC, 20 seconds) and elongation (72 ºC, 30 seconds). A final elongation step of five minutes at 72 ºC was allowed for the complete elongation of the amplicons. The quality of PCR reaction products were determined through electrophoresis on 1% ethidium bromide stained agarose gel and visualized under UV illumination.

Restriction Fragment Length Polymorphisms (RFLPs)

The IGS-1 amplicons were digested using the restriction endonuclease *Alu*I without prior purification (Harrington and Wingfield, 1995). PCR reaction mix (18 µL) was subjected

to digestion by 10 U of *Alu*I for three hours at 37 ºC. The resultant RFLP fragments were separated through electrophoresis on a 3% agarose gel stained with ethidium bromide and visualized under UV illumination. Fragments less than 100 base pairs in size were not scored because of their low visibility. RFLP fragment sizes were determined using the molecular size standard marker and compared to those of other Zimbabwean isolates previously characterised by Mwenje et al. (2003). Isolates CMW10165, CMW4457 and CMW10115 were used to represent RFLP Groups I, II and III respectively, previously characterised by Mwenje et al. (2003).

DNA Sequencing and analyses

The PCR amplicons of the IGS-1 region of isolates CMW10165, CMW9963, CMW9964, CMW9967, CMW4457, CMW11649, CMW11653, CMW3, CMW1, CMW11662, and CMW11650 (Table 1) were sequenced using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS (Perkin Elmer, Warrington, U.K.) according to the protocol of the manufacturer. The sequences were determined on an ABI PRISM™ 3100 (Applied Biosystems/HITACHI, Foster City, California, USA) automated DNA sequencer after purification of the sequencing reaction products using QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Primers P-1 and 5S-2B as well as internal primers MCP-2R, MCP-3, MCP-3R, 5S-3MC, 5S-3MCR, 5S, 4MC, 5S-4MCR, MCP-2A, MCP-2AR, 5S-5MC and 5S-5MCR (Coetzee et al., 2000b) were used to sequence both DNA strands.

Analysis of raw sequences was done using Sequence Navigator version 1.01 (ABI PRISM™). Resulting sequence data were compared by means of BlastN search with sequences already available on Genbank (http://www.ncbi.nlm.nih.gov). DNA sequence data were aligned using the program ClustalX version 1.8 (Thompson et al., 1997) and manually adjusted. Distance analysis was based on the HKY85 (Hasegawa et al., 1985) nucleotide substitution model with random addition of data. Phylogenetic trees were generated using the Neighbor-Joining tree building algorithm (Saitou and Nei, 1987) in PAUP* version 4.0b10 (Swofford, 1998). Missing or ambiguous data were excluded from the analysis. Two sets of bootstrap analyses (1000 replicates) (Felsenstein, 1985) were preformed to calculate the confidence intervals at the branch nodes. Isolates CMW9963 and CMW10165 representing RFLP Group I were used as outgroup taxa.

Amplified Fragment Length Polymorphism (AFLPs) analyses

AFLPs were generated from the extracted DNA using the method described by Vos et al. (1995) with minor modifications. Genomic DNA was diluted to approximately 10 ng/µl and digested with *Eco*RI and *Mse*I (2 U each) (New England Biolabs, Beverly, Massachusetts) following the manufacturer's instructions. The generated fragments were ligated to the following adapter sequences: *Eco*RI: (5' CTC GTA GAC TGC GTA CC/CAT CTG ACG CAT GGT TAA 5') and *Mse*I: (5' GAC GAT GAG TCC TGA G/TAC TCA GGA CTC AT 5') (Vos et al., 1995) using T4 DNA ligase (New England Biolabs, Beverly, Massachusetts). These DNA fragments were subjected to an initial pre-amplification step. The reaction mixture included 0.3 µM *Eco*RI + A (5' GAC TGC GTA CCA ATT CA 3') primer, 0.3 µM *Mse*I + C (5' GAT GAG TCC TGA GTA AC 3')

primer (Vos et al., 1995), 0.2 mM dNTPs, PCR buffer containing 1.5 mM $MgCl_2$, 0.6 U *Taq* Polymerase (Roche Diagnostics) and diluted Restriction- Ligation mix. Reaction conditions were 30 seconds at 72 ºC followed by 25 cycles of 30 seconds at 94 ºC; 30 seconds at 56 ºC; one minute + one second per cycle at 72 ºC and a final cycle of two minutes at 72 ºC. Product was diluted in low TE buffer and electrophoresed on 1% ethidium bromide stained agarose gel and visualized under UV.

The diluted product of the pre-amplification was used as template the for the subsequent and final amplification step. Reaction conditions for the final amplification step were 13 cycles for 10 seconds at 94 ºC; 30 seconds at 65 ºC with temperature decreasing by 0.7 ºC per cycle during subsequent cycles; one minute at 72 ºC; followed by 23 cycles of 10 seconds at 94 ºC; 30 seconds at 56 ºC; one minute + one second per cycle at 72 ºC and a final cycle of one minute at 72 ºC. The reaction mixture included the diluted pre-amplification product, 1x PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 µM Infrared dye (IRD) labelled (Li-COR, Lincoln, NE, USA) *Eco*RI + 2 (5'GAC TGC GTA CCA ATT C ACT/AGC 3') primer, 0.25 µM *Mse*I + 2 (5' GAT GAG TCC TGA GTA A CTC/CGC 3') primer and 0.6 U *Taq* Polymerase (Roche Diagnostics). The three primer combinations used were *Eco*RI + ACT/*Mse*I + CTC, *Eco*RI + ACT/*Mse*I + CGC and *Eco*RI + AGC/ *Mse*I + CTC. Formamide loading dye was added and the product was protected from light by wrapping the tubes in aluminium foil. Product was denatured at 94 ºC for three minutes prior to loading and run on 8% Long Ranger Polyacrylamide Gel (Cambrex Bioscience, Rockland. USA) on a LI-COR automated sequencer (LI-COR,

Lincoln, NE, USA) and the results were analysed using SAGA^{MX} software version 2.1 (Li-COR, Lincoln, NE, USA).

Bands were scored as present (1) or absent (0) and ambiguous bands were recorded as missing data. Data generated from the three primer combinations were combined to form a combined data matrix consisting of all the bands. A Dice coefficient was used to calculate the similarity between the isolates (Table 2). Genetic pairwise distances between the isolates were calculated from the similarity data. The distance matrix generated was then analysed using a Neighbor - Joining clustering algorithm (Saitou and Nei, 1987) in PAUP* version 4.0b10 (Swofford, 1998). A bootstrap analysis (Felsenstein, 1985) with 1000 replicates was performed to determine support at the branch nodes. Bootstrap values greater than 50% were retained in the dendrogram. Isolates CMW9960 and CMW9955 representing RFLP Group I were used to provide an outgroup.

RESULTS

DNA Amplification Sizes

All Zimbabwean isolates gave successful amplification of the IGS-1 region using primer pairs P-1 and 5S-2B. Three size groups were observed, 1200 base pairs (bp), 1000 bp and approximately 900 bp (Figure 1 and Figure 2).

Restriction Fragment Length Polymorphisms (RFLPs)

Five different RFLP patterns were observed for isolates included in this study and these were designated Groups I to V (Table 1; Figure 3 and Figure 4). The restriction maps for

these RFLP patterns were generated from the sequence data (Figure 5). The fragment and amplicon sizes for these five RFLP Groups are summarised in Figure 3 and Figure 4. RFLP Group I isolates had a restriction pattern with fragment sizes of approximately 380, 255 and 130 bp and amplicon size of 1200bp. Isolates of RFLP Group II had fragment sizes of approximately 480, 255 and 175 bp, those in RFLP Group III had fragment sizes of approximately 480, 230 and 175 and those in Group IV had fragment sizes of approximately 485, 255 and 170 bp RFLP Groups II, III and IV had an uncut amplicon size of 900bp. Isolates in RFLP Group V had RFLP pattern with fragment sizes of approximately 480, 300 and 175 bp and amplicon size of 1000 bp.

Analysis of DNA sequences

IGS-1 sequence data for the isolates under consideration (Table 1) gave a total of 1159 characters in the data set after alignment (Figure 8). A total of 471 missing and ambiguous characters were excluded from the dataset before subsequent analysis. The phylogenetic tree generated from the DNA sequences grouped isolates into four well supported groups (Figure 6). Isolates CMW9963 and CMW10165, representing RFLP Group I, had a 100% bootstrap support. Isolates CMW11662 and CMW11650 representing RFLP Group V formed a distinct group with a bootstrap support value of 100%. Isolates CMW4456, CMW11653 and CMW11649 belonging to RFLP Group II formed a cluster with a bootstrap support value of 88%. Isolates CMW11653 and CMW11649 further formed a sub-group within this group (100% bootstrap support). Isolates belonging to RFLP Groups III and IV formed one group in the DNA sequence analyses, but with a low bootstrap support (54%).

Amplified Fragment Length Polymorphism (AFLPs) analyses

A total of three primer combinations were employed and individual primer pairs produced different polymorphisms with bands either monomorphic or polymorphic. There were 413 bands and this represented an average of 137 bands per primer pair. The Neighbor-Joining tree generated from the distance matrix separated the isolates into three clusters (Figure 7). The first cluster included isolates belonging to RFLP Group I. The second cluster comprised isolates belonging to RFLP Group II, and the third cluster had isolates residing in RFLP Groups III, IV, and V. RFLP Group I isolates grouped together with bootstrap support of 96%. Within this group the isolates from Dombera (88% bootstrap support) and those from Pondo (71% bootstrap support) grouped in respective clusters. RFLP Group II isolates formed a cluster (98% bootstrap support) and were clearly separate from the other RFLP Groups. RFLP Group IV formed a cluster with 56% support value and further formed two subgroups. One sub-group had a support value of 65% with isolates CMW9964, CMW9967, CMW11672, CMW11589 and CMW3. Isolates CMW1 and CMW11666 formed another sub-group with a 64% support value. Isolates of RFLP Groups III and V formed a cluster having 80% support, with RFLP Group V isolates further forming a sub-group with a 97% support and those of RFLP Group III forming a cluster with a 64% support value.

After calculating the dice similarity coefficient, isolates of Group I had similarity values that ranged from 61% - 89%. Isolates of Group II had similarity values ranging from 68-75%, Group III isolates had a similarity value of 76%, Group IV isolates had similarity values that ranged from 60% -89% and Group V isolates had a similarity value of 81%. With the exception of Groups III and V, the similarity values between the groups were

less than 50%. The similarity between Groups III and V was the same as within group similarity.

DISCUSSION

In this study a collection of isolates from different parts of Zimbabwe were characterised using PCR - RFLPs, sequencing and AFLPs. Amplification of the IGS-1 region for the isolates included in this study demonstrate that there is variation in IGS-1 amplicon sizes. Furthermore, five different RFLP Groups, designated I to V, were obtained. Most of these RFLP Groups correlated with the separations observed based on AFLP data and IGS-1 sequence data. Three of the RFLP Groups have previously been identified based on their isozyme profiles (Mwenje and Ride, 1996) and further confirmed using IGS-1 sequences (Mwenje et al., 2003).

RFLP Group I isolates are identical to a species from South Africa identified as *A. fuscipes* (Coetzee et al., 2000b). This species has also been recently reported in Zimbabwe (Mwenje et al., 2003) and Ethiopia (Gezahgne et al., 2004). *Armillaria fuscipes* was originally described in Sri Lanka by Petch (1909). Based on IGS-1 and AFLP data RFLP Group I isolates were clearly separated from the other groups and formed a distinct group. This study provides conclusive evidence that this group represents at least one separate species and based on the DNA sequence data is quite distantly related to the fungi in the other RFLP Groups.

RFLP Group II isolates were less frequently encountered in Zimbabwe than RFLP Groups I and III. Only four isolates of this fungus have thus far been collected. RFLP Group II isolates have a significantly unique IGS-1 sequence and RFLP profile. In addition we have shown in this study with AFLP profiles that they are not closely related to the fungi in any of the other RFLP Groups. Our data confirm the findings of Mwenje et al. (2003), who suggests that these isolates represent an undescribed species.

Isolates representing RFLP Group III showed slight variation in their IGS-1 DNA sequences. Although significant sequence variation is not expected within the same species, the phenomenon is not without precedent and has also been reported in other fungi (Garbelotto et al., 1993). RFLP variation within a species has been reported previously in *Armillaria* (Harrington and Wingfield, 1995; Pérez-Sierra et al., 1999; Coetzee et al., 2000a; Dunne et al., 2002; Anderson and Stasovski, 1992) and this was also the case in the present study. RFLP Group III has previously been identified as representing an unnamed species (Mwenje and Ride, 1996).

RFLP Group IV isolates had similar IGS-1 PCR amplicon sizes to those in RFLP Groups II and III. However, these groups differ in their RFLP patterns. The IGS-1 phylogram derived from DNA sequence data showed that RFLP Group IV isolates are most closely related to isolates of RFLP Group III. However, based on AFLP profiles Group IV isolates seem to be distinct and probably represent a previously undescribed species.

Isolates residing in RFLP Group V either represent a previously undescribed species or they are variants of an existing species. BlastN searches showed that their IGS-1 sequences are most similar to those of Zimbabwean RFLP Group III. RFLP Groups III and V could represent the same species that exhibit high levels of intraspecies variation in their IGS-1 region of the rRNA operon. Based on the AFLP data Groups III and V have similar dice similarity coefficients, providing additional evidence that they represent the same species.

AFLPs have been used only once previously to characterise *Armillaria* spp. (Pérez-Sierra et al., 2004) but have been extensively used in other organisms (Majer et al., 1996; Abdel-Satar et al., 2003; Giannasi et al., 2001; Zeller et al., 2003). In this study, with the exception of RFLP Groups III and V, the similarity values were high within the RFLP Groups and very low between groups. This supports the separation of the isolates into different groups based on RFLPs. The AFLP results further support the suggestion (Mwenje et al., 2003) that there are at least three species of *Armillaria* in Zimbabwe.

In this study AFLPs as well as RFLPs and DNA sequences from the IGS-1 region were used to identify *Armillaria* isolates from different geographic areas and hosts in Zimbabwe. Similar results obtained for the AFLP data and IGS-1 sequence comparisons supported the potential use of AFLP technique in *Armillaria* characterisation. From the results obtained in this study we also conclude there are at least four distinct *Armillaria* taxa in Zimbabwe. These include the fungus that we believe is *A. fuscipes*, the two groups previously characterised based on RFLP analyses (RFLP Group III and Group III). The

fourth taxon has not previously been found but was clearly present based on IGS-1 and AFLP data.

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Table 1: List of Zimbabwean *Armillaria* isolates used in the study

Isolate number	Alternative culture collection number	Host	Origin	RFLP group	IGS-1 RFLP size
CMW9960	D1	*Prunus persica*	Dombera	I	380, 255, 130
CMW9961	D2	*Pr. persica*	Dombera	I	380, 255, 130
CMW9962	D3	*Pr. persica*	Dombera	I	380, 255, 130
CMW9963	D4	*Pr. persica*	Dombera	I	380, 255, 130
CMW9950	D6	*Pr. persica*	Dombera	I	380, 255, 130
CMW9966	D8	*Pr. persica*	Dombera	I	380, 255, 130
CMW9953	P1	*Pr. persica*	Pondo	I	380, 255, 130
CMW9955	P3	*Pr. persica*	Pondo	I	380, 255, 130
CMW9959	P4	*Pr. persica*	Pondo	I	380, 255, 130
CMW9956	P5	*Pr. persica*	Pondo	I	380, 255, 130
CMW9957	P6	*Pr. persica*	Pondo	I	380, 255, 130
CMW10165	P7	*Pr. persica*	Pondo	I	380, 255, 130
CMW9958	NP1	Citrus	Pondo	I	380, 255, 130
CMW4456	Z1	*Brachystegia utilis*	Eastern Highlands	II	480, 255, 175
CMW4457	40	*Camellia sinensis*	Nyanga	II	480, 255, 175
CMW11649	Z43	*C. sinensis*	Nyanga	II	480, 255, 175
CMW11653	Z45	*C. sinensis*	Nyanga	II	480, 255, 175
CMW9954	P21	*Pr. persica*	Pondo	III	480, 230, 175
CMW10115	56	*Newtonia buchananii*	Harare	III	480, 230, 175
CMW9964	D5	*Pr. persica*	Dombera	IV	485, 255, 170
CMW9967	D10	*Pr. persica*	Dombera	IV	485, 255, 170
CMW11672	P11	*Pr. persica*	Pondo	IV	485, 255, 170
CMW11589	P22	*Pr. persica*	Pondo	IV	485, 255, 170
CMW3	FB1	*Pr. persica*	Pondo	IV	485, 255, 170
CMW1	DFB	*Pr. persica*	Pondo	IV	485, 255, 170
CMW11666	M37	*Pinus elliotti*	Martin Forest	IV	485, 255, 170
CMW11662	JM2	*P. kaseyi*	Staplefords	V	480, 300, 175
CMW11650	JM3	*P. kaseyi*	Staplefords	V	480, 300, 175

CMW represents the culture collection of the of the Forestry and Agricultural Biotechnology Institute (FABI) University of Pretoria, South Africa; other culture numbers are alternative culture collection numbers.

Figure 1. A 1% agarose gel stained with ethidium bromide showing the PCR products of the IGS-1 region after amplification using the primers P-1 and 5S-2B. Lanes marked M shows a 100bp marker (sizes of the bands are indicated on the gel). Lanes 1-13 represent amplicons for isolates CMW10165, CMW9962, CMW9950, CMW9966, CMW9959, CMW9956, CMW9957, CMW11662, CMW 4457, CMW11653, CMW11589, CMW9954 and CMW10115.

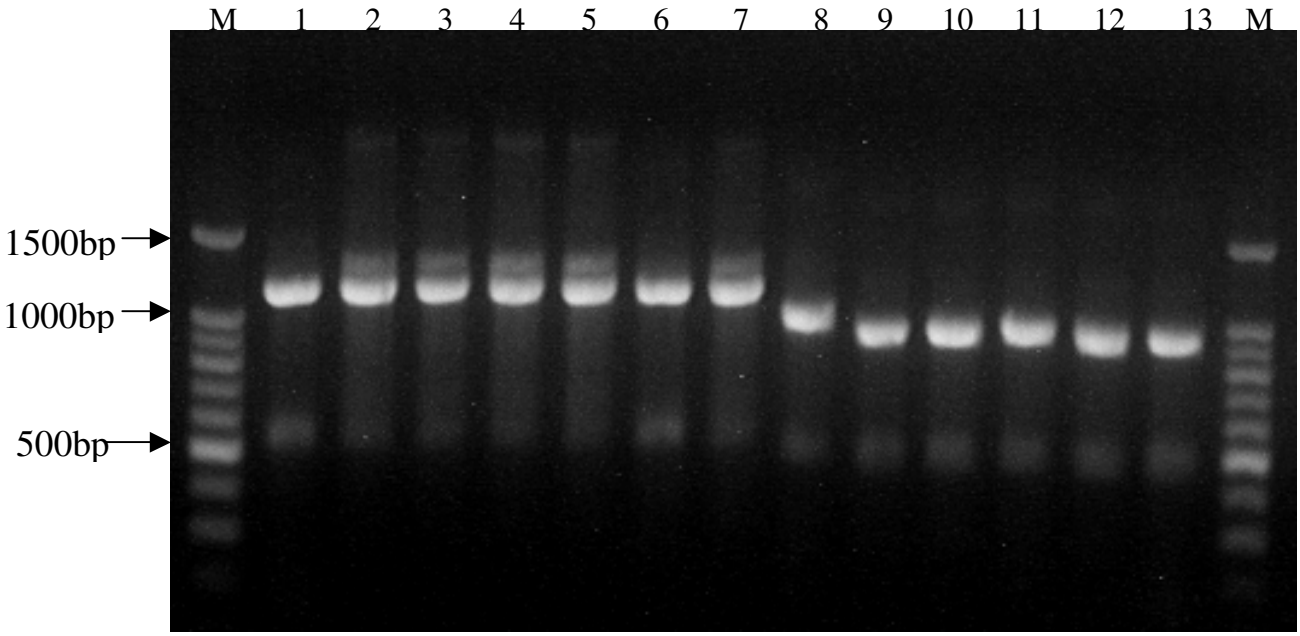
M
1
2
3
4
5
6
7
8
9
10
11
12
13
M
1500bp
1000bp
500bp

Figure 2. A 1% agarose gel stained with ethidium bromide showing the PCR products of the IGS-1 region after amplification with the primers P-1 and 5S-2B. Lanes marked M shows a 100bp molecular weight marker. Lanes 1-13 represent PCR products of the isolates CMW10165, CMW9960, CMW11650, CMW4457, CMW9964, CMW9967, CMW11649, CMW11666, CMW 3, CMW1, CMW11672, CMW9954 and CMW10115.

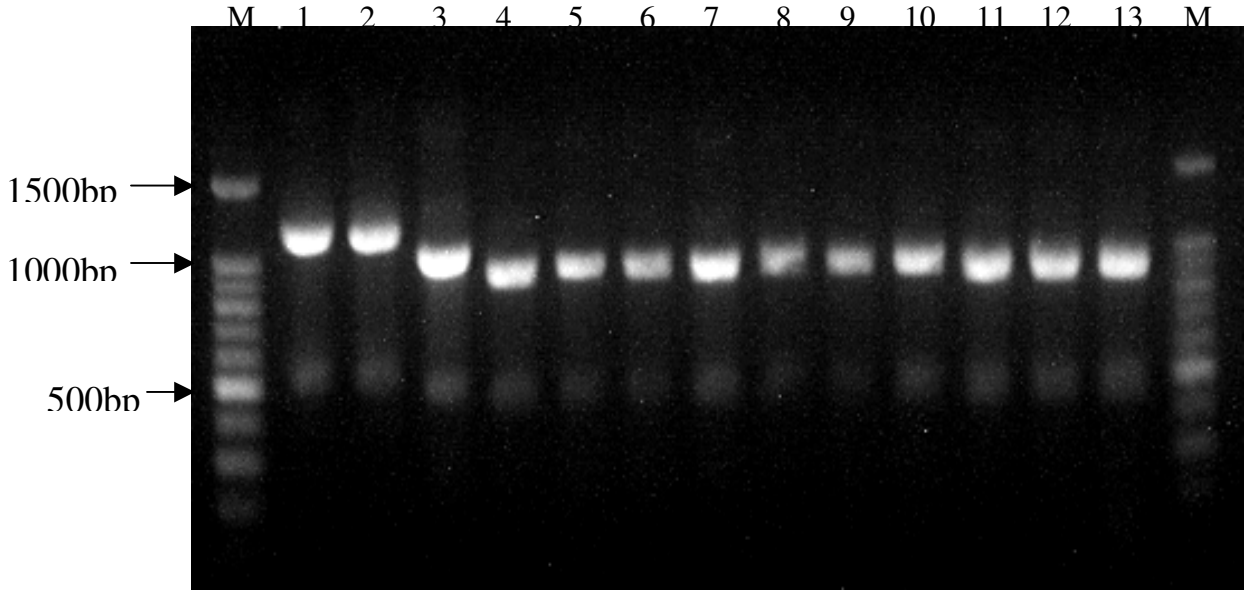
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1500bp
1000bp
500bp

Figure 3. A 3% agarose gel stained with ethidium bromide showing the RFLP patterns of *Alu*I digested PCR products of the IGS-1 region. Lanes marked M contain the 100bp marker. Lanes 1-13 represent the PCR products of the isolates CMW10165, CMW9962, CMW9950, CMW 9966, CMW9959, CMW9956, CMW9957, CMW11662, CMW4457, CMW11653, CMW11589, CMW9954 and CMW10115.

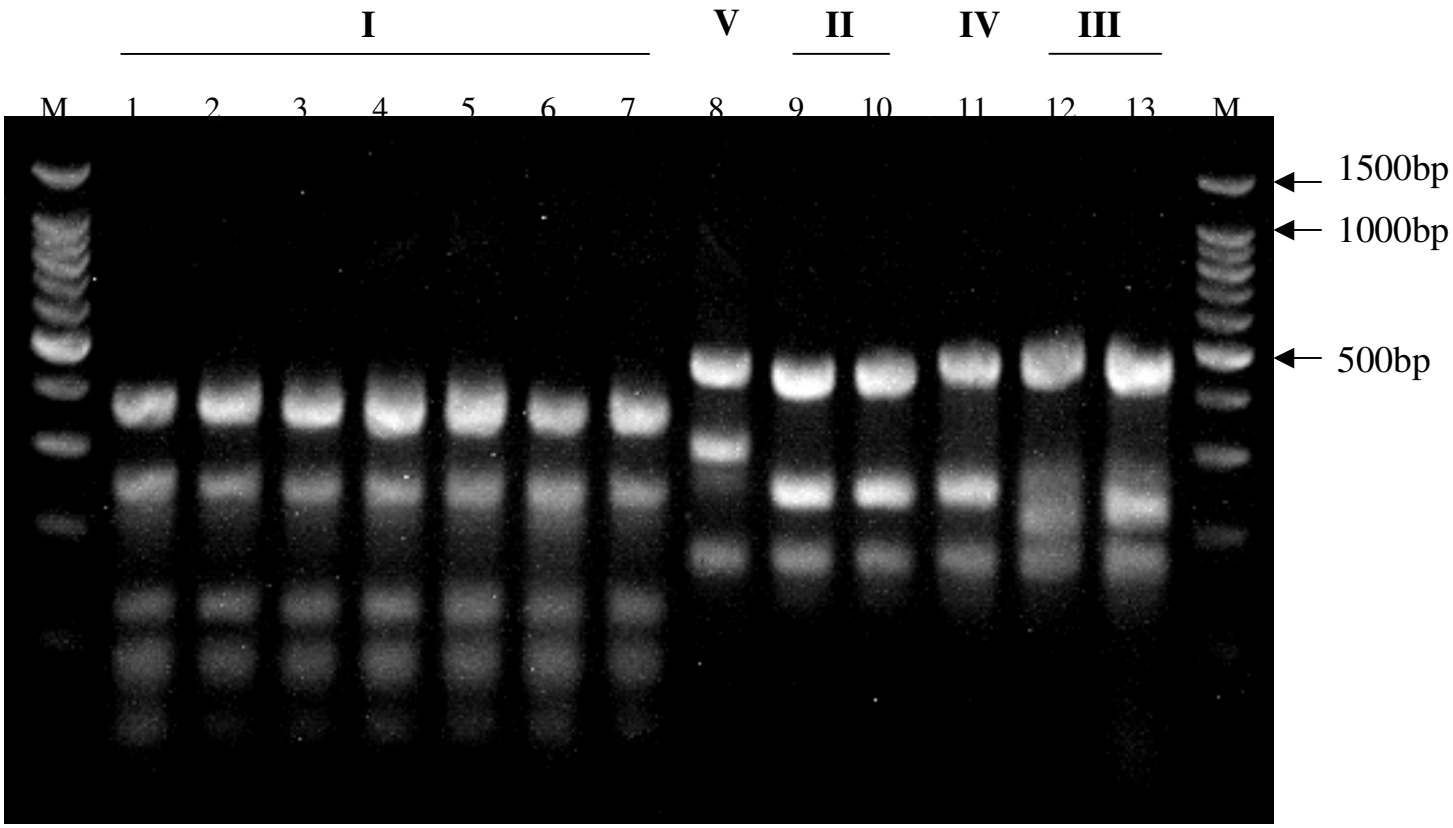
GROUPS
I
V
II
IV
III
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1500bp
1000bp
500bp

Figure 4. A 3% agarose gel stained with ethidium bromide showing the RFLPs of the *Alu*I digested PCR products of the IGS-1 region. Lanes marked M contain the 100bp marker. Lanes marked 1-13 represent the PCR products of the isolates CMW10165, CMW9960, CMW 11650, CMW4457, CMW9964, CMW9967, CMW11649, CMW11666, CMW3, CMW1, CMW9954, CMW9954 and CMW10115.

GROUPS

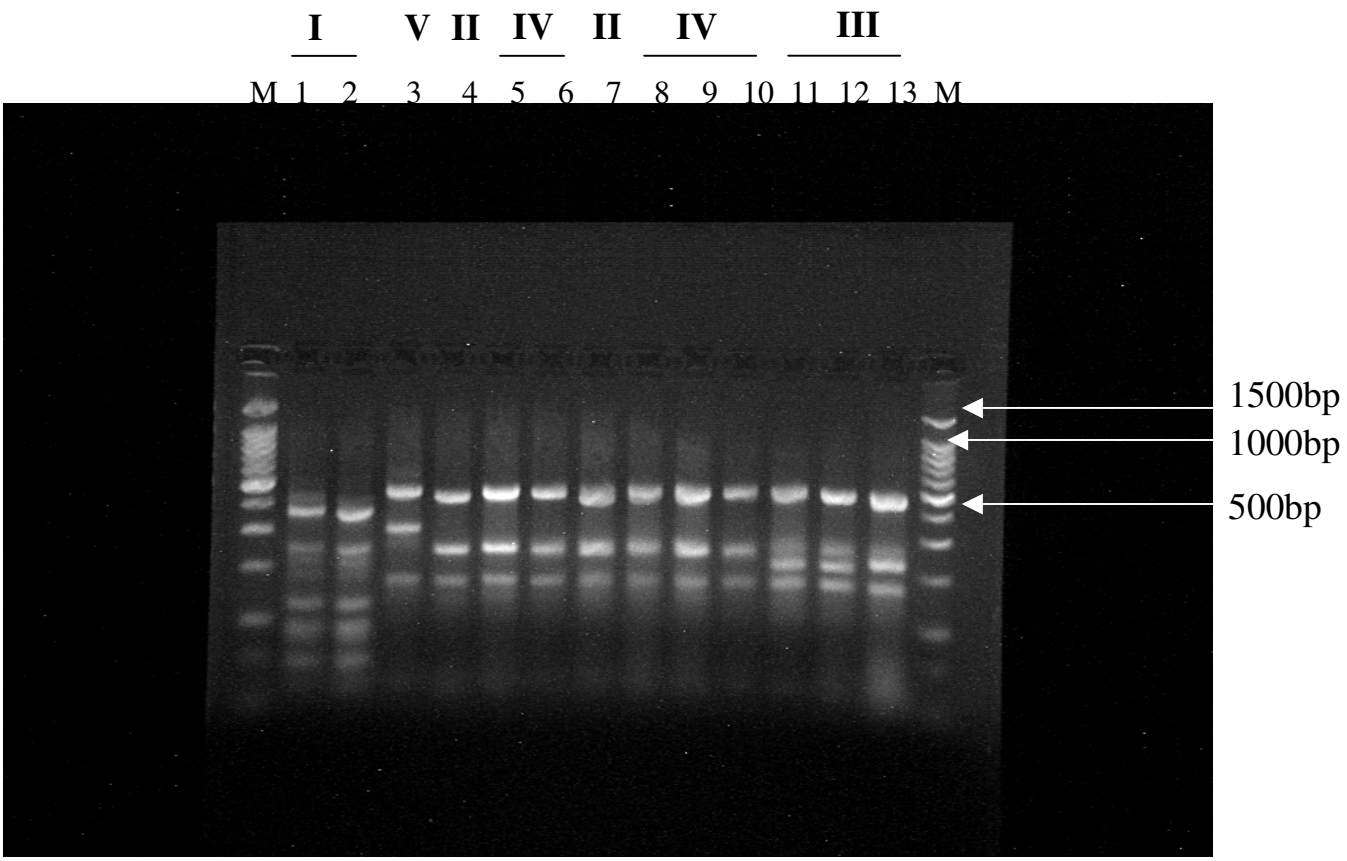

Figure 5. IGS-1 restriction maps generated for the enzyme *Alu*I based on the restriction patterns indicated in Table 1 for groups I, II, III, IV and V. Numbers designate the approximate length (bp) of the fragments after digestion.

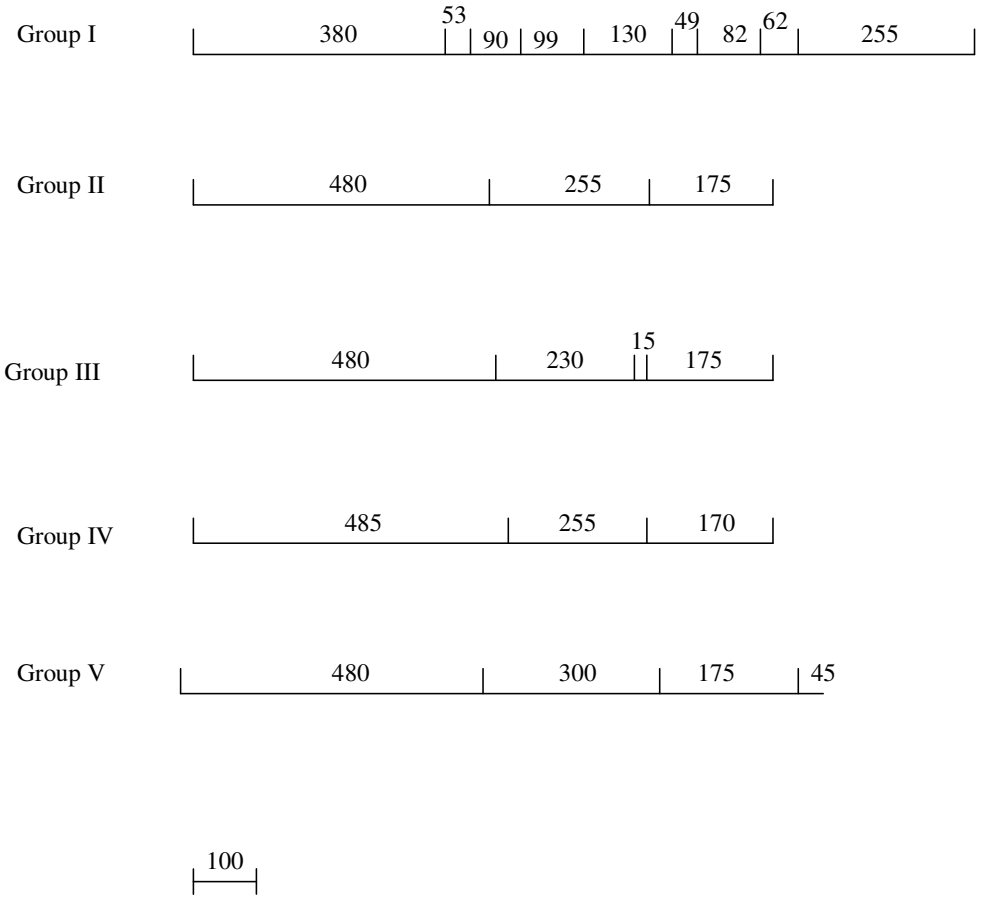
Group I
380
53
90
99
130
49
82
62
255
Group II
480
255
175
Group III
480
230
15
175
Group IV
485
255
170
Group V
480
300
175
45
100

Figure 6. Unrooted Neighbor- joining tree generated based on IGS-1 sequence data. Bootstrap values are indicated on the tree branch trees. Corresponding groups and areas of origin for the isolates are also indicated. The scale bar indicates the genetic distance between the isolates.

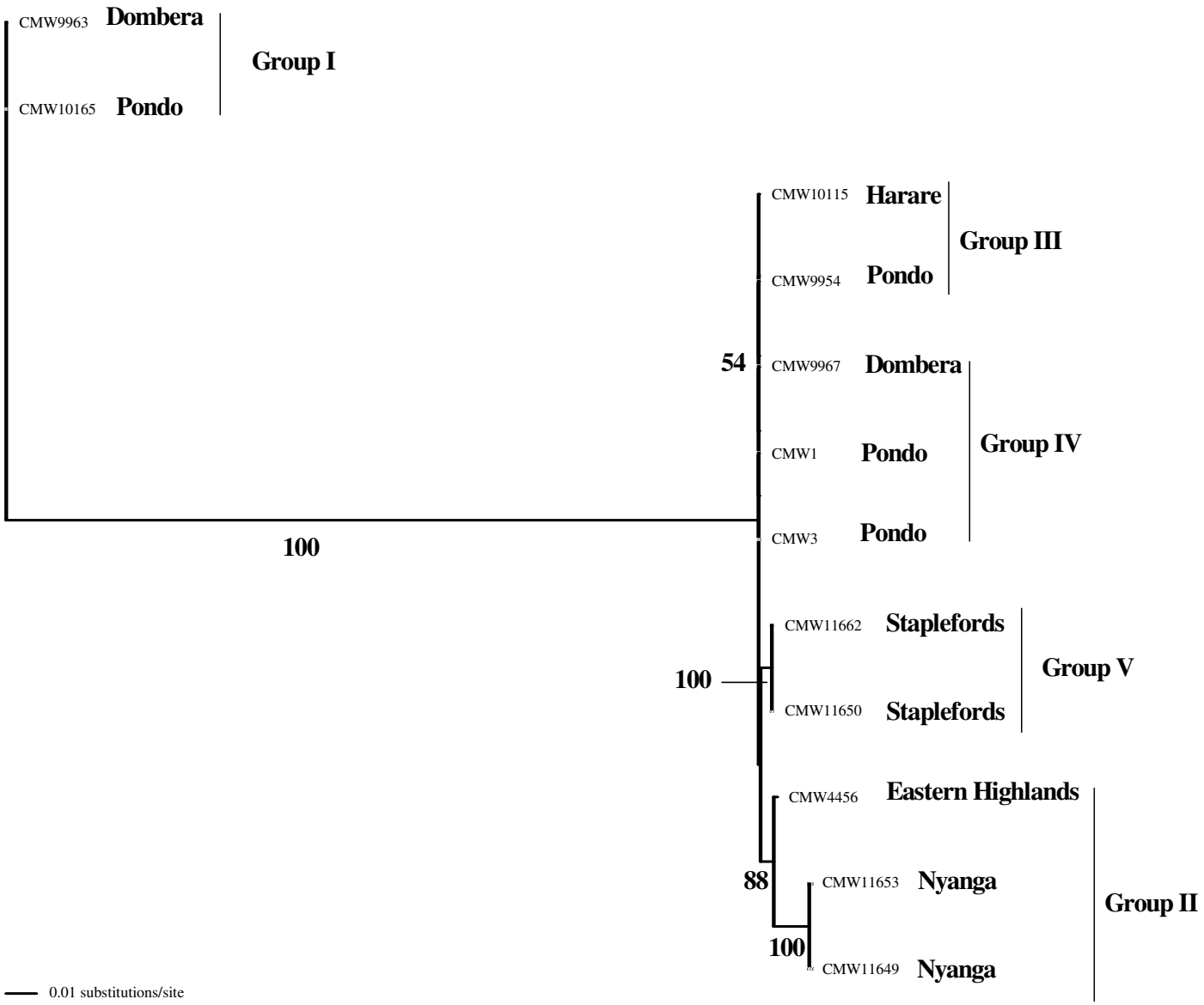
CMW9963 Dombera
CMW10165 Pondo
Group I
CMW10115 Harare
CMW9954 Pondo
Group III
54
CMW9967 Dombera
CMW1 Pondo
CMW3 Pondo
Group IV
100
CMW11662 Staplefords
100
CMW11650 Staplefords
Group V
CMW4456 Eastern Highlands
88
CMW11653 Nyanga
100
CMW11649 Nyanga
Group II
0.01 substitutions/site

Figure 7. Neighbor- Joining tree generated from AFLP characters of various *Armillaria* groups. Bootstrap values are shown on the branches and the scale bar corresponds to the to the distance between AFLP data for different isolates. The corresponding groups and areas of origin of the isolates are indicated next to the tree.

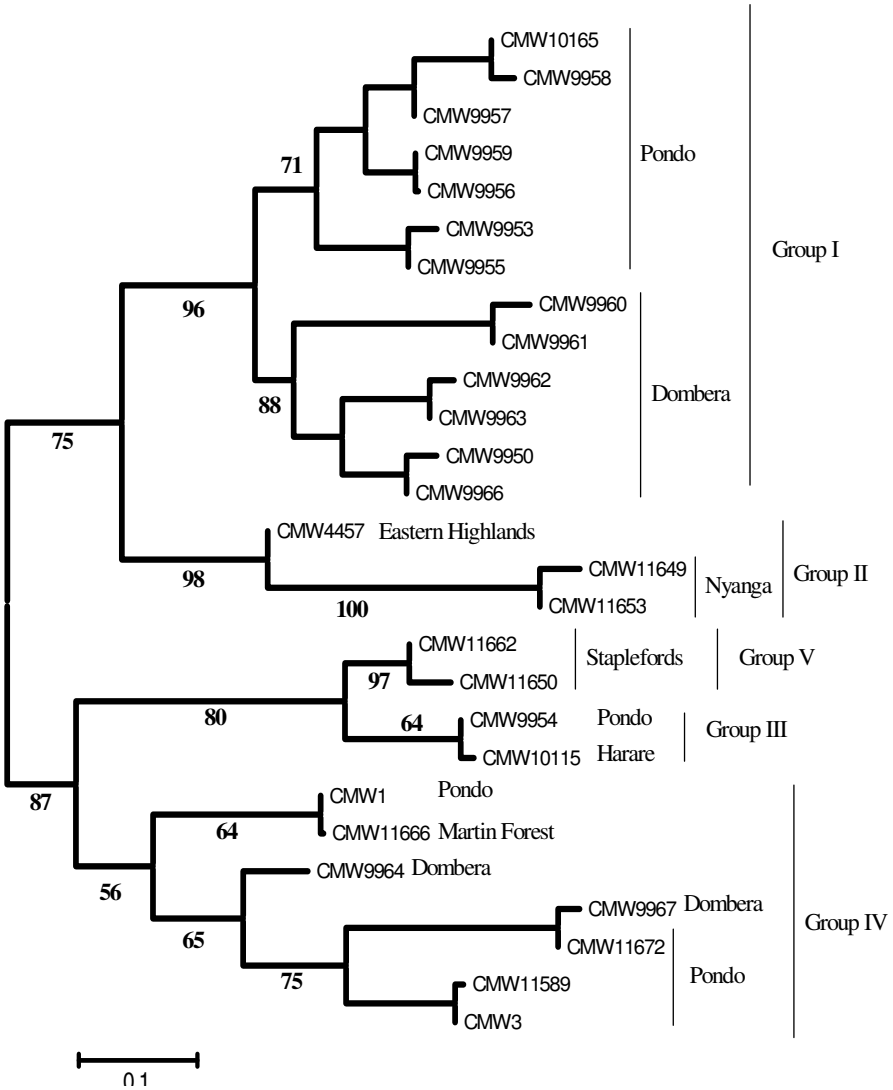
CMW10165
CMW9958
CMW9957
CMW9959
CMW9956
CMW9953
CMW9955
Pondo
Group I
71
96
CMW9960
CMW9961
CMW9962
CMW9963
CMW9950
CMW9966
Dombera
88
75
CMW4457 Eastern Highlands
98
CMW11649
CMW11653
Nyanga
Group II
100
CMW11662
CMW11650
Staplefords
Group V
97
80
64
CMW9954 Pondo
CMW10115 Harare
Group III
87
CMW1 Pondo
64
CMW11666 Martin Forest
CMW9964 Dombera
56
CMW9967 Dombera
65
CMW11672
Pondo
75
CMW11589
CMW3
Group IV
0.1

Table 2: Dice similarity coefficient calculated from AFLP fingerprint data of 27 *Armillaria* isolates.

	D1	D2	D3	D4	D6	D8	P1	P3	P4	P5	P6	P7	NP1	40	Z43	Z45	D5	D10	P11	P22	FB1	DFB	M37	JM2	JM3	P21	56
D1 (CMW9960)	1																										
D2 (CMW9961)	0.61	1																									
D3 (CMW9962)	0.65	0.79	1																								
D4 (CMW9963)	0.63	0.74	0.83	1																							
D6 (CMW9950)	0.65	0.75	0.8	0.86	1																						
D8 (CMW9966)	0.68	0.7	0.71	0.78	0.8	1																					
P1 (CMW9953)	0.61	0.68	0.73	0.74	0.73	0.81	1																				
P3 (CMW9955)	0.66	0.63	0.7	0.73	0.75	0.74	0.85	1																			
P4 (CMW9959)	0.63	0.69	0.69	0.74	0.67	0.73	0.72	0.86	1																		
P5 (CMW9956)	0.64	0.63	0.66	0.71	0.68	0.74	0.78	0.89	0.89	1																	
P6 (CMW9957)	0.63	0.63	0.67	0.69	0.65	0.7	0.76	0.84	0.86	0.88	1																
P7 (CMW10165)	0.63	0.63	0.64	0.68	0.61	0.71	0.72	0.76	0.79	0.79	0.88	1															
NP1 (CMW9958)	0.62	0.64	0.65	0.67	0.63	0.7	0.69	0.69	0.71	0.73	0.68	0.71	1														
40 (CMW4457)	0.4	0.32	0.31	0.31	0.31	0.37	0.32	0.31	0.36	0.35	0.36	0.35	0.32	1													
Z43 (CMW11649)	0.45	0.4	0.43	0.4	0.4	0.44	0.43	0.43	0.43	0.42	0.41	0.36	0.4	0.7	1												
Z45 (CMW11653)	0.41	0.32	0.41	0.39	0.35	0.4	0.37	0.34	0.29	0.32	0.34	0.38	0.39	0.68	0.73	1											
D5 (CMW9964)	0.13	0.15	0.14	0.13	0.07	0.13	0.17	0.15	0.11	0.12	0.15	0.18	0.17	0.21	0.2	0.28	1										
D10 (CMW9967)	0.14	0.16	0.17	0.15	0.12	0.21	0.17	0.14	0.15	0.13	0.13	0.21	0.19	0.26	0.29	0.36	0.64	1									
P11 (CMW11672)	0.18	0.23	0.32	0.26	0.28	0.26	0.27	0.22	0.23	0.2	0.25	0.27	0.35	0.25	0.32	0.39	0.63	0.62	1								
P22 (CMW11589)	0.16	0.26	0.28	0.24	0.25	0.27	0.26	0.23	0.22	0.2	0.25	0.24	0.27	0.25	0.3	0.37	0.63	0.61	0.69	1							
FB1 (CMW3)	0.38	0.35	0.42	0.36	0.36	0.4	0.43	0.42	0.37	0.36	0.37	0.42	0.41	0.35	0.39	0.41	0.6	0.6	0.6	0.6	1						
DFB (CMW1)	0.37	0.36	0.42	0.39	0.36	0.4	0.41	0.34	0.35	0.33	0.33	0.34	0.39	0.45	0.4	0.41	0.61	0.63	0.6	0.63	0.89	1					
M37 (CMW11666)	0.31	0.26	0.29	0.39	0.29	0.29	0.28	0.28	0.25	0.25	0.26	0.3	0.28	0.3	0.34	0.34	0.6	0.61	0.6	0.62	0.62	0.63	1				
JM2 (CMW11662)	0.21	0.28	0.34	0.31	0.31	0.29	0.3	0.3	0.25	0.25	0.29	0.27	0.28	0.27	0.33	0.33	0.24	0.33	0.41	0.43	0.41	0.44	0.46	1			
JM3 (CMW11650)	0.2	0.27	0.32	0.3	0.28	0.27	0.33	0.31	0.28	0.27	0.3	0.3	0.31	0.29	0.33	0.33	0.24	0.27	0.42	0.43	0.42	0.43	0.44	0.81	1		
P21 (CMW9954)	0.22	0.29	0.34	0.31	0.31	0.26	0.34	0.32	0.29	0.27	0.3	0.29	0.28	0.25	0.2	0.2	0.19	0.29	0.43	0.4	0.42	0.43	0.42	0.8	0.78	1	
56 (CMW10115)	0.29	0.34	0.36	0.36	0.37	0.36	0.4	0.4	0.35	0.32	0.38	0.34	0.28	0.3	0.4	0.4	0.27	0.27	0.44	0.41	0.39	0.42	0.42	0.72	0.76	0.77	1

Figure 8. Aligned nucleotide sequences for the IGS-1 region for isolates used in this study. Dashes (-) indicate gaps and unknown bases are indicated by N.

```
                10        20        30        40        50        60        70
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW10165  NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW10115  NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW9954   NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW11662  NNTGAATGGGAACGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW11650  NNTGAATGGGAACGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW9967   NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW3      NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW1      NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW4456   NNNNNNNNNNNNNGGGGTACTGTACGTGGTAGAGTAGCCTTGTTGCTACGATCCACTGAGGTTAAGCCCT
CMW11653  CTTGAATGGGAACGGGGTAGTCTTACGTGTAGAGTAGCCT-GT-GCTACGATCCACTGAGGTTAAGCCCT
CMW11649  CTTGAATGGGAACGGGGTAGTCTTACGTGTAGAGTAGCCT-GT-GCTACGATCCACTGAGGTTAAGCCCT

                80        90       100       110       120       130       140
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   TGTTCTAAAGATTTGTTCAACTGTGTGTTGGGCGTACATGCTGGGCTGGTTGAGGGCGGGGAATGTAACC
CMW10165  TGTTCTAAAGATTTGTTCAACTGTGTGTTGGGCGTACATGCTGGGCTGGTTGAGGGCGGGGAATGTAACC
CMW10115  TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW9954   TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW11662  TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW11650  TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW9967   TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW3      TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW1      TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW4456   TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW11653  TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC
CMW11649  TGTTCTAAAGATTTGTTCAACGAAATGTTGGACGTACATGCTGGGCTCGTTGAGGGCGGGAAATGTAACC

              150       160       170       180       190       200       210
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   TATGCGCTCATAAACAGCATGTTGAATGGAGGGGTATGGATCCAAGCGTATTG-TATATACGGTGTATGG
CMW10165  TATGCGCTCATAAACAGCATGTTGAATGGAGGGGTATGGATCCAAGCGTATTG-TATATACGGTGTATGG
CMW10115  TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW9954   TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW11662  TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW11650  TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW9967   TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW3      TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW1      TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW4456   TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW11653  TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
CMW11649  TATGCGCTCATAAACAGCATGTTTAATGGAAGCCTATTGTGTATAA--TATTGGTATATACGGTGTACGG
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                220       230       240       250       260       270       280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    AGTGCGGGTATACAGAAGAGAAGAGTATACAGTGCAGTACACAGTATATATATATATATATATTATGTAATC
CMW10165   AGTGCGGGTATACAGAAGAGAAGAGTATACAGTGCAGTACACAGTATATATATATATATATTATGTAATC
CMW10115   AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW9954    AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW11662   AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW11650   AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW9967    AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW3       AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW1       AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW4456    AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW11653   AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC
CMW11649   AGTACGGGTATACAGAAGAG-----TATACAGTACAGTAGACAGTATATATATATATA--TTATAT-ATC


                290       300       310       320       330       340       350
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    TACATGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGGTC----------TTGGGTTGAAATCA
CMW10165   TACATGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGGTC----------TTGGGTTGAAATCA
CMW10115   TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW9954    TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW11662   TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW11650   TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW9967    TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW3       TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW1       TA--TGACTTGGACTTGGACTTGTACTTGGACTTGGATCTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW4456    TA--TGACTTGGACTTGGAT----------------CTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW11653   TA--TGACTTGGACTTGGAT----------------CTTGGATCACAATGCAAGTAAGGTAGTAGGCA
CMW11649   TA--TGACTTGGACTTGGAT----------------CTTGGATCACAATGCAAGTAAGGTAGTAGGCA

                360       370       380       390       400       410       420
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    ----------------------CCAAAGACAATGCAAGGAA--------------------------
CMW10165   ----------------------CCAAAGACAATGCAAGGAA--------------------------
CMW10115   ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW9954    ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW11662   ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW11650   ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW9967    ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAaTGCAAGGCAATGCAAGGC
CMW3       ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW1       ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW4456    ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW11653   ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
CMW11649   ATGCAACGCAAGGCTAGTAGACAACGCAAGGCAATGCAAGGATAGTAGACAATGCAAGGCAATGCAAGGC
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                 430       440       450       460       470       480       490
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   -------------------------GCTAGTAGACAAGAC--GACAAGTAAGCTACCAGGCAGACTTGT
CMW10165  -------------------------GCTAGTAGACAAGAC--GACAAGTAAGCTACCAGGCAGACTTGT
CMW10115  TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW9954   TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW11662  TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW11650  TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW9967   TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW3      TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW1      TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAAGGCAAGTAAGCTAGCAGGCAGACTTGT
CMW4456   TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAACGCAAGTAAGCTAGCAGGCAGACTTGT
CMW11653  TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAACGCAAGTAAGCTAGCAGGCAGACTTGT
CMW11649  TAGTAGACAACGCAACGCAATGCAAGGCTAGTAGACAACGCAACGCAAGTAAGCTAGCAGGCAGACTTGT

                 500       510       520       530       540       550       560
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   GAGTCTTGAGAGCTTGTACGCATCTCTTAGTTGGCGCGCATAGAGTCTTTGGACTTGGGACTTGGACACC
CMW10165  GAGTCTTGAGAGCTTGTACGCATCTCTTAGTTGGCGCGCATAGAGTCTTTGGACTTGGGACTTGGACACC
CMW10115  GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW9954   GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW11662  GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW11650  GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW9967   GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW3      GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW1      GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW4456   GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW11653  GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------
CMW11649  GAG--TTGAGAGCTTGTACGCATGTCTTAGTTGGTGTGCA-----------------------------

                 570       580       590       600       610       620       630
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   CAATGGATTGCGGACTTGGACAGAATTGCAAGCTGCATTGAGCGCTCGTACGCATGCATGCCTTACTTGT
CMW10165  CAATGGATTGCGGACTTGGACAGAATTGCAAGCTGCATTGAGCGCTCGTACGCATGCATGCCTTACTTGT
CMW10115  -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW9954   -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW11662  -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW11650  -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW9967   -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW3      -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW1      -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW4456   -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW11653  -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
CMW11649  -------TTGCGGACTTGG---------------GCATTGAGGGCTTGTATGCACGCA--CCTTA-CGGA
```

```
                640       650       660       670       680       690       700
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   CTTGCA-----AGCTGCATCCATG--ACTTGCCCTCAAGCAAATG--CATTGAATGCGTCGACTTGCAAG
CMW10165  CTTGCA-----AGCTGCATCCATG--ACTTGCCCTCAAGCAAATG--CATTGAATGCGTCGACTTGCAAG
CMW10115  CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW9954   CTTGGACATTGAGGTGTATGCACGGACATT--------------------GAG----------------
CMW11662  CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW11650  CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW9967   CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW3      CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW1      CTTGGACATTGAGGTGTATGCACGCACCTTACGGACTT------GGACATTGAG----------------
CMW4456   CTTGGACATTGAGGTGTATGCACGC---TT--------------GGACATTGAG----------------
CMW11653  CTTGGACATTGAGGTGTATGCACGC---TT--------------GGACATTGAG----------------
CMW11649  CTTGGACATTGAGGTGTATGCACGC---TT--------------GGACATTGAG----------------

                710       720       730       740       750       760       770
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   CTAGTGTTGCGCATATTATGCATGTCTTACTTGCATTTCGCTAGTTAGCACATTGAC-TTGCAAGC---C
CMW10165  CTAGTGTTGCGCATATTATGCATGTCTTACTTGCATTTCGCTAGTTAGCACATTGAC-TTGCAAGC---C
CMW10115  ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW9954   ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW11662  ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW11650  ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW9967   ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW3      ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW1      ---GTGT----------ATGCA----------------CGG--------ACATTGAGG-TGTATGCACG-
CMW4456   ---GTGT----------ATGCA----------------CGCT----TGGACATTGAGGGTGTATGCACG-
CMW11653  ---GTGT----------ATGCA----------------CGCT----TGGACATTGAGGGTGTATGCACG-
CMW11649  ---GTGT----------ATGCA----------------CGCT----TGGACATTGAGGGTGTATGCACG-

                780       790       800       810       820       830       840
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963   AAGTTACGCTAGTTAGTTAGACAACCTTGGTTTGACTTTGGCAAATGCGTTCACTTGCAAGCTTAGTTGG
CMW10165  AAGTTACGCTAGTTAGTTAGACAACCTTGGTTTGACTTTGGCAAATGCGTTCACTTGCAAGCTTAGTTGG
CMW10115  ----------------------------------------------C------ACCT--------------
CMW9954   ----------------------------------------------C------ACCTT-------------
CMW11662  -------------------GAC---ATTGAGGTGTA--TGCACGG------ACATTGAGGTGTATGCACG
CMW11650  -------------------GAC---ATTGAGGTGTA--TGCACGG------ACATTGAGGTGTATGCACG
CMW9967   -------------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
CMW3      -------------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
CMW1      -------------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
CMW4456   --CTTG-------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
CMW11653  --CTTG-------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
CMW11649  --CTTG-------------GAC---ATTGAGGTGTA--TGCACGC------ACCTT--------------
```

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                850       860       870       880       890       900       910
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    ACTATGATTTCGTGCATTGAAATACAAGTCAACATGCTAGCTAGCACTTCACGAATGGAACTTGGTTTAT
CMW10165   ACTATGATTTCGTGCATTGAAATACAAGTCAACATGCTAGCTAGCACTTCACGAATGGAACTTGGTTTAT
CMW10115   ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW9954    ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW11662   GACATTGAGGTGTATGCACGGACATTGAGGTGTATGCACGCACCTTACGGACTTGGACATTGAGGGCTTG
CMW11650   GACATTGAGGTGTATGCACGGACATTGAGGTGTATGCACGCACCTTACGGACTTGGACATTGAGGGCTTG
CMW9967    ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW3       ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW1       ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW4456    ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW11653   ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG
CMW11649   ----------------------------------------------ACGGACTTGGACATTGAGGGCTTG

                920       930       940       950       960       970       980
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    AGCAAGTATGCCACCTATAGCCAAGTACGAAAGCATTGACTTGCAAGCTAAGCTTCGTTGGATTCTCTAT
CMW10165   AGCAAGTATGCCACCTATAGCCAAGTACGAAAGCATTGACTTGCAAGCTAAGCTTCGTTGGATTCTCTAT
CMW10115   TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW9954    TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW11662   TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW11650   TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW9967    TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW3       TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW1       TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW4456    TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW11653   TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------
CMW11649   TACGCACGCACCTTACTTTGTTGGCGCAAAAAT-AAAGACTTGCAAGCTAAGCTTGATTGGACT------

                990       1000      1010      1020      1030      1040      1050
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    TAGTTACATCTACTTGGACTATGGCTGACAGGCAAAAAGCAAAGGGGGACTTGTTGGCAGAATTGAACTT
CMW10165   TAGTTACATCTACTTGGACTATGGCTGACAGGCAAAAAGCAAAGGGGGACTTGTTGGCAGAATTGAACTT
CMW10115   ---------------GGAGT---------------------------------------CAGACTTGA----
CMW9954    ---------------GGAGT---------------------------------------CAGACTTGA----
CMW11662   ---------------GGAGT---------------------------------------CAGACTTGA----
CMW11650   ---------------GGAGT---------------------------------------CAGACTTGA----
CMW9967    ---------------GGAGT---------------------------------------CAGACTTGA----
CMW3       ---------------GGAGT---------------------------------------CAGACTTGA----
CMW1       ---------------GGAGT---------------------------------------CAGACTTGA----
CMW4456    ---------------GGAGT---------------------------------------CAGACTTGA----
CMW11653   ---------------GGAGT---------------------------------------CAGACTTGA----
CMW11649   ---------------GGAGT---------------------------------------CAGACTTGA----
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                1060      1070      1080      1090      1100      1110      1120
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CMW9963    TTTCTTCGTTTACAGCGTGCACCGTG-GCCGTGCTGGGTCAGACTTAATGCCATGTTACTATCAAAAACC
CMW10165   TTTCTTCGTTTACAGCGTGCACCGTGTGCCGTGCTGGGTCAGACTTAATGCCATGTTACTATCAAAAACC
CMW10115   --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW9954    --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW11662   --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW11650   --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW9967    --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW3       --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW1       --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW4456    --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTGACTATCAAAAACC
CMW11653   --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC
CMW11649   --TATTCGT--------ACTTAATGCT-ATCTTGCTATCTTACTATCTTACTATCTTACTATCAAAAACC


            1130      1140      1150
           ....|....|....|....|....|....|....|....
CMW9963    ACAGCACCCAGGATTCCCGCATGGTCCCC-ACCGTGGTA
CMW10165   ACAGCACCCAGGATTCCCGCATGGTCCCCCACCGTGGTA
CMW10115   ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW9954    ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW11662   ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGAGGNN
CMW11650   ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGAGGNN
CMW9967    ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW3       ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW1       ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW4456    ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGTGGTA
CMW11653   ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGGGNNN
CMW11649   ACAGCACCCAGGATTCCCGCGTGGTCCCCCACCGGGNNN
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Chapter 3:

Phylogenetic relationships among *Armillaria* species based on partial Elongation Factor 1 alpha (EF 1-α) DNA sequence data.

Phylogenetic relationships among *Armillaria* species based on partial Elongation Factor 1 alpha (EF 1-α) DNA sequence data.

Abstract

Armillaria spp. are important root rot pathogens with a wide host range and a world wide distribution. The taxonomy of these fungi has been problematic for many years but the understanding of relationships has been substantially improved through the application of DNA sequence comparisons. In this study, the relationships between different *Armillaria* spp. was, for the first time, determined using EF 1-α DNA sequence data. A total of 42 isolates, representing the majority of *Armillaria* spp., with diverse geographic distribution and hosts were included in this study. PCR amplification yielded products of 600 base pairs for all the isolates. Phylogenetic trees resulting from parsimony analysis showed that this gene region is useful for studying relationships between species. Generally, results were similar to those emerging from previous comparisons using ITS and IGS-1 sequence data. This is the first time a single copy gene has been used to study phylogenetic relationships in *Armillaria* and overall, the data support previously held views regarding the relationships between species.

INTRODUCTION

Species of *Armillaria* represent important plant pathogens that may cause serious root disease problems in plantations, natural forests and in agriculture. These pathogens cause the plant disease known as Armillaria root rot (Hood et al., 1991). The taxonomy of these fungi has been surrounded by considerable confusion and debate. The genus name was disputed for many years, with the name *Armillariella* being considered valid by some authors (Volk and Burdsall, 1995). There has also been much uncertainty regarding species that should appropriately be included in the genus (Watling et al., 1991). The taxonomic status of *Armillaria* is now reasonably well recognised and there are at least 38 species included in the genus based on morphological characteristics or reproductive isolation (Volk and Burdsall, 1995).

Newly established plants on lands formerly occupied by forest or orchards are often seriously damaged due to infection by *Armillaria* species. These infections typically result from rhizomorphs that grow out from residual stumps of dead trees that had previously been infected with the fungus (Hood et al., 1991). The extent of disease development depends on the environment, the pathogenicity of the species involved and the resistance of the host (Blodgett and Worrall, 1992). Some species are apparently host specific whilst others cause disease on large numbers of different plant species (Hood et al., 1991). The impact of Armillaria root rot on plantation and forest trees, as well as other crops, justifies the need to effectively identify and characterise the species involved.

A number of techniques have been employed to identify *Armillaria* species. Traditionally, morphological characters were used for this purpose. Morphological identification largely focuses on the macro- and micro-morphology of the basidiocarps (sexual structures) to differentiate among species.

Although this method is reasonably easy to apply, the rare occurrence of basidiocarps in nature and their relatively short life cycle limits the use of morphology for identification (Swift, 1972; Kile and Watling, 1981). In addition to these problems some species, for example *A. gemina* and *A. ostoyae*, have identical basidiocarp morphologies (Bérubé and Dessureault, 1989). These limitations led to the introduction of mating compatibility tests to facilitate identification (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979). This method is, however, time consuming and interpretation of results is commonly ambiguous. Yet, despite their disadvantages morphology and mating tests have played an important role in *Armillaria* taxonomy and are still commonly used.

In an attempt to overcome the problems associated with morphology and mating compatibility tests, identification methods/ techniques employing biochemical and genotypic characteristics have emerged. Biochemical characters obtained from isozyme and protein profiles (Whalström et al., 1991; Mwenje and Ride, 1996) as well as monoclonal and polyclonal antibodies (Burdsall et al., 1990) have been used. Genotypic characters from mtDNA, nDNA and amplified IGS-1 as well as ITS region RFLP analyses (Anderson et al., 1989; Harrington and Wingfield, 1995; Coetzee et al., 2000b), DNA-DNA hybridisation (Miller et al., 1994) and AFLP (Pérez-Sierra et al., 2004) have yielded useful results.

Comparisons of DNA sequence data are increasingly being used for the identification of *Armillaria* spp. in order to gain knowledge concerning their phylogenetic relationships. Sequence data for this purpose have largely emerged from the IGS-1 (Anderson and Stasovski, 1992; Coetzee et al., 2000a/b; 2001; Pérez-Sierra et al., 2004) and ITS regions (Coetzee et al., 2000a; 2001; Chillali et al., 1998; Pérez-Sierra et al., 2004). Piercey-Normore et al. (1998) used combined sequence data of four anonymous DNA regions to determine the phylogeny of North American Biological Species (NABS) of *Armillaria*. The

combined anonymous data set gave a more resolved phylogenetic tree than those based on ITS and IGS-1 sequence data. No studies have been reported employing DNA sequence data for a protein-coding gene for phylogenetic analyses including a wide variety of *Armillaria* species.

The objectives of this study were to generate DNA sequence data for the Translational Elongation Factor 1-α (EF 1-α) gene for the majority of *Armillaria* spp. from different parts of the world. This gene is involved in protein synthesis in eukaryotes through transporting amino-acyl tRNAs to the ribosomes (Slobin, 1980) and has been successfully used in taxonomic and phylogenetic studies on ascomycetes and basidiomycetes, both at the intra and inter-specific levels (Jiménez-Gasco et al., 2002; Baayen et al., 2000, Kauserud and Schumacher, 2001). These data would provide an additional gene region on which to test taxonomic groupings and phylogenetic relationships previously identified using other gene regions.

MATERIALS AND METHODS

Cultivation of Isolates

Isolates included in this study were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Isolates were grown on MYA (2% Biolab Malt extract, 0.2% Biolab Yeast Extract and 1.5% Biolab Agar) in Petri dishes for two weeks at 23 ºC in the dark.

DNA Extraction

Isolates were transferred to liquid MY (2% Biolab Malt extract and 0.2% Biolab Yeast extract) in 500 mL Erlenmeyer flasks and allowed to grow for three weeks at 23 ºC in the dark. Mycelium was harvested through filtration, freeze-dried and ground into a fine powder in liquid nitrogen. Approximately 0.6 g of powdered mycelium was added to one mL of extraction buffer (200 mM Tris-HCl pH 8; 25 mM EDTA; 250 mM NaCl and 0.5% SDS) and incubated at 57 ºC for one hour. The aqueous phase was separated from cell debris by centrifugation (15300 *g*, 30 minutes). Phenol - chloroform (1:1) extractions were performed until a clean interphase was obtained. Excess phenol was removed through a final chloroform extraction. DNA was precipitated overnight at –20 °C using cold Ethanol (2:1 v/v) and collected by centrifugation (15300 *g*, 15 minutes). The precipitated DNA was washed with 70% ethanol and recollected by centrifugation. The precipitated DNA was dried at 55 °C and resuspended in sterile distilled water. DNA concentrations were determined using a Beckman Du Series 7500 UV Spectrophotometer following the procedure outlined in Maniatis et al. (1982).

Amplification of the partial (EF 1-α) gene region

Approximately 100 ng of DNA extracted from the *Armillaria* isolates was used as a template for amplification of a region of the EF 1-α gene. Amplicons were generated using primer EF595F (5' CGT GAC TTC ATC AAG AAC ATG 3') that binds at the 5' end of the exon and primer EF1160R (5' CCG ATC TTG TAG ACG TCC TG 3') that is complimentary to the 3' end of the exon (Kauserud and Schumacher, 2001) (Figure 1). The PCR reaction mixture included one mM of each dNTP; 2.5 mM $MgCl_2$; PCR buffer containing $MgCl_2$ and supplied with the polymerase enzyme; 0.01 µM of each primer and 100 ng of DNA and 2.5 U of *Taq* Polymerase (Boehringer Mannheim, South Africa). The

final reaction volume was 50 µl. The PCR reaction conditions were, an initial denaturation at 94 °C for two minutes; followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds and an extension step at 72 °C for 30 seconds. The final elongation step was allowed to proceed for seven minutes at 72 °C. PCR products were electrophoresed on a 1% (w/v) ethidium stained agarose gel and the bands were visualized under UV illumination.

DNA Sequencing

PCR products were purified prior to sequencing using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Primers EF595F and EF1160R were used in separate reactions to sequence both DNA strands. Sequencing reactions were done using a ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Warrington, U.K.) according to the manufacturer's instructions. DNA sequences for the partial EF 1-α gene were determined using an ABI PRISM™ 3100 automatic DNA sequencer (Applied Biosystems/HITACHI, Foster City, California, USA).

DNA Sequence Analysis

Sequencing results were analysed using Sequence Navigator version 1.01 (ABI PRISM™). The DNA sequences were aligned using the program ClustalX version 1.8 (Thompson et al., 1997) and manually adjusted. Phylogenetic analysis was done using PAUP* version 4.0b10 (Swofford, 1998). DNA sequences from the basidiomycete *Schizophyllum commune* (GenBank accession number X94913) were used for an outgroup. Missing and ambiguous characters were excluded from the analysis. Phylogenetic signal (Hillis and Huelsenbeck, 1992) was determined for 1000 random trees. Phylogenetic trees were generated based on parsimony using Heuristic searches with random stepwise addition of sequences (10

replicates) and TBR (tree bisection reconnection) branch swapping with MulTree active. Characters were reweighted according to the mean consistency index (CI) after each tree search until the number of trees stabilised to reduce homoplasy. Confidence at the branch points was obtained through bootstrap analysis (1000 replicates) (Felsenstein, 1985). Settings were the same as above except that simple addition of sequences was used and groups were retained with bootstrap values greater than 60%.

RESULTS

DNA Amplification

A DNA amplicon was successfully amplified for all the isolates used in this study. All amplifications yielded a single fragment. The amplification products for the isolates were approximately 600 base pairs in length (Figure 2).

Sequence data and analysis

The total number of characters included in the data matrix was 562 after alignment by inserting gaps (Figure 4). Twenty-five missing or ambiguously aligned characters and 329 constant characters were excluded from the analysis. The number of parsimony informative characters was 173. A *g*1 value of –1.40443 was obtained indicating that there is phylogenetic signal. Heuristic searches yielded eight most parsimonious trees with a length of 462 steps. The CI and Retention Index (RI) were 0.680 and 0.852, respectively. Five most parsimonious trees were generated (length = 314 steps, CI = 0.787 and RI = 0.897) after reweighting. The trees had similar topologies but differed in branch length. One of the five most parsimonious trees (Figure 3) was chosen for representation.

Bootstrap values supported the separation of species into distinct clades. In the analyses, two major and well supported groups were detected. One of these represented isolates from Africa (100% bootstrap support) and the other incorporated all isolates from other parts of the world. These are referred to as the African and Non-African clades. The northern hemisphere species grouped within a well supported sub-clade (92% bootstrap support) within the Non-African clade.

Isolates within the African clade formed two sub-clades. Isolates CMW3164 and CMW4953 representing *A. fuscipes* grouped within one sub-clade with 76% bootstrap support. The remainder of the isolates CMW4455, CMW4456, CMW10115 and CMW9954 representing Zimbabwean groups II and III (Mwenje and Ride, 1996) formed the second sub-clade with a 99% bootstrap support. Isolates CMW9954 and CMW10115 formed a group within the second sub-clade with 61% bootstrap support.

Isolates representing *A. borealis, A. gallica*, *A. nabsnona, A. cepistipes* and *A. tabescens* grouped together in one clade with a 63% support value. This clade comprised of three less well supported clades namely the *A. borealis* clade, *A. tabescens* clade and a clade comprising *A. gallica*, *A. nabsnona,* and *A. cepistipes. Armillaria gemina* and *A. ostoyae* grouped in a sister clade with a 100% bootstrap support. These two major clades had an 83% support.

Isolates representing *A. mellea* formed a monophyletic group within the northern hemisphere clade. Isolates CMW3956 and CMW4605 from eastern North America formed a separate sub-clade with a 97% bootstrap support value. Isolates CMW3964 and CMW4620 from western North America grouped together with 100% bootstrap support. *Armillaria mellea* isolates CMW4613 and CMW11231 from

Europe resolved into a clade with 100% bootstrap support. Isolates CMW3961, CMW4610 and CMW4611 from Asia formed a separate clade with 100% bootstrap support.

Southern hemisphere isolates CMW4955 and CMW4960 representing *A. fumosa* and isolate CMW4971 representing *A. pallidula* grouped together with a 100% bootstrap support. The clade with *A. hinnulea* isolates CMW4980 and CMW4981 had a 100% bootstrap support. An unknown isolate CMW5446 grouped with *A. luteobubalina* isolates CMW8876 and CMW4977 and *A. limonea* isolates CMW4680 and CMW4991 resolved into a clade with 98% support. Isolates CMW4967, CMW4722 and CMW5448 representing *A. novae-zelandiae* and CMW4143 representing an unknown species formed a clade with a bootstrap value of 100%. Two isolates CMW4994 and CMW5597 of unknown identity formed a distinct sub-clade with 100% bootstrap support.

DISCUSSION

DNA sequence data for the EF 1-α gene were successfully generated and analysed for the first time for a wide range of *Armillaria* spp. in this study. All isolates yielded PCR products of similar size. This indicated that the amplified gene region does not include large indels and is therefore a suitable choice of gene region for phylogenetic studies. The aligned sequences showed considerable homology among *Armillaria* spp. but various species specific nucleotide substitution and indels were observed. Little sequence variation was observed within species with noticeable variation between different species. This is consistent with various studies employing IGS-1 and ITS rDNA operon DNA sequence data in taxonomic studies of *Armillaria* spp. (Anderson and Stasovski, 1992; Chillali et al., 1998; Coetzee et al., 2001; 2003).

Phylogenetic comparisons based on EF 1-α sequence data showed that *Armillaria* spp. thought to be native to Africa reside in a clade strongly separated from all other species. This so-called African clade has previously been identified based on isozyme analysis (Mwenje and Ride, 1997) and IGS-1 sequence data (Mwenje et al., 2003). Sequence data for a new gene region and particularly a protein-coding gene reflect the same patterns that have emerged from previous molecular based comparisions.

Isolates from Africa that have previously been shown to represent different taxonomic groups (Mwenje et al., 2003) resided in a strong monophophyletic assemblage and are regarded as the African *Armillaria* group. These isolates were previously thought to represent a single species treated as *A. heimii sensu lato* and shown to have high levels of intraspecific variation (Mohammed et al., 1989). Recent studies based on IGS-1 sequence and AFLP data, however, suggested that the African isolates represent at least two different species, *A. fuscipes* (syn. *A. heimii*) and a unnamed species (Coetzee et al., 2000b, Mwenje et al., 2003). Results of the present study also show that the African isolates reside in sub-clades representing the three taxonomic groups suggested by Mwenje et al. (2003).

Isolates within the *A. mellea* clade formed strongly supported monophyletic groups consistent with the geographical origin of the isolates. These were isolates from Asia, Europe, eastern North American and western North America. Differences between geographically separated isolates of *A. mellea* have been observed in a number of previous studies. Anderson et al. (1989) showed that *A. mellea* isolates from Europe and eastern North America differ in their *Eco*RI, *Bam*HI and *Sal*I digestion patterns of the rRNA operon. Intraspecies variation pertaining to IGS-1 RFLP patterns was similarly reported for this species by Harrington and Wingfield (1995). Likewise, differences have been observed in the mating systems of isolates from Europe, North America and Japan (Anderson et al., 1980; Ota et al., 1998).

The sub-division of isolates of *A. mellea* according to their origin is congruent with the study of Coetzee et al. (2000a) showing that isolates of *A. mellea* from various Northern Hemisphere origins represent Asian, European, eastern North American and western North American lineages. Of these, isolates from Europe, North America and Asia have been shown to be sexually compatible and thus reported to be the same biological species (Anderson et al., 1980; Anderson et al., 1989; Ota et al., 1998). The separation of the isolates into geographic groups may reflect intraspecific variation due to allopatric separation. Alternatively, these lineages may represent sibling species in the process of allopatric speciation (Coetzee et al., 2000a) with incompletely developed intrinsic genetic isolation mechanisms. Inclusion of sequence data from a gene region not previously considered adds strong additional support for the view that *A. mellea* from different geographic areas are genetically distinct.

Results of this study showed that isolates in the *A. ostoyae* clade included those representing *A. ostoyae* and *A. gemina*. *Armillaria ostoyae* and *A. gemina*, have previously shown to be phylogenetically closely related (Anderson and Stasovski, 1992; Miller et al., 1994). These two species also have identical basidiocarp morphology (Bérubé and Dessureault, 1989). They can however, be differentiated from other species based on vegetative features (Bérubé and Dessureault, 1989) and on mating tests (Anderson and Ullrich, 1979).

Isolates representing *A. borealis A. gallica, A. nabsnona, A. cepistipes* and *A. tabescens* grouped in the *A. gallica* clade. This is consistent with the fact that *A. gallica* and *A. cepistipes* have previously been shown to be phylogenetically closely related based on DNA data (Anderson and Stasovski, 1992; Miller et al., 1994, Chillali et al., 1998). *Armillaria nabsnona* has also been found to be related to *A. gallica* based on DNA reassociation data (Miller et al., 1994) and our new sequence data confirmed this.

Armillaria gallica and *A. cepistipes* are ecologically (Korhonen, 1995) and morphologically (Termoshuizen and Arnolds, 1987; Marxmüller, 1992; Korhonen, 1995) similar and can only be differentiated using mating tests (Termoshuizen and Arnolds, 1987).

The grouping of *A. borealis* in the *A. gallica* clade in this study is an interesting result. Previous studies based on ITS and IGS-1 sequence data as well as RFLP analysis of the rDNA operon showed that this species is phylogenetically most closely related to *A. ostoyae* and *A. gemina* (Anderson and Stasovski, 1992; Chillali et al., 1998). Also, Korhonen (1995) placed *A. borealis* with *A. ostoyae* based on morphological similarities with *A. ostoyae* and *A. gemina*. The lack of correlation between the ribosomal phylogeny and that of the EF 1-α gene suggests that the evolutionary histories of these two regions are not the same. This highlights the danger of using single gene phylogenies to infer phylogenetic relationships.

The grouping of *A. tabescens* within the *A. gallica* clade was unexpected. Previous work based on DNA reassociation showed that *A. mellea* and *A. tabescens* are most closely related (Miller et al., 1994). Chillali et al. (1998) further showed, based on ITS sequence data, that *A. mellea* and *A. tabescens* are basal to the rest of northern hemisphere species. Miller et al. (1994) contended that *A. tabescens* is the more basal species and therefore more ancient than *A. mellea*. These studies, however, did not include species from the Southern Hemisphere. The grouping of *A. tabescens* with *A. gallica, A. cepistipes* and *A. nabsnona* may be explained by different rates at which ribosomal and protein-coding genes evolve. The different evolutionary rates then lead to these species grouping together when ribosomal genes are used and differently when protein-coding genes are employed as was observed in this study.

Armillaria fumosa, *A. pallidula*, *A. novae-zelandiae*, *A. luteobubalina, A. limonea, A. hinnulea* and the undescribed species from New Zealand, have only been reported from the southern hemisphere (Kile and Watling, 1983; 1988). Cladograms generated from EF1-α sequences confirm that these species are closely related and that they group basal to those from the Northern Hemisphere. This is consistent with the results of previous studies based on IGS-1 and LSU sequence data, suggesting that the Southern Hemisphere species are ancestral to those from the Northern Hemisphere (Coetzee et al., 2001, Dunne et al., 2002). The new sequence data set therefore provides further evidence for the hypothesis that *Armillaria* or the ancestor of this genus originated in Gondwanaland (Coetzee et al., 2001, Dunne et al., 2002).

Armillaria fumosa and *A. pallidula* grouped together in a one clade in this study. This supports the findings of Coetzee et al. (2001) who reported that these species are closely related and cannot be distinguished using ITS sequence information. Kile and Watling (1988) using interfertility tests and morphology showed that these are distinct species although they share some morphological similarities. The results of the present study together with those of Coetzee et al. (2001) indicate that the species have recently split from a common ancestor and have not accumulated sufficient differences at the DNA level to differentiate between them.

The grouping of *A. hinnulea* outside the Northern Hemisphere clade in this study was of particular interest. This species has been reported only from New Zealand and it is therefore to be expected that it should be phylogenetically closely related to those species occurring in the southern hemisphere. Phylogenetic studies based on ITS sequence data, however, showed that it is most closely related to species from the northern Hemisphere (Coetzee et al., 2001; Dunne et al., 2002). Dunne et al. (2002)

suggested that it may have evolved from a common ancestor with *A. cepistipes* [= *A. bulbosa*]. This result is supported by the findings of Kile and Watling (1983) who showed that the basidiocarp morphology of *A. hinnulea* is in various aspects similar to those of the European species *A. cepistipes*. The fact that the EF 1-α gene sequences gave results different from those from studies based on the ITS region might be due to the fact that EF 1-α has evolved more slowly than the ITS region. More rapid evolution of the ITS region could have resulted in the inclusion of synapomorphic characters leading to *A. hinnulea* grouping with northern hemisphere species. In contrast, the EF 1-α gene nucleotides for this species may have retained the ancestral character states of species in the southern hemisphere, resulting in the grouping of this species basal to those from the Northern Hemisphere.

Isolates representing *A. luteobubalina* from Chile and Australia had similar DNA sequences and grouped together in one clade. An isolate from Chile, tentatively identified as *A. luteobubalina* based on IGS-1 and ITS sequences (Coetzee et al., 2003), grouped with the isolates representing this species. The identification of this isolate as *A. luteobubalina* has been controversial as this species has never before been reported from South America. Results of the current study provide additional evidence that the isolates from Chile represent A. *luteobubalina* and that the species is present in South America. Coetzee et al. (2003) also showed that the isolates in this clade, despite their large geographic separation, retained a high level of ITS and IGS-1 sequence similarity. These researchers therefore postulated that this is an ancient species with its origin in the Gondwana supercontinent.

Phylogenetic trees obtained in this study showed that *A. luteobubalina* is closely related to *A. limonea*. Isolates representing the latter species grouped in a monophyletic clade. This relationship supports the

findings of Kile and Watling (1988) that the two species share some morphological characters such as a yellow pigment in their pileus.

An unidentified isolate from Indonesia grouped with *A. novae-zelandiae* in a strongly supported clade. This isolate represents a set of isolates that were obtained from infected *Eucalyptus grandis* trees but for which no basidiocarps were found. A description based on morphology or identification using mating tests was thus not possible (Coetzee et al., 2003). The set of isolates were considered by Coetzee et al. (2003) who attempted to identify them. In their study the authors showed that the isolates either represent *A. novae-zelandiae* or a previously undescribed species that is closely related to *A. novae-zelandiae*. The phylogenetic trees generated in this study thus support the finding of Coetzee et al. (2003).

Armillaria novae-zelandiae is common in New Zealand and Australia (Kile and Watling, 1983). Isolates from both areas appear to represent a single species (Coetzee et al., 2001). These isolates are morphologically similar (Kile and Watling, 1983) and are sexually compatible (Kile and Watling, 1983). Our results using sequence data from a new gene region support the view that these isolates represent the same taxon.

Two undescribed isolates from New Zealand formed a distinct clade and showed no relationship to any known species, but fell in the southern hemisphere group. These isolates were shown by Coetzee et al. (2001) to probably represent an undescribed species. Results of the present study also provide additional support for the view that these isolates represent a discrete taxon that awaits description.

This study presents the first EF 1-α DNA sequence data for *Armillaria* species. It is also the first protein coding gene and first single copy gene to be presented for this genus. Sequence data from the majority of isolates belonging to the different species showed unique species specific substitutions and thus could be differentiated into clades representing the species. Results of this study demonstrate that the EF 1-α region is useful for phylogenetic analysis and classification of *Armillaria* species.

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Table 1: List of *Armillaria* isolates used in the study.

Isolate Number	Alternative culture collection number	Species	Collector/ Supplier	Origin
CMW4455	40	Group II	E. Mwenje	Zimbabwe
CMW4456	Z1	Group II	M. Ivory	Zimbabwe
CMW10115	56	Group III	E. Mwenje	Zimbabwe
CMW9954	P21	Group III	E. Mwenje	Zimbabwe
CMW3164	B933	*A. fuscipes*	J.M Sung	La-Reunion
CMW4953	LR2	*A. fuscipes*	C. Fabregue	La-Reunion
CMW3172	B370	*A. borealis*	K. Korhonen	Finland
CMW3182	B373	*A. borealis*	K. Korhonen	Germany
CMW3158	B898	*A. tabescens*	T. Volk	USA
CMW3165	B531	*A. tabescens*	J. Guillaumin	France
CMW6901	21A	*A. gallica*	M. T. Banik	USA
CMW3171	B110	*A. gallica*	K.J Smereka	USA
CMW6909	33/82144	*A. nabsnona*	D. Morrison	USA
CMW6905	28/HB-20	*A. cepistipes*	M.T. Banik	USA
CMW3162	B481	*A. ostoyae*	J. Anderson	USA
CMW6888	5/JJW223	*A. gemina*	Worrall	USA
CMW3181	B485	*A. gemina*	J. Anderson	USA
CMW3956	B497	*A. mellea*	J. Anderson	East USA
CMW4605	B282	*A. mellea*	T.C. Harrington	East USA
CMW11231	426	*A. mellea*	-	Europe
CMW4613	B1205	*A. mellea*	M. Saber	Europe
CMW3961	B730	*A. mellea*	T. Terashita	Asia
CMW4610	B916	*A. mellea*	J.M. Sung	Asia
CMW4611	B917	*A. mellea*	J.M. Sung	Asia
CMW3964	B927	*A. mellea*	T. Bruns	West USA
CMW4620	B1218	*A. mellea*	-	West USA
CMW4960	Q/COLL.9.4	*A. fumosa*	C. Mohammed	Australia
CMW4955	123/1	*A. fumosa*	C. Mohammed	Australia
CMW4971	3984	*A. pallidula*	C. Mohammed	Australia
CMW4980	119/DAR	*A. hinnulea*	C. Mohammed	Australia
CMW4981	LOT3/2	*A. hinnulea*	C. Mohammed	Australia
CMW5446	7348/10	unknown	R. H. Petersen	Australia
CMW8876	Chile-1	*A. luteobubalina*	M.J. Wingfield	Chile
CMW4977	SA(6)	*A. luteobubalina*	C. Mohammed	Australia
CMW4680	C3.28/0.1	*A. limonea*	I.A. Hood	New Zealand
CMW4991	3522/2	*A. limonea*	G.S Ridley	New Zealand
CMW4967	NSW3(4)	*A. novae-zelandiae*	C. Mohammed	Australia
CMW4722	G3.0.34.4	*A. novae-zelandiae*	I.A. Hood	New Zealand
CMW5448	7365/2	*A. novae-zelandiae*	R.H. Petersen	Australia
CMW4143	-	unknown	M.J. Wingfield	Indonesia
CMW4994	4698/10	unknown	G.S. Ridley	New Zealand
CMW5597	A35-4	unknown	I.A. Hood	New Zealand

Figure 1. Diagram showing the structure of the elongation factor 1-α gene from the basidiomycete *Schizophyllum commune*. Exons and introns are presented with black and white boxes, respectively. The binding positions for primers EF595F and EF1160R are indicated on the enlarged diagram. The numbers indicate the positions of the bases in the EF 1-α gene open reading frame of *S. commune*.

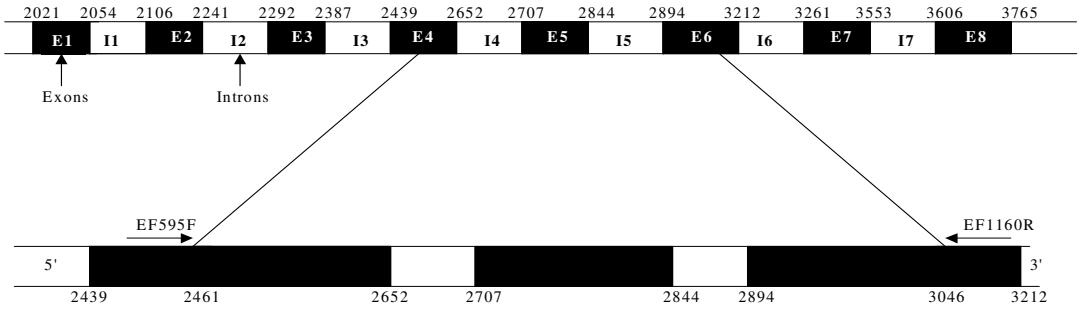
2021 2054 2106 2241 2292 2387 2439 2652 2707 2844 2894 3212 3261 3553 3606 3765
E1 I1 E2 I2 E3 I3 E4 I4 E5 I5 E6 I6 E7 I7 E8
Exons
Introns
EF595F
EF1160R
5'
3'
2439 2461 2652 2707 2844 2894 3046 3212

Figure 2. A 1% Ethidium stained agarose gel showing the EF 1-α PCR products for some *Armillaria* species. Lanes marked M indicate a 100bp molecular standard marker. EF1-α amplification products for isolates CMW4455; CMW10115; CMW3956; CMW4722; CMW6909 and CMW3172 are shown in lanes 1 to 6.

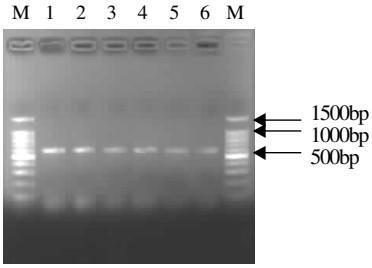
M 1 2 3 4 5 6 M
1500bp
1000bp
500bp

Figure 3. One of the most parsimonious trees generated after a heuristic search using the EF1-α DNA sequence data with ambiguous and missing data excluded. Branch length values are shown above the tree branches and percentage bootstrap values (1000 replicates) are shown below the tree branches. Number of parsimony informative characters =173, Tree length = 314, CI = 0.787 and RI = 0.897.

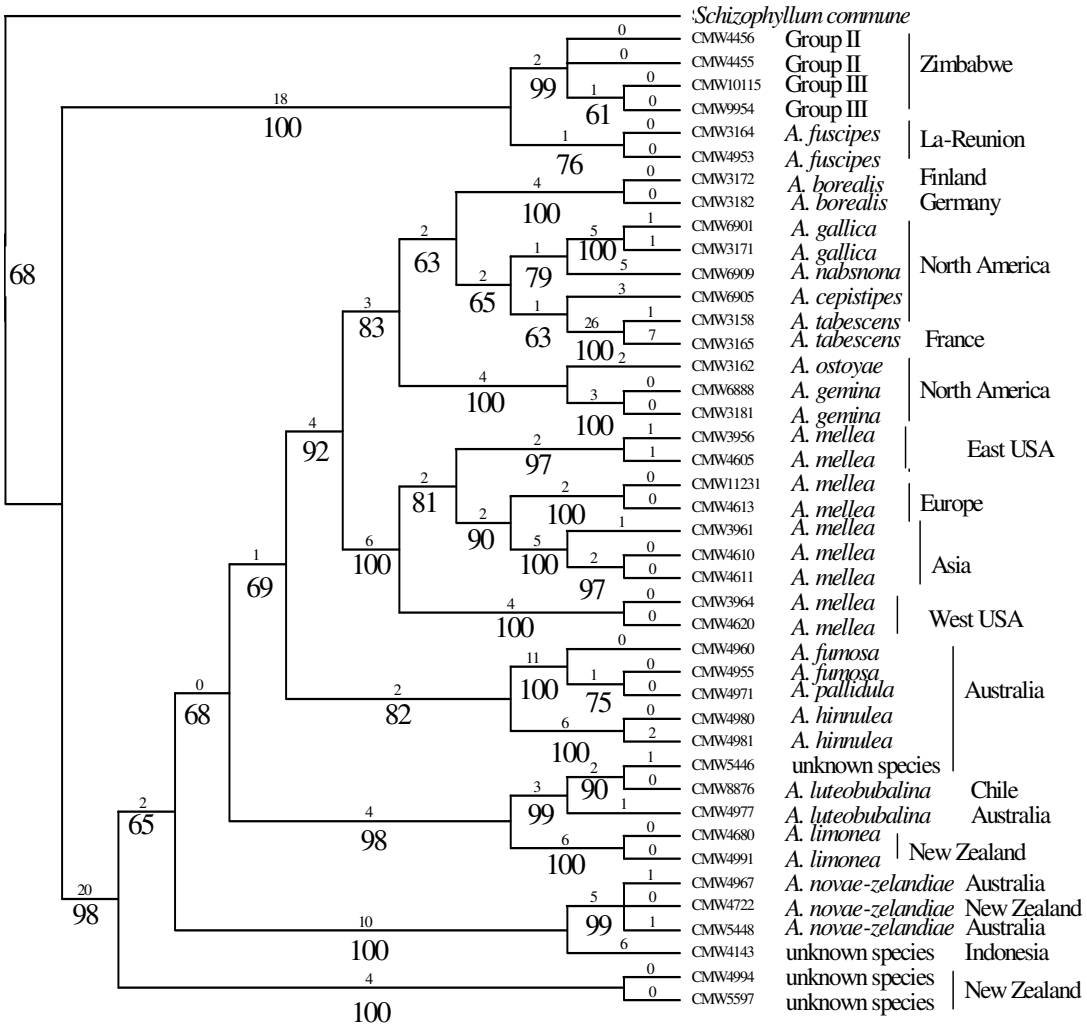
Schizophyllum commune
CMW4456 Group II
CMW4455 Group II
CMW10115 Group III
CMW9954 Group III
Zimbabwe
CMW3164 A. fuscipes
CMW4953 A. fuscipes
La-Reunion
CMW3172 A. borealis
Finland
CMW3182 A. borealis
Germany
CMW6901 A. gallica
CMW3171 A. gallica
CMW6909 A. nabsnona
North America
CMW6905 A. cepistipes
CMW3158 A. tabescens
CMW3165 A. tabescens
France
CMW3162 A. ostoyae
CMW6888 A. gemina
North America
CMW3181 A. gemina
CMW3956 A. mellea
East USA
CMW4605 A. mellea
CMW11231 A. mellea
CMW4613 A. mellea
Europe
CMW3961 A. mellea
CMW4610 A. mellea
CMW4611 A. mellea
Asia
CMW3964 A. mellea
CMW4620 A. mellea
West USA
CMW4960 A. fumosa
CMW4955 A. fumosa
CMW4971 A. pallidula
CMW4980 A. hinnulea
CMW4981 A. hinnulea
Australia
CMW5446 unknown species
CMW8876 A. luteobubalina
Chile
CMW4977 A. luteobubalina
Australia
CMW4680 A. limonea
CMW4991 A. limonea
New Zealand
CMW4967 A. novae-zelandiae
Australia
CMW4722 A. novae-zelandiae
New Zealand
CMW5448 A. novae-zelandiae
Australia
CMW4143 unknown species
Indonesia
CMW4994 unknown species
CMW5597 unknown species
New Zealand

Figure 4. Aligned nucleotide sequences for the EF1-α region for isolates used in this study. Dashes (-) indicate gaps and unknown bases are indicated by N.

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                        10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum       AGAACATGATCACCGGTACCTCCCAGGCTGACTGCGCTATCCTCACCATCGCCGGTGGCA
CMW4456             NGAACATGATCACCGGTACCTCTCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW4455             AGAACATGATCACCGGTACCTCTCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW10115            AGAACATGATCACCGGTACCTCTCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW9954             AGAACATGATCACCGGTACCTCTCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW3164             AGAACATGATCACCGGTACCTCCCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW4953             AGAACATGATCACCGGTACCTCCCAGGCTGACTGTGCCATTCTTATCATCGCTGGTGGAA
CMW3172             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3182             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3162             NGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW6888             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3181             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW6901             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3171             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW6905             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATTATCGCTGGTGGAA
CMW6909             NNNNNNNNATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATTATCGCTGGTGGAA
CMW3956             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATTGCTGGCGGAA
CMW4605             NNNNNNNNATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGCGGAA
CMW3964             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4620             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW11231            AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4613             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3961             AGAACATGATCACCGGTACCTAGCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4610             AGAACATGATCACCGGTACCTAGCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4611             AGAACATGATCACCGGTACCTAGCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW3158             AGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATCCTTATCATTGCTGGTGGTA
CMW3165             NNAACATGATCACTGGTACCTCCCAGGCTGATTGTGCCATCCTTATCATTGCTGGTGGTA
CMW4994             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW5597             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4960             AGAACATGATTACGGGTACCTCTCAGGCTGATTGTGCTATCCTCATCATCGCTGGTGGAA
CMW4955             AGAACATGATTACGGGTACCTCTCAGGCTGATTGTGCTATCCTCATCATCGCTGGTGGAA
CMW4971             AGAACATGATTACGGGTACTTCTCAGGCTGATTGTGCTATCCTCATCATCGCTGGTGGAA
CMW4980             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4981             NNNNNNNNNNNNNNNGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW5446             AGAACATGATCACCGGTACTTCCCAGGCTGATTGTGCTATTCTCATCATCGCTGGTGGAA
CMW4967             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4722             NNNNNNNNNNNNACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW5448             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4143             NGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4680             NNNNNNNNNNNNACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4991             AGAACATGATCACCGGTACCTCCCAGGCTGATTGTGCCATTCTCATCATCGCTGGTGGAA
CMW4977             NNNNNNNNNNNNACCGGTACCTCCCAGGCTGATTGTGCTATTCTCATCATCGCTGGTGGAA
CMW8876             AGAACATGATCACCGGTACTTCCCAGGCTGATTGTGCTATTCTCATCATCGCTGGTGGAA
```

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                          70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum       CTGGTGAATTCGAGGCTGGTATCTCCAAGGATGGCCAGACCC-GCGAGCACGCTCTCCTT
CMW4456             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW4455             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW10115            CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW9954             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW3164             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW4953             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGACAGACCC-GAGAGCATGCTCTCCTT
CMW3172             CTGGTGAGTTCGAAGCCGGTATTTCCAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTT
CMW3182             CTGGTGAGTTCGAAGCCGGTATTTCCAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTT
CMW3162             CTGGTGAGTTCGAAGCCGGTATTTCCAAGGACGGCCAGACCC-GAGAGCATGCCCTCCTT
CMW6888             CTGGTGAATTCGAAGCCGGTATTTCAAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTT
CMW3181             CTGGTGAATTCGAAGCCGGTATTTCAAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTT
CMW6901             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAACACGCCCTCCTT
CMW3171             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAACACGCCCTCCTT
CMW6905             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCACGCCCTCCTT
CMW6909             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCACGCCCTCCTT
CMW3956             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW4605             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW3964             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTC
CMW4620             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCACGCCCTCCTC
CMW11231            CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW4613             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW3961             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW4610             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW4611             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGGCAGACCC-GAGAGCACGCCCTCCTT
CMW3158             CTGGTGAATTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCACGCCCTTCTT
CMW3165             CTGGTGAATTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCACGCCCTTCTT
CMW4994             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACTC-GAGAGCATGCCCTCCTT
CMW5597             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACTC-GAGAGCATGCCCTCCTT
CMW4960             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4955             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4971             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4980             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCATGCCCTCCTT
CMW4981             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCCTGAGAGCATGCCCTCCTT
CMW5446             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCACGCCCTCCTT
CMW4967             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCATGCCCTCCTT
CMW4722             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCATGCCCTCCTT
CMW5448             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGCCAGACCC-GAGAGCATGCCCTCCTT
CMW4143             CTGGTGAGTTCGAAGCGGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4680             CTGGTGAGTTTGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4991             CTGGTGAGTTTGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW4977             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
CMW8876             CTGGTGAGTTCGAAGCCGGTATCTCCAAGGACGGTCAGACCC-GAGAGCATGCCCTCCTT
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                        130       140       150       160       170       180
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum      GCCTTCACCCTCGGTGTCCGTCAGCTCATCGTCGCCGTCAACAAGATGGACACGACCAAG
CMW4456            GCCTTTACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW4455            GCCTTTACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW10115           GCCTTTACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW9954            GCCTTTACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW3164            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW4953            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW3172            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACTAAG
CMW3182            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACTAAG
CMW3162            GCCTTCACCCTCGGTGTCAGGCAGCTCATCGTCGCCGTCAACAAAATGGACACCACCAAG
CMW6888            GCCTTCACCCTCGGTGTCAGGCAGCTCATCGTCGCCGTCAACAAGATGGACACCACCAAG
CMW3181            GCCTTCACCCTCGGTGTCAGGCAGCTCATCGTCGCCGTCAACAAGATGGACACCACCAAG
CMW6901            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW3171            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW6905            GCCTTCACTCTCGGTGTCAGGCAGCTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW6909            GCCTTCACCCTCGGTGTCAGGCAGCTTATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW3956            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4605            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW3964            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW4620            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW11231           GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4613            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW3961            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4610            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW4611            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW3158            GCCTTCACTCTTGGTGTCAGGCAGCTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW3165            GCCTTCACTCTTGGTGTCAGGCAGCTCATTGTTGCCGTCAACAAGATGGACACCACCAAG
CMW4994            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW5597            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4960            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4955            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4971            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4980            GCCTTTACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW4981            GCCTTTACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW5446            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCTCCGTCAACAAGATGGACACCACCAAG
CMW4967            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW4722            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW5448            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW4143            GCCTTCACCCTCGGTGTCAGGCAACTCATTGTCGCCGTCAATAAGATGGACACCACCAAG
CMW4680            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCTCCGTCAACAAGATGGACACCACTAAG
CMW4991            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCTCCGTCAACAAGATGGACACCACTAAG
CMW4977            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCGCCGTCAACAAGATGGACACCACCAAG
CMW8876            GCCTTCACCCTCGGTGTCAGGCAGCTCATTGTCTCCGTCAACAAGATGGACACCACCAAG
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                    190       200       210       220       230       240
                ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum   GTAAGCATACGACAGTAAATATTCCGTCATCGACTCAGGCTTATATTCTCTACAGTGGAG
CMW4456         GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW4455         GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW10115        GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW9954         GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW3164         GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW4953         GTACATGATCCTCTATTTCATCCTTT-CTTTGGCTAA-CCTCATTTTTGTTTTAGTGGAG
CMW3172         GTACGAGATCTGCCGCTTTGC-TTTTACTTTAGTCAAATCTGACTGGTATCTCAGTGGAG
CMW3182         GTACGAGATCTGCCGCTTTGC-TTTTTCTTTAGTCAAATCTGACTGGTATCTCAGTGGAG
CMW3162         GTACGAGATCTACTGTTTTACCTTTTTCCTTAGGCAAATCTGACTGTCATCTCAGTGGAG
CMW6888         GTACGAGATCTACTGTTTTACCTTTTTCCTTAGGCAAATCTGACTGTCATCTCAGTGGAG
CMW3181         GTACCAGATCTACTGTTTTACCTTTTTCCTTAGGCAAATCTGACTGTCATCTCAGTGGAG
CMW6901         GTACGAGATCTGTTGCTTTGCCTTGTG-TTTAGCCAAATCTAACTGTTATCTCAGTGGAG
CMW3171         GTACGAGATCTGTTGCTTTGCCTTGTG-TTTAGCCAAATCTAACTGTTATCTCAGTGGAG
CMW6905         GTACGAGATCTGCTGCTTTGCCTTTTG-TTTAGCCAAATCTGACTGTTATCTCAGTGGAG
CMW6909         GTACGAGATCTGCTGCTTTACCTTTTG-TTTAGCCTAATCTGATTGTTATCTCAGTGGAG
CMW3956         GTACGGGATCTGCTGTTTCAGCTTTT-CTTTAGTCAAATCTGATTGTTATCTCAGTGGAG
CMW4605         GTACGGGATCTGCTGTTTCAGCTTTT-CTTTAGTCAAATCTGATTGTTATCTCAGTGGAG
CMW3964         GTACGGAATCTGCTGTTTCACCTTTT-CTTTCGTCAGATCTGATTGTTATCTCAGTGGAG
CMW4620         GTACGGAATCTGCTGTTTCACCTTTT-CTTTCGTCAGATCTGATTGTTATCTCAGTGGAG
CMW11231        GTACAGGATCTGCTGTTTCAG-TTTTTCTTTAGTCAAATCTGATTGTTATCTCAGTGGAG
CMW4613         GTACAGGATCTGCTGTTTCAG-TTTTTCTTTAGTCAAATCTGATTGTTATCTCAGTGGAG
CMW3961         GTACGGGATCTGCTGTTTCAG-TTT---TTTAGTCAAATATGATTGTTATCTCAGTGGAG
CMW4610         GTACGGGATCTGCTGTTTCAG-TTTTT---TAGTCAAATATGATTGTTATCTCAGTGGAG
CMW4611         GTACGGGATCTGCTGTTTCAG-TTTTT---TAGTCAAATATGATTGTTATCTCAGTGGAG
CMW3158         GTACGAACCCTACCCCATCGC-TTTTTCTTTCGCGAAGTCTGACATTTATCTTAGTGGAG
CMW3165         GTACGAACCCTACCCCATCGC-TTTTTCTTTCGCGAAGTTCGACATTTATCTTAGTGGAG
CMW4994         GTACGAGATCTGCTGTTTCACCTTTT-CTTTAACTGAATCTGATTGTTATCCCAGTGGAG
CMW5597         GTACGAGATCTGCTGTTTCACCTTTT-CTTTAACTGAATCTGATTGTTATCCCAGTGGAG
CMW4960         GTACAAGATCTGCTGTTTCACCTGTT-CTTTAGCTAAATTTGACTGTTATCACAGTGGAG
CMW4955         GTACAAGATCTGCTGTTTCACCTGTT-CTTTAGCTAAATTTGACTGTTATCACAGTGGAG
CMW4971         GTACAAGATCTGCTGTTTCACCTGTT-CTTTAGCTAAATTTGACTGTTATCACAGTGGAG
CMW4980         GTACAAGATTTGCTGTTTCACCTTTT-CTTTAGCCAAATCTGACTGTTATATCAGTGGAG
CMW4981         GTACAAGATTTGTTGTTTCACCTTTT-CTTTAGCCAAATCTGACTGTTATATCAGTGGAG
CMW5446         GTTCGAGATCTGATGTTTCACCTTTT-CTTTAGTCAAATCTGACTGTTATCTTAGTGGAG
CMW4967         GTACGAGATCTGCTTTCTCACCATTT-CTTGAGCTAAATCTGACTGTTATCTCAGTGGAG
CMW4722         GTACGAGATCTGCTTTCTCACCATTT-CTTGAGCCAAATCTGACTGTTATCTCAGTGGAG
CMW5448         GTACGAGATCTGCTTTCTCACCATTT-CTTGAGCCAAATCTGACTGTTATCTCAGTGGAG
CMW4143         GTACAAGATCTGCTTTTTCACCATTC-CTTAAGCCAAATCTGACTGTTATCTCAGTGGAG
CMW4680         GTTCGAGATCTGATGTTTCACCTTTT-CTTTAGCCAAATCTGACTGTTATCTTAGTGGAG
CMW4991         GTTCGAGATCTGATGTTTCACCTTTT-CTTTAGCCAAATCTGACTGTTATCTTAGTGGAG
CMW4977         GTTCGAGATCTGATGTTTCACCTCTT-CTTTAGTCAAATCTGACTGTTATCTTAGTGGAG
CMW8876         GTTCGAGATCTGATGTTTCACCTCTT-CTTTAGTCAAATCTGACTGTTATCTTAGTGGAG
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                        250       260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum      CGAGGACCGTTTCAACGAAATCGTCAAGGAGACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4456            CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW4455            CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW10115           CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW9954            CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW3164            CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW4953            CGAGGACCGATTCAACGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW3172            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW3182            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW3162            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW6888            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW3181            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW6901            CGAGGACCGGTTCAACGAAATTGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW3171            CGAGGACCGGTTCAACGAAATTGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW6905            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW6909            CGAGGACCGGTTCAACGAAATTGTCAAGGAAACTTCCACCTTCATCAAGAAGGTCGGCTA
CMW3956            CGAGGACCGATTCAATGAAATTGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW4605            TGAGGACCGTTTCAATGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW3964            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGTTA
CMW4620            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGTTA
CMW11231           CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGTTA
CMW4613            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGTTA
CMW3961            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW4610            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW4611            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCTACCTTCATCAAGAAGGTCGGCTA
CMW3158            TGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW3165            TGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4994            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW5597            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4960            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4955            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4971            CGAGGACCGATTCAATGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4980            CGAGGACCGATTCAATGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4981            CGAGGACCGATTCAATGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW5446            CGAGGACCGATTCAACGAAATCGTCAAGGAAACTTCCACTTTCATCAAGAAGGTCGGCTA
CMW4967            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4722            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW5448            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTCGGCTA
CMW4143            CGAGGACCGGTTCAACGAAATCGTCAAGGAAACGTCCACCTTCATCAAGAAGGTCGGCTA
CMW4680            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTTGGCTA
CMW4991            CGAGGACCGATTCAACGAAATCGTCAAGGAAACCTCCACCTTCATCAAGAAGGTTGGCTA
CMW4977            CGAGGACCGATTCAACGAAATCGTCAAGGAAACTTCCACCTTCATCAAGAAGGTCGGCTA
CMW8876            CGAGGACCGATTCAACGAAATCGTCAAGGAAACTTCCACTTTCATCAAGAAGGTCGGCTA
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                        310       320       330       340       350       360
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum      CAACCCGAAGACCGTCGCCTTCGTCCCGATCTCCGGCTGGCACGGCGACAACATGTTGGA
CMW4456            TAACCCTAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4455            TAACCCTAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW10115           TAACCCTAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW9954            TAACCCTAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3164            TAACCCCAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4953            TAACCCCAAGGCTGTCGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3172            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3182            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3162            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW6888            CAACCCCAAGGCTGTTGCTTTCGTCCCTATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3181            CAACCCCAAGGCTGTTGCTTTCGTCCCTATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW6901            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3171            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW6905            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW6909            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3956            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4605            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3964            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4620            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW11231           CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4613            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3961            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4610            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4611            CAACCCCAAGGCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3158            CAACCCCAAGTCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW3165            CAACCCCAACTCTGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGACAACATGTTGGA
CMW4994            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW5597            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4960            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4955            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4971            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4980            CAACCCTAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4981            CAACCCTAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW5446            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGACAACATGTTGGA
CMW4967            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4722            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW5448            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4143            CAACCCCAAGGCCGTTGCCTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4680            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4991            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGATAACATGTTGGA
CMW4977            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGACAACATGTTGGA
CMW8876            CAACCCCAAGGCCGTTGCTTTCGTCCCCATCTCTGGATGGCACGGTGACAACATGTTGGA
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                        370       380       390       400       410       420
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum      GGAGTCCACCAAGTACGTC------CCGATGCCAATTTTTCCTCGCAT-GT--CGCTT-A
CMW4456            GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-GATCTATGATTAATGGTAGATCTTGA
CMW4455            GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-GATCTATGATTAATGGTAGATCTTGA
CMW10115           GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-GATCTATGATTAATGGTAGATCTTGA
CMW9954            GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-GATCTATGATTAATGGTAGATCTTGA
CMW3164            GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-AATCTATGATTAATGATAGATCTTGA
CMW4953            GGAGTCCACCAAGTAAGCTT-ACATCCGACTAT-AATCTATGATTAATGATAGATCTTGA
CMW3172            GGAGTCCGCCAAGTAAGTCC-TTACCCAAGTAT-GACC-------AGTACTGCCTCTTAA
CMW3182            GGAGTCCGCCAAGTAAGTCC-TTACCCAAGTAT-GACC-------AGTACTGCCTCTTAA
CMW3162            GGAATCCGCCAAGTAAGTCCCTTACCCAACTAT-GACC-------AGTGCTGGCTCTTAA
CMW6888            GGAATCCGCCAAGTAAGTCCCTTACCCAACTAT-GATC-------AGTGCTGGCTCTTAA
CMW3181            GGAATCCGCCAAGTAAGTCCCTTACCCAACTAT-GATC-------AGTGCTGGCTCTTAA
CMW6901            GGAGTCCGCCAAGTAAGTCT-TTACCTAACTAT-GATC-------AGTGCTGCCTCTTAA
CMW3171            GGAGTCCGCCAAGTAAGTCT-TTACCCAACTAT-GATC-------AGTGTTGCCTCTTAA
CMW6905            GGAGTCTGCCAAGTAAGTCCTTTACCCAACTATTGATC-------AGTGCTGCCTCTTAA
CMW6909            GGAGTCTGCCAAGTAAGTCT-TTACCCAACTAG-GATC-------AGTGCTGCCTCTTAA
CMW3956            GGAGTCCGCCAAGTACGTCC-TTACTCAACTCT-GATC-------CGTACTGGGTCTGAA
CMW4605            GGAGTCCGCCAAGTACGTCC-TTACTCAACTCT-GATC-------CGTACTGGGTCTGAA
CMW3964            GGAGTCCGCCAAGTACGTCC-TTACTCAACTCT-GATC-------CGTACTGGGTCTTAA
CMW4620            GGAGTCCGCCAAGTACGTCC-TTACTCAACTCT-GATC-------CGTACTGGGTCTTAA
CMW11231           GGAGTCCGCCAAGTACGTCC-TTACTTAACTAT-GATC-------CGTACTGAGTCTTAA
CMW4613            GGAGTCCGCCAAGTACGTCC-TTACTTAACTAT-GATC-------CGTACTGAGTCTTAA
CMW3961            GGAGTCCGCCAAGTACGTCC-TTACTTAACTAT-GATC-------CGTATTGTATCTTAA
CMW4610            GGAGTCCGCCAAGTACGTCC-TTACTTAACTAT-GATC-------CGTATTGTATCTTAA
CMW4611            GGAGTCCGCCAAGTACGTCC-TTACTTAACTAT-GATC-------CGTATTGTATCTTAA
CMW3158            GGAGTCCGCCAAGTAAGTCA-TTACCATATTAT-GAGC-------GATACGGCTTCTTAA
CMW3165            GGAGTCCGCCAAGTAAGTCA-TTACCATATTAT-GAGC-------GATGCGGCGGCTTAA
CMW4994            GGAGTCCGCCAAGTAAGTCC-TTATCCAACTAT-GATC-------AGTACTACCTCTTAA
CMW5597            GGAGTCCGCCAAGTAAGTCC-TTATCCAACTAT-GATC-------AGTACTACCTCTTAA
CMW4960            GGAATCTGTCAAGTAAGACC-TAATCCAACTCATAATC----------ACTACCTCTTAA
CMW4955            GGAATCTGTCAAGTAAGACC-TAATCCAACTCATCATC----------ACTACCTCTTAA
CMW4971            GGAATCTGTCAAGTAAGACC-TAATCCAACTCATCATC----------ACTACCTCTTAA
CMW4980            GGAGTCCGCCAAGTAAGTCC-TTATCCAAGTTATGATC-------AGTACTATCTCTTAA
CMW4981            GGAGTCCGCCAAGTAAGTCC-TTATCCAA-TTATGATC-------AGTACTATCTCTTAA
CMW5446            GGAGTCCGCCAAGTATGTCCCTTATCCAGCTAT-GATC-------AGTACTACTTCTTAA
CMW4967            GGAGTCCGCCAAGTAAGTTC-TCATCCAACCAT-GATC-------AGTACCACCTCTTAA
CMW4722            GGAGTCCGCCAAGTAAGTTC-TTATCCAACCAT-GATC-------AGTACCACCTCTTAA
CMW5448            GGAGTCCGCCAAGTAAGTTC-TTATCCAACCAT-GATC-------AGTACCACCTCTTAA
CMW4143            AGAGTCCGCCAAGTAAGTTC-TTATCCAACCAT-GATC-------AGTACCACCTCTTAA
CMW4680            GGAGTCCGCCAAGTAAGG---TTATCCAGCTAT-GGTC-------AGTACTACCTCTTAA
CMW4991            GGAGTCCGCCAAGTAAGG---TTATCCAGCTAT-GGTC-------AGTACTACCTCTTAA
CMW4977            GGAGTCTGCCAAGTATGTCC-TTATCCAGCTAT-GATC-------AATACTACTTCTTAA
CMW8876            GGAGTCCGCCAAGTATGTCCCTTATCCAGCTAT-GATC-------AGTACTACTTCTTAA
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                    430       440       450       460       470       480
                ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum   CCTAGACTGCAGCATGCCGTGGTACAAGGGCTGGACCAAGGAGACCAAGGCTGGTGTCGT
CMW4456         CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
CMW4455         CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
CMW10115        CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
CMW9954         CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
CMW3164         CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
CMW4953         CCTTCTCTGTAGCATGCCCTGGTACAAGGGTTGGACCAAAGAGACCAAGGCCGGTGTCGT
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CMW3182         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW3162         CGTGCTCTGTAGTATGCCATGGTACAAGGGCTGGACCAAGGAGACTAAGGCTGGTGTCGT
CMW6888         CGTGCTCTGCAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW3181         CGTGCTCTGCAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW6901         CGTGTTTTGTAGTATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW3171         CGTGTTTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
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CMW6909         CATTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW3956         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACTAAGGCCGGTGTCGT
CMW4605         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACTAAGGCCGGTGTCGT
CMW3964         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAATAAGGCCGGTGTCGT
CMW4620         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAATAAGGCCGGTGTCGT
CMW11231        CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAGTAAGGCTGGTGTCGC
CMW4613         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAGTAAGGCTGGTGTCGC
CMW3961         CGTTCTCTGTAGCATGCCATGGTACAAGGGTTGGACCAAGGAGACTAAGGCCGGTGTCGT
CMW4610         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACTAAGGCCGGTGTCGT
CMW4611         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACTAAGGCCGGTGTCGT
CMW3158         CGTTGTTGAAAGCATGCCATGGTACAAGGGTTGGACGAAGGAGACCAAGGCTGGTGTCGT
CMW3165         CGTTGTTGAAAGCATGCCATGGTACAAGGGCTGGACGAAGGAGACCAAGGCCGGTGTCGT
CMW4994         CGTTCTCTGTAGCATGCCGTGGTACAAGGGCTGGACCAAGGAGACCAAGGCTGGCGTCGT
CMW5597         CGTTCTCTGTAGCATGCCGTGGTACAAGGGCTGGACCAAGGAGACCAAGGCTGGCGTCGT
CMW4960         CATTATCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW4955         CATTATCTGTAGCATGCCATGGTACAAGGGTTGGACTAAGGAGACCAAGGCCGGTGTCGT
CMW4971         CATTATCTGTAGCATGCCATGGTACAAGGGCTGGACTAAGGAGACCAAGGCCGGTGTCGT
CMW4980         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGACACCAAGGGCGGTGTCGT
CMW4981         CGTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGACACCAAGGGCGGTGTCGT
CMW5446         CCTTATCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW4967         CCTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAACAAGTCCGGTGCGGT
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CMW5448         CCTTCTCTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAACAAGTCCGGTGCGGT
CMW4143         CTTTCTTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGATACCAAGGCTGGTGTGGT
CMW4680         CCTTATTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW4991         CCTTATTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
CMW4977         CCTTATTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGAACAAGGCCGGTGTCGT
CMW8876         CCTTATTTGTAGCATGCCATGGTACAAGGGCTGGACCAAGGAGACCAAGGCCGGTGTCGT
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                          490       500       510       520       530       540
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Schizophyllum       CAAGGGCAAGACCCTCCTCGATGCCATCGACGCCATCGAGCCCCCCGTTCGTCCCTCCGA
CMW4456             CAAGGGCAAGACTCTCCTTGATGCCATTGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW4455             CAAGGGCAAGACTCTCCTTGATGCCATTGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW10115            CAAGGGCAAGACTCTCCTTGATGCCATTGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW9954             CAAGGGCAAGACTCTCCTTGATGCCATTGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW3164             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW4953             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCTGA
CMW3172             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW3182             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW3162             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW6888             CAAGGGCAAGACTCTCCTTGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW3181             CAAGGGCAAGACTCTCCTTGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW6901             TAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW3171             TAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW6905             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW6909             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAGCCCCCTGTCCGTCCCTCCGA
CMW3956             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW4605             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW3964             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW4620             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW11231            CAAAGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW4613             CAAAGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW3961             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
CMW4610             CAAGGGCAAGACTCTCCTCGATGCCATTGACGCCATTGAACCCCCTGTTCGTCCCTCCGA
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CMW3158             CAAGGGCAAGACTCTCCTCGATGCCATTGATGCCATTGAGCCCCCTGTCCGACCCTCCGA
CMW3165             CAAGGGCAAGACTCTCCTCGATGCTATTGATGCCATTGAGCCCCCTGTC-GA-CCTCCGA
CMW4994             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCCATTGAGCCCCCTGTTCGTCCCTCCGA
CMW5597             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCCATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4960             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4955             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4971             CAAGGGCAAGACTCTCCTTGATGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4980             CAAGGGCAAGACTCTCCTTGACGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4981             CAAGGGCAAGACTCTCCTTGACGCCATCGACGCTATTGAGCCCCCTGTTCGTCCCTCCGA
CMW5446             CAAGGGCAAGACTCTCCTTGACGCCATCGACGCCATCGAGCCCCCTGTTCGTCCCTCCGA
CMW4967             CAAGGGAAAGACTCTCCTTGATGCCATCGACGCCATTGAGCCACCTGTTCGTCCCTCCGA
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CMW5448             CAAGGGAAAGACTCTCCTTGATGCCATCGACGCCATTGAGCCACCTGCTCGTCCCTCCGA
CMW4143             CAAGGGAAAGACTCTCCTTGATGCCATCGACGCCATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4680             TAAGGGCAAGACTCTCCTTGATGCCATTGACGCCATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4991             TAAGGGCAAGACTCTCCTTGATGCCATTGACGCCATTGAGCCCCCTGTTCGTCCCTCCGA
CMW4977             CAAAGGCAAGACTCTCCTTGACGCCATCGACGCCATCGAGCCCCCTGTTCGTCCCTCCGA
CMW8876             CAAAGGCAAGACTCTCCTTGACGCCATCGACGCCATCGAGCCCCCTGTTCGTCCCTCCGA
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                      550       560
                 ....|....|....|....|..
Schizophyllum    CAAGCCCCTCCGTCTCCCCCTC
CMW4456          CAAGCCTCTCCGTCTCCCTCTC
CMW4455          CAAGCCTCTCCGTCTCCCTCTC
CMW10115         CAAGCCACTCCGTCTCCCTCTC
CMW9954          CAAGCCACTCCGTCTCCCTCTC
CMW3164          CAAGCCTCTCCGTCTCCCTCTC
CMW4953          CAAGCCTCTCCGTCTCCCTCTC
CMW3172          CAAGCCTCTCCGTCTCCCTCTC
CMW3182          CAAGCCTCTCCGTCTCCCTCTC
CMW3162          CAAGCCTCTCCGTCTCCCTCTC
CMW6888          CAAGCCTCTCCGTCTCCCTCTC
CMW3181          CAAGCCTCTCCGTCTCCCTCTC
CMW6901          CAAGCCTCTCCGTCTCCCTCTC
CMW3171          CAAGCCTCTCCGTCTCCCTCTC
CMW6905          CAAGCCTCTCCGTCTTCCTCTC
CMW6909          CAAGCCTCTCCGTCTCCCTCTC
CMW3956          CAAGCCTCTCCGTCTTCCTCTC
CMW4605          CAAGCCTCTCCGTCTTCCTCTC
CMW3964          CAAGCCTCTCCGTCTTCCTCTC
CMW4620          CAAGCCTCTCCGTCTTCCTCTC
CMW11231         CAAGCCTCTCCGTCTTCCTCTC
CMW4613          CAAGCCTCTCCGTCTTCCTCTC
CMW3961          CAAGCCTCTCCGCCTTCCTCTC
CMW4610          CAAGCCTCTCCGCCTTCCTCTC
CMW4611          CAAGCCTCTCCGCCTTCCTCTC
CMW3158          CAAGCCTCTCCGTCTTCCTCTC
CMW3165          -AAGCCTCTCCGNNNNNNNNNN
CMW4994          CAAGCCTCTCCGTCTCCCTCTC
CMW5597          CAAGCCTCTCCGTCTCCCTCTC
CMW4960          CAAGCCTCTCCGTCTCCCTCTC
CMW4955          CAAGCCTCTCCGTCTCCCTCTC
CMW4971          CAAGCCTCTCCGTCTCCCTCTC
CMW4980          CAAGCCTCTCCGTCTCCCTCTC
CMW4981          CAAGCCTCTCCGTCTCCCTCTC
CMW5446          CAAGCCTCTCCGTCTCCCTCTC
CMW4967          CAAGCCTCTCCGACTCCCTCTC
CMW4722          CAAGCCTCTCCGACTCCCTCTC
CMW5448          CAAGCCTCTCCGACTCCCTCTC
CMW4143          CAAGCCTCTCCGACTCCCTCTC
CMW4680          CAAGCCTCTCCGTCTCCCTCTC
CMW4991          CAAGCCTCTCCGTCTCCCTCTC
CMW4977          CAAGCCTCTCCGTCTCCCTCTC
CMW8876          CAAGCCTCTCCGTCTCCCTCTC
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Chapter 4:

Characterisation of *Armillaria spp.* based on pectic isozyme analyses.

Characterisation of *Armillaria spp.* based on pectic isozyme analyses.

Abstract

Armillaria spp. are the casual agents of Armillaria root rot on a wide variety of mainly woody plants. Identification of these fungi using morphological characteristics is complicated by the fact that fruiting structures are uncommon and often ephemeral. Although DNA sequences are very effective for characterising species, there is a need for simple and inexpensive identification protocols. In this regard, pectic isozyme analysis has been successfully applied to identify a limited number of *Armillaria* species. In the present study, 39 *Armillaria* isolates, representing 17 *Armillaria* spp. from different hosts and geographic regions were characterised using isozyme patterns for pectin lyase (PL), pectin methylesterase (PME) and polygalacturonase (PG). Isozyme patterns were determined directly from culture filtrates through electrophoresis in polyacrylamide gels stained in ruthenium red. The species could be clearly separated from each other and isolates belonging to the same species had a more or less identical banding pattern and grouped together after cluster analysis. Overall, the Northern hemisphere species of *Armillaria* could be distinguished from those originating in the Southern hemisphere. *Armillaria borealis* isolates produced an isozyme profile closely related to those of *A. ostoyae. Armillaria gemina, A. nabsnona, A. cepistipes* and *A. gallica* had related patterns. Related patterns were also observed for *A. pallidula* and *A. fumosa*. *Armillaria mellea* isolates had related isozyme patterns but could be separated according to their geographic origins. This study has shown that pectic enzyme analysis can be an effective tool in the identification of *Armillaria* species.

INTRODUCTION

Species of *Armillaria* are Basidiomycetous root pathogens of a wide range of woody plants. These fungi have a wide global distribution and they also include some of the most important pathogens of trees (Hood et al., 1991). In this regard they are especially important in forest and fruit crops.

Various techniques have been used to identify and group *Armillaria* species. Earlier work relied exclusively on pairing tests and morphology (Hintikka, 1973; Korhonen, 1978; Anderson and Ullrich, 1979; Bérubé and Dessureault, 1988). Morphological identification is based primarily on fruiting body characteristics (e.g. Bérubé and Dessureault, 1988; 1989; Watling et al., 1982). However, the seasonal nature and short life span of fruiting bodies limits the use of this method for identification. Mating tests, while not dependant on fruiting structures, rely on biological compatibility of isolates of the same species (Hintikka, 1973; Korhonen, 1978; Anderson and Ullrich, 1979). They are however, time consuming and are only applicable to heterothallic and sexual species.

Qualitative DNA based methods including comparisons of sequences of the IGS-1 and ITS regions of the ribosomal DNA (Anderson and Stasovski, 1992; Chillali et al., 1997, 1998; Coetzee et al., 2000; 2001; 2003), RFLPs (Jahnke et al., 1987; Anderson et al., 1989; Smith and Anderson, 1989) and AFLPs (Pérez-Sierra et al., 2004) have recently been increasingly used to identify *Armillaria* species. Protein based techniques have also been employed in *Armillaria* taxonomy (Morrison, 1982; Morrison et al., 1985; Lin et al., 1989; Whalström et al., 1991; Mwenje and Ride, 1996). Despite limited resolution of protein-based methods in comparison to DNA techniques, they can potentially provide an inexpensive

diagnostic tool for identification of large numbers of new isolates and in the absence of DNA sequencing facilities.

Isozymes are multiple forms of the same enzyme and they differ in molecular weight, regulation, isoelectric points and electrophoretic mobilities (D'Ovidio et al., 2004). Isozymes arise as the result of the presence of multiple genes coding for a protein or as a result of post-translational modification of the enzymes (D'Ovidio et al., 2004). Pectic isozymes have been used widely in fungal taxonomy (Johansson, 1988; Karlsson and Stenlid, 1991; Chang and Mills, 1992). The technique has also been applied successfully for the identification of *Armillaria* spp. (Whalström et al., 1991; Mwenje and Ride, 1996).

Morrison et al. (1985) used esterase and polyphenol oxidases to study isolates from British Columbia and separated the isolates into *A. bulbosa*, North American Biological species (NABS) IX (= *A. nabsnona*), NABS X, and group F clustered with NABS V (= *A. sinapina*). Esterase patterns were also used to differentiate four North American Biological Species of *Armillaria* by Lin et al. (1989). Whalström et al. (1991) analysed the pectic esterase and polygalacturonases isozyme patterns of five European species and found that the patterns differed among the species. *Armillaria mellea* had two specific polygalacturonase bands, which were absent in the other species. *Armillaria ostoyae* and *A. borealis* had very similar profiles. *Armillaria* isolates from Zimbabwe resided in three groups using isozymes (Mwenje and Ride 1996). Mwenje and Ride (1997) also identified four taxonomic groups in Africa based on pectin lyase and pectin methylesterase isozyme patterns. Despite the usefulness of pectic enzymes in distinguishing some species of *Armillaria*, a comparison of the pectic enzymes profiles of most species is not available.

The aim of this study was to analyse the relationships between a wide range of *Armillaria* spp. using pectic enzyme profiles. These relationships were then compared with those of previously published (Coetzee et al., 2001; this dissertation) DNA based comparisons. In this way the value of the pectic enzyme profiles could be assessed as a rapid and inexpensive method for distinguishing large numbers of isolates emerging from large field collections.

MATERIALS AND METHODS

Origin of Isolates

In total, 17 species of *Armillaria* represented by 39 isolates from many different origins were considered in this study (Table 1). These isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), and a representative set of isolates has been deposited with the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Cell wall preparation

In order to produce cell walls, fresh stem segments of Msasa (*Brachystegia spiciformis*) were ground into fine sawdust using a mill as described by Mwenje and Ride (1996). This sawdust was soaked for one hour in 100% ethanol, filtered through Whatman filter paper (#1) and rinsed in 100% ethanol for 10 minutes. This was followed by washing twice in acetone (10 minutes for each wash) after which it was air dried. The crude extract was then stored at room temperature until further use.

Enzyme production

Isolates were grown in duplicate at 25 °C under stationary conditions in 250 ml conical flasks containing 50 mL Vogel's medium (Vogel, 1956) amended with one g cell walls. After 30-35 days of incubation in the dark, the cultures were harvested by filtration using Whatman filter paper (#1). Filtrate (30-40 mL) was concentrated overnight by dialysis to approximately one mL using 12.5% (w/v) Polyethylene Glycol dissolved in sodium acetate buffer (pH 5.5). The concentrate was stored at –20 ºC.

Native gel electrophoresis

Electrophoresis was performed in 10% polyacrylamide resolving gels, using a high pH, non-dissociating discontinuous system (Hames, 1987). Pectin was incorporated at 0.5% (w/v) into resolving and plug gels, but not in the stacking gels, to enable the detection of pectic enzymes. Equal amounts of double strength buffer and enzyme solution were mixed prior to loading the gel.

Detection of Pectin Lyases (PL) and Pectin Methylesterases (PME)

The method used by Mwenje and Ride (1996) was employed to detect PLs and PMEs. Gels were incubated for 10 minutes at 5 °C in 10 mM $CaCl_2$ after electrophoresis. They were then incubated for 40 minutes at room temperature in 20 mM Tris-HCl (pH 8.5) containing 10 mM $CaCl_2$. Gels were stained overnight in 0.03 (w/v) ruthenium red. Excess ruthenium red was removed using at least three changes of distilled water. Pectin lyase bands appeared as white bands, while PME bands appeared as dark, red/ purple bands.

Detection of Polygalacturonases (PGs) and Pectin Methylesterases (PMEs)

After electrophoresis, gels were washed briefly in water and incubated for 1.5 hours at 25 °C in 100 mM malic acid without shaking. Gels were rinsed in water for five minutes and stained overnight in ruthenium red (0.03 w/v in water). Stained gels were washed in water to remove excess ruthenium red. Polygalacturonases were visualised as white bands and PMEs appeared as dark bands.

Numerical Analyses

Gels were scored for presence or absence of bands with 1 and 0 representing present and absent bands, respectively. The combined data set for polygalacturonase and pectin methylesterase was analysed using the Number Cruncher Statistical Systems (NCSS) software (Hintze, 1998). Ward's method (Wishart, 1987) was used to calculate the Euclidean distance from which a dendrogram was generated.

RESULTS

Isozyme patters for various pectic enzymes

Pectin Lyase (PL) isozyme patterns are indicated in Figure 1 and Table 1 and the data matrix is shown in the appendix (Table 2). A total of eight different PL isozymes, designated PL1 –PL8, were detected (Figure 1). European *A. mellea* strains (CMW4615, CMW11231, CMW11265 and CMW11266) yielded six PL bands, and thus the largest number of PL isozymes. PL1 and PL2 bands were present in these isolates, as well as in *A. novae-zelandiae* and *A. gallica* isolates. PL3 was present only in the species from Africa. Band PL4 was detected only in Zimbabwean group III isolates (CMW9954 and CMW10115). All *A. mellea* isolates except one from Japan had band PL5, which they shared with *A. novae-zelandiae* and *A. tabescens* isolates. Band PL6 was common in all *A. mellea* isolates but was also detected in *A. novae-zelandiae* and *A. tabescens*. Bands PL7 and PL8 were common in all *A. mellea*

isolates but were also detected in *A. tabescens*, *A. luteobubalina*, *A. ostoyae*, *A. borealis* and the unnamed *Armillaria* sp. from New Zealand (CMW5597).

Pectin methylesterase (PME) bands are shown in Table 1 and the banding pattern is shown in Figure 1 and appendix (Table 2). Four different pectin methylesterase bands, designated PME1-PME4, were found in this study (Figure 1). PME1 was detected only in the isolate of the unnamed *Armillaria* sp. from New Zealand (CMW 5597). Band PME2 was absent in *A. tabescens,* the unnamed species from New Zealand and all *A. mellea* isolates. Band PME3 was detected in *A. borealis*, the unnamed species from New Zealand, *A. limonea*, *A. luteobubalina*, *A. novae-zelandiae*, *A. pallidula*, *A. tabescens, A. mellea* from Japan, *A. fumosa* and *A. ostoyae*. Band PME 4 was present in all isolates from Africa, *A. gallica*, *A. fumosa* and *A. pallidula.*

Polygalacturonase (PG) isozymes had eight different bands (PG1 – PG8) as shown in Figure 1, Table 1 and Table 2 (appendix). All African isolates were identical in their PG banding patterns; having PG1, PG2 and PG3. The PG1 band was also present in *A. luteobubalina*, *A. novae-zelandiae*, *A. limonea*, *A. tabescens, A. mellea* isolates from North America and Japan, *A. ostoyae* and unnamed isolate (CMW5597) from New Zealand. Band PG2 was present in the African isolates and *A. pallidula,* and PG3 was exclusive to the African isolates. Band PG4 was detected in *A. cepistipes*, *A. borealis, A. nabsnona, A. ostoyae*, *A. gemina* and *A. gallica* and band PG5 was present in *A. gallica, A. gemina, A. nabsnona,* and *A. cepistipes.* Bands PG6 and PG7 were present in *A. borealis*, *A. tabescens*, *A. ostoyae*, *A. cepistipes*, *A. nabsnona*, *A. gemina*, *A. gallica* and all *A. mellea* isolates. Band PG8 was present in unnamed species from New Zealand, *A. gallica, A. limonea*, *A. luteobubalina*, *A. tabescens* and North America and Japanese *A. mellea*.

Numerical Analyses and grouping of isolates

All isolates included in this study could be grouped according to the *Armillaria* spp. that they represented, in the dendrogram generated based on combined data of pectin lyase, polygalacturonases and pectin methylesterases (Figure 2). Isolates also resided in two major groups. One of these included all *Armillaria* spp. found only in the Northern Hemisphere and the second group included all species from the Southern hemisphere.

Armillaria borealis grouped very close to *A. ostoyae,* forming one group within the Northern Hemisphere collection of isolates. This group was connected to a cluster that included *A. gallica, A. gemina, A. nabsnona* and *A. cepistipes*. A second major cluster within the Northern Hemisphere group was formed by *A. mellea* and *A. tabescens. Armillaria mellea* isolates formed clusters, comprising of isolates from Japan, Europe and North America, respectively. The cluster comprising the North American isolates was connected to the *A. tabescens* group and this was further connected to the cluster comprising of Japanese *A. mellea* isolates. The European group formed two connected subclusters.

The Southern Hemisphere group included isolates representing the unknown isolate from New Zealand, *A. limonea, A. luteobubalina, A. novae-zelandiae, A. pallidula, A. fumosa, A. fuscipes* and the Zimbabwean groups I to III as defined by Mwenje and Ride (1996). Isolates of *A. luteobubalina* clustered closely with the isolate (CMW5597) representing the unknown species from New Zealand in one subgroup, which was related, to a subgroup of *A. limonea*. The *A. luteobubalina* and *A. limonea* group was connected to the group comprising *A. pallidula* and *A. fumosa*. Isolates representing *A. novae-zelandiae* and African isolates formed distinct clusters.

DISCUSSION

In this study we have shown that the pectic enzymes PL, PG and PME can be used to separate all the species of *Armillaria* tested. These included 17 species and thus the majority of those that are commonly encountered. We have further been able to show that Northern hemisphere isolates are completely different to those from the Southern hemisphere. Southern hemisphere species have been found to group basally in a DNA based phylogeny, hence ancestral to those from the Northern Hemisphere (Coetzee et al, 2001, Dunne et al., 2002). DNA sequence data has shown that species from the Southern hemisphere are more closely related to each other and very distantly from those from the Northern hemisphere. Isozyme comparisons in this study confirm this.

Pectic enzymes have previously been used for the identification of *Armillaria* spp. (Whalström et al., 1991; Mwenje and Ride, 1996). However, in those studies only few species were considered and they were from limited geographic areas. The broad range of species considered in the present study has shown that this technique can be used as an alternative to DNA sequence analyses, where such facilities are not available. Using pectic enzymes is also reasonably cheap and reproducible.

In this study, isolates from Zimbabwe and La-Reunion representing African *Armillaria* group II (Mwenje and Ride, 1996; Mwenje et al., 2003) and *A. fuscipes* had identical banding patterns. These isolates also clustered together in the dendrogram. This result is in contrast to IGS-1 sequence data (Mwenje et al., 2003) which separates these isolates into different groups. Isolates of African *Armillaria* group III (Mwenje and Ride, 1996; Mwenje et al., 2003) differed slightly in their enzyme profile from groups I and III. This is also consistent with analyses of IGS-1 sequence data where these isolates have been shown to represent different, but closely related, groups (Mwenje et al., 2003).

Armillaria fumosa and *A. pallidula* had different banding patterns and they grouped closely in cluster analysis. Previous reports have indicated that these two species are phylogenetically closely related. For example, Coetzee et al. (2001) could not differentiate between the two species based on sequences of the ITS region data. The species could also not be separated based on EF 1-α sequence data in a previous study (Chapter 3, this dissertation). However, *A. fumosa* and *A. pallidula* were previously shown to be distinct species based on morphology and mating type tests (Kile and Watling, 1988).

Armillaria luteobubalina and *A. limonea* grouped in the same cluster and had slightly different banding patterns. *Armillaria luteobubalina* is of Australian origin and *A. limonea* originates from New Zealand. Using compatibility tests, Kile and Watling (1988) concluded that these represent different biological species. The grouping of these species based on pectic enzymes in this study is consistent with the findings of Coetzee et al. (2003) who showed that these two species are phylogenetically closely related based on their ITS sequence data. Also present in this cluster was an unnamed isolate from New Zealand. The fact that this isolate grouped with isolates representing *A. luteobubalina* indicated that it might be related to *A. luteobubalina*. Coetzee et al. (2003) showed that this isolate has ITS sequence data that is not identical to *A. luteobubalina* but is phylogenetically related. This isolate also had distinct EF 1-α sequences (Chapter 3, this dissertation) providing further evidence that it represents a previously undescibed species.

Armillaria novae-zelandiae isolates from Chile, New Zealand and Australia grouped together regardless of geographic origin. This result is consistent with the report of Kile and Watling (1983) who showed that *A. novae-zelandiae* from Australia and New Zealand are sexually compatible and belong to the

same biological species. Using ITS sequence data Coetzee et al. (2001) showed that these isolates grouped in a single clade further proving that they represent a single species.

Armillaria mellea isolates had different banding patterns and formed three subclusters corresponding to their biogeographic distributions. Thus, isolates from Japan, Europe and North America formed separate subclusters which are consistent with those emerging from DNA sequence data. The European isolates were further separated into two closely related groups, which is also similar to that found by Coetzee et al. (2000). Anderson et al. (1989) using RFLP data from the rRNA operon showed that *A. mellea* from Europe and North America have different restriction patterns. Harrington and Wingfield (1995) also showed that North American and European isolates of *A. mellea* have different IGS-1 RFLP profiles after digestion with *Alu*I. Likewise, Coetzee et al. (2000) using ITS and IGS-1 data, separated *A. mellea* isolates according to geographic origin. Western and Eastern North America *A. mellea* isolates have been shown to be different (Coetzee et al., 2000).

Isolates representing *A. tabescens* had banding patterns different to those of *A. mellea*. Isolates representing these two species clustered closer together and this grouping is consistent with the findings of Coetzee et al. (2000) who showed that *A. tabescens* resides in a clade basal to that of *A. mellea*. It has similarly been shown, using DNA re-association data, that *A. tabescens* is very closely related to *A. mellea* (Miller et al., 1994). Although these species are clearly different, we have added evidence that *A. tabescens* and *A. mellea* are closely related species.

Armillaria ostoyae had a unique overall banding pattern. The profile of this species was most closely related to that of *A. borealis* and they clustered closely together on the dendrogram obtained in this

study. The IGS-1 RFLP patterns for these two species are very similar (Harrington and Wingfield, 1995) and they have previously been shown to be closely related based on their isozyme profiles (Whalström et al., 1991). Furthermore, Anderson et al. (1989) also concluded that these two species are closely related using rDNA operon data and Anderson and Stasovski (1992) showed that the intergenic region sequences for *A. borealis* and *A. ostoyae* are very similar.

Armillaria gemina, *A. nabsnona* and *A. cepistipes* had identical banding profiles and these species clustered together in the dendrogram. They shared most of the bands with *A. gallica* although the latter species was clearly different. The grouping of *A. gallica*, *A. nabsnona* and *A. cepistipes* excluding *A. gemina* concurs with previous studies that showed that these species are phylogenetically closely related (Anderson and Stasovski, 1992; Miller et al., 1994, Chillali et al., 1998). The grouping of *A. gemina* within this group was unexpected as it has been shown to be more closely related to *A. ostoyae* in IGS-1 DNA sequence and rDNA data (Smith and Anderson, 1989; Anderson and Stasovski, 1992), RFLP patterns (Harrington and Wingfield, 1995) and morphology (Bérubé and Dessureault, 1989). In this study *A. gemina*, *A. nabsnona* and *A. cepistipes* grouped in a closely related subcluster with *A. gallica*.

Results of this study have demonstrated that combined pectin lyase, polygalacturonase and pectin methylesterase data can be used to differentiate between many *Armillaria* species. The technique, however, failed to separate closely related species. A major advantage of isozyme analyses is that a large number of isolates can be analysed together relatively rapidly. A major disadvantage is that they provide little phyogenetic inference and they cannot be used to consider questions relating to origin or evolution. They are however, relatively inexpensive and in laboratories where DNA sequence analyses are not available, they provide an alternative approach for the recognition of discrete species.

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Table 1**:** List of isolates analysed for isozyme patterns.

Isolate number	Species or Taxonomic Group	Origin	Bands Present
CMW3172	*A. borealis*	Finland	PL7, PL8, PME2, PME3, PG4, PG6, PG7
CMW3182	*A. borealis*	West Germany	PL7, PL8, PME2, PME3, PG4, PG6, PG7
CMW5597	Unnamed species	New Zealand	PL7, PL8, PME1, PME3, PG1, PG8
CMW6901	*A. gallica*	USA	PL1, PL2, PME2, PME4, PG4, PG5, PG6, PG7, PG8
CMW6902	*A. gallica*	USA	PL1, PL2, PME2, PME4, PG4, PG5, PG6, PG7, PG8
CMW6888	*A. gemina*	USA	PME2, PG4, PG5, PG6, PG7
CMW6889	*A. gemina*	USA	PME2, PG4, PG5, PG6, PG7
CMW10165	Group I	Zimbabwe	PL3, PME2, PME4, PG1, PG2, PG3
CMW4456	Group II	Zimbabwe	PL3, PME2, PME4, PG1, PG2, PG3
CMW4457	Group II	Zimbabwe	PL3, PME2, PME4, PG1, PG2, PG3
CMW10115	Group III	Zimbabwe	PL3, PL4, PME2, PME4, PG1, PG2, PG3
CMW9954	Group III	Zimbabwe	PL3, PL4, PME2, PME4, PG1, PG2, PG3
CMW4953	*A. fuscipes*	La Reunion	PL3, PME2, PME4, PG1, PG2, PG3
CMW4680	*A. limonea*	New Zealand	PME2, PME3, PG1, PG8

Isolate number	Species or Taxonomic Group	Origin	Bands Present
CMW4991	*A. limonea*	New Zealand	PME2, PME3, PG1, PG8
CMW4977	*A. luteobubalina*	Australia	PL7, PL8, PME2, PME3, PG1, PG8
CMW4967	*A.novae-zelandiae*	Australia	PL1, PL2, PL5, PL6, PME2, PME3, PG1
CMW4722	*A. novae-zelandiae*	New Zealand	PL1, PL2, PL5, PL6, PME2, PME3, PG1
CMW8876	*A .luteobubalina*	Chile	PL7, PL8, PME2, PME3, PG1, PG8
CMW5448	*A. novae-zelandiae*	Chile	PL1, PL2, PL5, PL6, PME2, PME3, PG1
CMW4966	*A. novae-zelandiae*	Australia	PL1, PL2, PL5, PL6, PME2, PME3, PG1
CMW4968	*A.pallidula*	Australia	PME2, PME3, PME4, PG2
CMW4971	*A. pallidula*	Australia	PME2, PME3, PME4, PG2
CMW3165	*A. tabescens*	France	PL5, PL6, PL7, PL8, PME3, PG1, PG6, PG7, PG8
CMW3946	*A. tabescens*	USA	PL5, PL6, PL7, PL8, PME3, PG1, PG6, PG7, PG8
CMW6909	*A. nabsnona*	USA	PME2, PG4, PG5, PG6, PG7
CMW6905	*A .cepistipes*	USA	PME2, PG4, PG5, PG6, PG7
CMW4605	*A mellea*	East USA	PL5, PL6, PL7, PL8, PG6, PG7
CMW4603	*A. mellea*	East USA	PL5, PL6, PL7, PL8, PG6, PG7

Isolate number	Species or Taxonomic Group	Origin	Bands Present
CMW3964	*A. mellea*	West USA	PL5, PL6, PL7, PL8, PG1, PG6, PG7, PG8
CMW4620	*A .mellea*	West USA	PL5, PL6, PL7, PL8, PG1, PG6, PG7, PG8
CMW4615	*A. mellea*	Europe	PL1, PL2, PL5, PL6, PL7, PL8, PG6, PG7
CMW11265	*A. mellea*	Europe	PL1, PL2, PL5, PL6, PL7, PL8, PG6, PG7
CMW11266	*A. mellea*	Europe	PL1, PL2, PL5, PL6, PL7, PL8, PG6, PG7
CMW11231	*A. mellea*	Europe	PL1, PL2, PL5, PL6, PL7, PL8, PG6, PG7
CMW3961	*A. mellea*	Japan	PL6, PL7, PL8, PME3, PG1, PG6, PG7, PG8
CMW3967	*A. mellea*	Japan	PL6, PL7, PL8, PME3, PG1, PG6, PG7, PG8
CMW4960	*A. fumosa*	Australia	PME2, PME3, PME4
CMW3162	*A. ostoyae*	USA	PL7, PL8, PME2, PME3, PG1, PG4, PG6, PG7

PL= Pectin Lyase; PME= Pectin methylesterase; PG= Polygalacturonase

Figure 1. Diagram showing the type and number of Pectin lyase, Pectin methylesterase and Polygalacturonase patterns produced by the *Armillaria* isolates. Columns 1-2: CMW3172 and CMW3182 (*A. borealis*); 3-4: CMW6901 and CMW6902 (*A. gallica*); 5-6: CMW6888 and CMW6889 (*A. gemina*); 7-8: CMW3165 and CMW3946 (*A. tabescens*); 9: CMW6909 (*A. nabsnona*); 10: CMW6905 (*A. cepistipes*); 11-20: CMW3964, CMW4620, CMW4605, CMW4603, CMW4615, CMW11265, CMW11266 and CMW11231 (*A. mellea*); 21-22: CMW4953 and CMW10165 (*A. fuscipes*); 23-24: CMW4456 and CMW 4457 (Group II); 25-26: CMW9954 and CMW10115 (Group III); 27-28: CMW4680 and CMW4991 (*A. limonea*); 29-30: CMW4977 and CMW8876 (*A. luteobubalina*); 31-34: CMW4967, CMW4722, CMW5448 and CMW4966 (*A. novae-zelandiae*); 35: CMW5597 (Unknown); 36-37: CMW4968 and CMW4971 (*A. pallidula*); 38: CMW4960 (*A. fumosa*) and 39: CMW3162 (*A. ostoyae*).

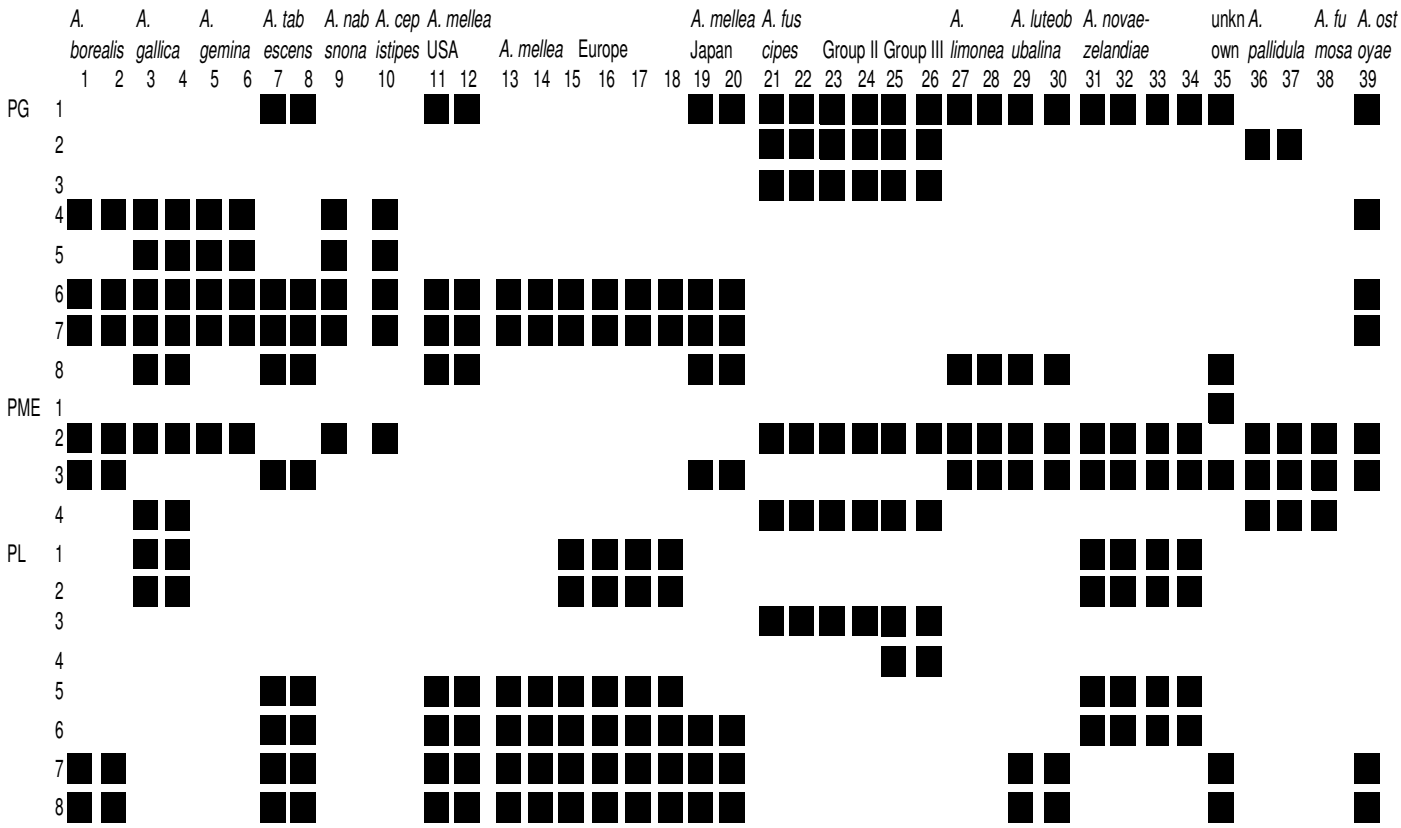
A. borealis
A. gallica
A. gemina
A. tab escens
A. nab snona
A. cep istipes
A. mellea USA
A. mellea Europe
A. mellea Japan
A. fus cipes
Group II
Group III
A. limonea
A. luteob ubalina
A. novae-zelandiae
unkn own
A. pallidula
A. fu mosa
A. ost oyae
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
PG
PME
PL

Figure 2. A dendrogram generated after a cluster analysis of 39 *Armillaria* isolates based on isozyme patterns. The dendrogram is based on the Euclidean distances, calculated using Ward's method, using presence or absence of isozyme bands. Dissimilarity values are shown on the scale.

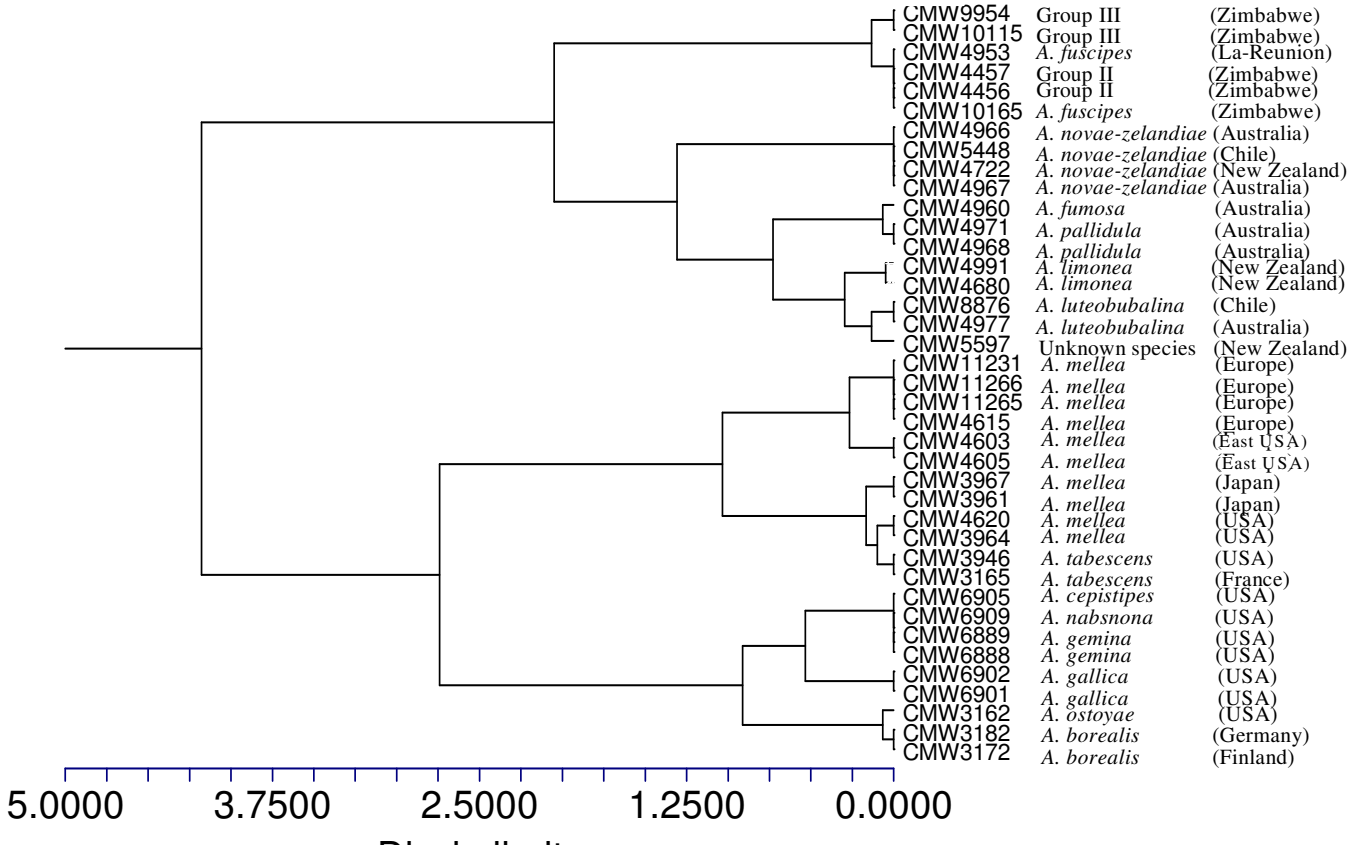
CMW9954 Group III (Zimbabwe)
CMW10115 Group III (Zimbabwe)
CMW4953 A. fuscipes (La-Reunion)
CMW4457 Group II (Zimbabwe)
CMW4456 Group II (Zimbabwe)
CMW10165 A. fuscipes (Zimbabwe)
CMW4966 A. novae-zelandiae (Australia)
CMW5448 A. novae-zelandiae (Chile)
CMW4722 A. novae-zelandiae (New Zealand)
CMW4967 A. novae-zelandiae (Australia)
CMW4960 A. fumosa (Australia)
CMW4971 A. pallidula (Australia)
CMW4968 A. pallidula (Australia)
CMW4991 A. limonea (New Zealand)
CMW4680 A. limonea (New Zealand)
CMW8876 A. luteobubalina (Chile)
CMW4977 A. luteobubalina (Australia)
CMW5597 Unknown species (New Zealand)
CMW11231 A. mellea (Europe)
CMW11266 A. mellea (Europe)
CMW11265 A. mellea (Europe)
CMW4615 A. mellea (Europe)
CMW4603 A. mellea (East USA)
CMW4605 A. mellea (East USA)
CMW3967 A. mellea (Japan)
CMW3961 A. mellea (Japan)
CMW4620 A. mellea (USA)
CMW3964 A. mellea (USA)
CMW3946 A. tabescens (USA)
CMW3165 A. tabescens (France)
CMW6905 A. cepistipes (USA)
CMW6909 A. nabsnona (USA)
CMW6889 A. gemina (USA)
CMW6888 A. gemina (USA)
CMW6902 A. gallica (USA)
CMW6901 A. gallica (USA)
CMW3162 A. ostoyae (USA)
CMW3182 A. borealis (Germany)
CMW3172 A. borealis (Finland)
5.0000
3.7500
2.5000
1.2500
0.0000

Table 2. (Appendix) A data matrix showing presence and absence of PL, PME and PG bands.

	PL1	PL2	PL3	PL4	PL5	PL6	PL7	PL8	PME1	PME2	PME3	PME4	PG1	PG2	PG3	PG4	PG5	PG6	PG7	PG8
CMW3172	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	1	1	0
CMW3182	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	1	1	0
CMW5597	0	0	0	0	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	1
CMW6901	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1
CMW6902	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1
CMW6888	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
CMW6889	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
CMW10165	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW4456	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW4457	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW10115	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW9954	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW4953	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0
CMW4680	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1
CMW4991	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1
CMW4977	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0	1
CMW4967	1	1	0	0	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0
CMW4722	1	1	0	0	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0
CMW8876	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0	1
CMW5448	1	1	0	0	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0
CMW4966	1	1	0	0	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0
CMW4968	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0
CMW4971	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0
CMW3165	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1
CMW3946	0	0	0	0	1	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1
CMW6909	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
CMW6905	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
CMW4605	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW3964	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0	1	1	1
CMW4620	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0	1	1	1
CMW4603	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW4615	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW11265	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW11266	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW11231	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0
CMW3961	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1
CMW3967	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	1	1	1
CMW4960	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
CMW3162	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	1	0	1	1	0

Summary

Armillaria spp. are important root pathogens that cause considerable plant mortality throughout the world. The nomeclatural and taxonomic placement of the genus has been intensely debated for a long time. Early identification relied exclusively on mating tests and morphological similarities. The introduction of DNA and protein based methods has greatly increased the understanding of the phylogeny of *Armillaria* species.

The literature surrounding *Armillaria*, Armillaria root disease, characters that are distinct to *Armillaria*, means of disease spread and techniques that have been used to identify *Armillaria* spp. are considered in this thesis. The controversy surrounding the proper genus name and which species should be included in the genus is also discussed.

In this study a collection of isolates obtained from Zimbabwean plantations are characterized. IGS-1 sequence data and AFLP data grouped these isolates into four groups while RFLP data separated them into five groups. One group has been tentatively identified as *A. fuscipes* whilst the remaining ones have not been described due to scarcity of basidiocarps in the field.

A broad selection of *Armillaria* spp. representing most of the known species were characterized using EF 1-α DNA sequences and pectic enzymes. Isolates from the Southern Hemisphere were clearly separate from those originating in the Northern Hemisphere. Within these two large clades, isolates formed subclades indicating their relatedness. Both techniques confirm relationships between species reported previously using other techniques. This is however the first study that presents the molecular phylogeny of *Armillaria* based on a single copy protein coding gene.

The identification techniques used in this study were valuable for species characterisation. Absence of fruiting bodies however, made morphological classification impossible. The results of this thesis should be useful in the process of developing future disease management strategies for Armillaria root rot in Zimbabwe.