DIVERSITY PATTERNS AND HOST SHIFTS IN ECTOMYCORRHIZAL FUNGI ACROSS AN AGRICULTURAL DISTURBANCE GRADIENT IN THE MID-ZAMBEZI AREA, ZIMBABWE

By

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Abstract

Fallowing creates land mosaics characterised by vegetation communities at different stages of succession. Such mosaics are expected to harbour mycorrhizal associations that reflect host species composition and diversity. Ectomycorrhizal fungi are often a neglected group of the ecosystem despite their importance as bioindicators of disturbance. The present study investigated the diversity of ectomycorrhizal fungi and their host associations across a fallow chrono-sequence of 1-14 years in the mid-Zambezi Valley area, Zimbabwe. A total of ten ectomycorrhizal fungaltaxa that included *Lactarius gymnocarpus*, *Lactarius* sp., *Boletus* sp., *Thelephora terrestris* and *Amphinema byssoides* were recorded from 13 tree species from six families (Combretaceae, Ebenaceae, Fabaceae, Simaroubaceae, Rhamnaceae and Tiliaceae). The Spearman rank correlation test showed no significant correlation (p=0.002) between fallow age and ectomycorrhizal fungi status of host tree species. The generalised linear model (GLM) showed no significant relationship (p=0.079) among ectomycorrhizal fungi in any tree species and fallow age. The Raup-Crick similarity index indicated that there was an interaction between fallow age and the mycorrhizal status of the tree species, as opposed to host specificity. These results support previously reported low host specificity for ectomycorrhizal fungi among tropical African plant communities. Theyfurther indicate that selective tree felling negatively impacts host specific ectomycorrhizal fungi. The results also indicate that an increase in host tree species does not necessarily lead to increased ectomycorrhizal species diversity, thus implying the influence of other factors. Genetic similarity tests (using Nei and Li similarity coefficients) based on RAPDs and RFLPs showed varying relationships among the collected fungi. PCR-RFLPs confirmed genetic polymorphisms among samples of the recorded taxa. The intra- and inter-specific genetic diversity among the sampled ectomycorrhizal fungi partly explains the low host specificity among the collected ectomycorrhizal fungi and the dominance of a few ectomycorrhizal species in the study area.

Keywords: Ectomycorrhizal fungi, fallows, mid-Zambezi valley, succession,RAPD, PCR-RFLP, Zimbabwe

Definition of terms

1. **Ectomycorrhizal fungi**: are fungi thatproduce a system of hyphae, called the Hartig net, in between cells of the root of a plant. The mycorrhizae do not enter the actual cells of the roots, travelling instead between the root cells. They also form a mantle around plant root tips.

2. **Endomycorrhizal fungi**: are fungi that grow in between the cells of a plant root but also have structures that allow the fungus to penetrate the actual cells in the plant root, making them more invasive than ectomycorrhizal fungi.

a. **Arbuscular fungi**: are endomycorrhizal fungi characterised by the formation of unique structures, arbuscules and vesicles, which penetrateroot cortical cells.

3. **Fallow**: cultivated land left for a period without being sown, usually in order to retain its fertility.

4. **Seral**: relating to or being in an ecological sere, which is a stage found in ecological succession in an ecosystem towards its climax community state.

5. **Mantle**: a hyphal sheath enveloping the plant root.

6. **Epigeous sporocarps**: fungal fruiting bodies appearing above ground, and thus visible to the naked eye, as opposed to **hypogeous sporocarps** which occur below ground and are thus invisible.

7. **Polymorphism**: the presence of genetic variation within a population, upon which natural selection can operate.

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Declaration 1: Originality

I, Joshua Tsamba, declare that the thesis, which I hereby submit for the degree of Master of Philosophy at the University of Zimbabwe, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. I further declare that all sources cited or quoted are indicated by means of a comprehensive list of references.

SIGNATURE:

DATE:

Declaration 2: Publication

Details that form part and/or include research presented in this thesis include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication.

Publication 1

Tsamba, J.[1], Kativu, S.[1]and Sithole-Niang, I.[2] (2015).Diversity and host associations of ectomycorrhizaefungi in fallow lands of the mid-Zambezi valley area**,**Zimbabwe**.***Transactions of the Royal Society of South Africa* 70 (1): 71-77.

This work was carried out by the first author under the guidance and supervision of the second and third author.

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CHAPTER 1: INTRODUCTION

Regimes and stages of anthropogenic disturbances have negative effects on soil physical, chemical and biological properties (Onguene and Kuyper, 2002). Agricultural practices that involve vegetation clearance, burning, fallowing, and mono-cropping all have the effect of reducing biodiversity at primary and detritivore levels (Asbjornsen and Montaguini, 1994; Egerton-Warburton and Allen, 2000; Helgason*et al*., 1998; Johnson, 1993; Thompson, 1987). Overall, this has the effect of delaying vegetation regeneration. At the detritivore level, these anthropogenic activities reduce or even eliminate fungal communities. Among fungal communities are symbiotic mycorrhizae which form mutualistic plant–fungus associations that play a major role in maintenance and sustenance of biodiversity and ecosystem functioning (Smith and Read, 2008). These fungal associations play a vital role in the biology and ecology of vegetation communities, affecting tree growth, water and nutrient absorption and protection against pathogens. Mycorrhizae are the most widespread symbionts among forest and cultivated ecosystems (Brundrett, 2009). An estimated 80% of terrestrial plant species are mycorrhizal (Wang and Qiu, 2006). These mycorrhizae are classified into two major groups: endomycorrhizae and ectomycorrhizal fungi (ECMs). Ectomycorrhizal fungi form symbiotic relationships with many trees and shrubs that dominate boreal forests, temperate forests and tropical and subtropical forests and woodlands (Bâ*et al.*, 2011).

ECMs consist of the following: a sheath or mantle enclosing the root, a labyrinth hyphal network between root epidermal and cortical cells (called the Hartig net), an extrametrical mycelium sometimes aggregated in linear organs called rhizomorphs that form essential connections with the soil, and sometimes fruiting bodies. Approximately 5000-6000 species of Basidiomycota and Ascomycota form ectomycorrhizal associations with tree or shrub species (Buscot *et al*., 2000). This relationship is usually considered to be obligatory (Smith and Read, 1997). The fungus benefits by receiving photosynthetically derived carbon compounds, while the plant has an increased uptake of mineral nutrients facilitated by the fungus.

1.1. Ectomycorrhizal fungi and community succession

ECMs express minute differences in community composition, differences that would otherwise be difficult to detect when assessed at the primary trophic level of vascular plants (Paoletti, 1999). They, therefore, indicate changes at much finer and earlier stages of community succession. It thus becomes important to observe changes that occur below ground in order to understand above ground reflections. In essence, ECMs can therefore be used as bio-indicators of disturbance (Paoletti, 1999). Such bio-indicators are species which are sensitive to slight changes in a predictive manner that allow for the detection and measurement of impacts of various anthropogenic pressures on ecosystems (Paoletti, 1999; Bouyer *et al*., 2007).

Many tropical soils are considered nutrient-poor owing to strong acidity, high clay content, high exchangeable aluminium and low available phosphorous (Sanchez and Salinas, 1981). In view of the low nutrient availability, it is not surprising that few tropical woody species are non-mycorrhizal (Alexander, 1989; Janos, 1980). Many of the symbiotic fungi produce powerful toxic alkaloids that confer some protection from herbivores (Clay, 1990) and, perhaps even more important, deter seed predators. Thus the early death of some *Brachystegia* and *Julbernardia*

seedlings in Miombo woodlands may be attributed to seedlings' failure to establish an association with mycorrhizae by the time they shed their cotyledons (Frost, 1996).

1.2. Ectomycorrhizal fungi and fallowing

Cultivation has a direct impact on woody species community structure and diversity. This results from vegetation clearance and subsequent abandonment of cleared land, and selective harvesting of trees for various purposes. Thus, land clearance for crop cultivation affects biodiversity directly through habitat conversion and indirectly through fragmentation and alteration of energy flows and biotic composition and structure (Giller*et al*., 1997). The direct impact of vegetation clearance and subsequent abandonment of cleared land on fungal communities is not fully understood, particularly in semi-arid tropical environments.

1.3. Primary objective

The study sought to understand diversity patterns and host associations of ectomycorrhizal fungi across an agricultural disturbance gradient in the mid-Zambezi area of Zimbabwe.

1.3.1 Specific objectives

Specific objectives of the study were to establish:

- the ectomycorrhizal flora of fallow lands in the mid-Zambezi area;
- whether ectomycorrhizae are host specific or generalists;
- whether ectomycorrhizal fungi diversity varies with fallow age;
- whether there are any shifts in ectomycorrhizal fungal hosts across fallow age;
- whether dominant ectomycorrhizae species vary genetically across fallows and across hosts.

1.3.2. Research Questions

The study attempted to answer the following questions:

- Which ectomycorrhizal species are associated with fallow lands of the mid-Zambezi area?
- Are the ectomycorrhizal fungi host specific?
- Does fallow age determine ectomycorrhizal diversity?
- Is there any infra-specific fungal genetic variation across hosts or fallow ages?

1.3.3. Justification for the study

Human population density rose significantly following tsetse fly eradication in parts of the Mid-Zambezi area of Zimbabwe (Chizarura, 2003). This was associated with an expansion of the agricultural landscape through conversion of natural woodland to farmland (Murwira*et al*., 2010). In areas where human population density is relatively low, shifting cultivation is practiced(Tambara *et al*., 2012a). This creates a gradient of disturbance from densely populated areas - where land pressure does not permit shifting cultivation - to less densely populated areas where fallowing is practised, with fallow age determined by regularity of clearance. Hence, fallowing facilitates regeneration of woody plant species(Tambara *et al*., 2012a). In this way, cultivation creates a mosaic of land units, characterised by vegetation communities at different levels of succession. Some woody species disappear, new ones emerge, yet others persist and become dominant up to climax community stage(Tambara *et al*., 2012a). Similarly, fungal species composition is expected to change with age of fallowing in response to host species composition. Diversity changes and host shifts among fungal communities have never been studied across a human disturbance gradient under semi-arid conditions like those experienced in the Mid-Zambezi valley. The present study sought to examine biodiversity and successional changes at the detritivore (decomposition) trophic level across a human disturbance gradient. It

is expected that increased woody species will be associated with increased ectomycorrhizal fungal diversity(Bâ *et al*., 2011).

1.4. Thesis outline

This thesis consists of eight chapters. Chapter 1 is the general introductionto the thesis. Chapter 2 provides an outline of literature related to the subject area within the region and particularly in Zimbabwe. In Chapter 3 the study area is defined, the materials and methods are described and the sampling protocol for the study outlined. In Chapter 4 the study objectives are addressed.While in Chapter 5 findings from Chapter 4 are discussed. Chapter 6is a synthesis of the whole study. Specifics on Chapters 4 are as follows:

Section 4.1: This sectionprovides a record of ectomycorrhizal fungi speciesin the area across a fallow chrono-sequence of 1 to 14 years.

Section 4.2:In this section fungal species richness in fallows of all ages are compared,as a way of determining changes associated with succession.

Section 4.3: In this section,thegenetic characteristics of the identified dominant ectomycorrhizal fungi of the area across fallows and host species are established.

Section 4.4:This part establisheswhether the age of a fallow or host specificity determines the mycorrhizal status of any mycorrhizal tree species.

Chapter 5: In this chapter, with sections 5.1 to 5.4, findings from sections 4.1 to 4.4 in Chapter 4 outlined above are discussed.

Chapter 6:In this chapter, the findings of the thesis are synthesised, and the implications of these with regards to agricultural activities and biodiversity conservation in the mid-Zambezi valley are discussed.

CHAPTER 2. LITERATURE REVIEW

2.1. Diversity of ectomycorrhizal trees and ectomycorrhizal fungi in Africa

Most recorded ectomycorrhizal tree species are located in temperate and boreal zones (Smith and Read, 2008). However, in tropical Africa, ectomycorrhizal tree species mainly occur in open gallery and rainforests of the Guineo-Congolian basin, the Zambezian Miombo woodlands of East and South-Central Africa, and theSudanian savanna woodlands of sub-Saharan Africa(Figure2.1) (Newbery*et al*., 1988; Thoen and Bâ, 1989; Sanon *et al.*, 1997; Onguene and Kuyper, 2002; Ducousso *et al.*, 2004; Rivière *et al*., 2007; Ducousso *et al*., 2008). ECMs are found mainly on Caesalpinioid legumes (Amherstieae, Detarieae, Sarcolaenaceae, Dipterocarpaceae, Asteropeiaceae, Phyllantaceae, Sapotaceae, Papilionoideae, Gnetaceae and Proteaceae (Bâ *et al*., 2011). Bâ *et al*. (2011) further established that among the tropical African tree species recorded as being ectomycorrhizal,only 26% have been confirmed asharbouring ectomycorrhizal fungi. Also, within the tropical African forests, tree species are generally associated with arbuscular mycorrhizae, and whenever ectomycorrhizaeoccur, they usually co-occur with arbuscular mycorrhizae (Moyersoen*et al*., 1998a, b). Studies on Africanflora confirm the scarcity of ectomycorrhizal fungi despite the abundance of mycorrhizal tree species. In Senegal, Thoen and Bâ (1989) reported the presence of only two ectomycorrhizal native tree species. Redhead (1968a, b) noted that of the 51 plant species recorded in Nigeria, only three were associated with ectomycorrhizae, the rest being associated with arbuscular mycorrhizae. Rambelli (1973) also recorded few ectomycorrhizal species in Ivory Coast, while in Tanzania, Högberg and Nylund (1981) noted that of the 47 native species recorded, 40 were associated

with arbuscular mycorrhizae, six with ectomycorrhizal fungi and one with dual mycorrhizal associations. In Cameroon, Newbery *et al*., (1988)reported that of the 55 plant species studied, 32 were associated with arbuscular mycorrhizae and 23 were with ectomycorrhizae. Onguene and Kuyper (2002) also confirmed this trend in Cameroon. A low number of ectomycorrhizal tree species was also observed in open and gallery forests of Burkina Faso (Sanon *et al*., 1997) and in rainforests of Guinea (Thoen and Ducousso 1989; Rivière *et al*., 2007; Diédhiou *et al*., 2010). Although not as extensive as mostly arbuscular mycorrhizal forests, some tropical forests can be dominated by ectomycorrhizal tree species. These include the open miombo woodland communities dominated by *Brachystegia*, *Isoberlinia* and *Julbernardia* in East Africa (Högberg and Nylund, 1981) and the rainforest communities of *Gilbertiodendron dewevrei* in the Congo basin (Tortie*t al*., 2001).

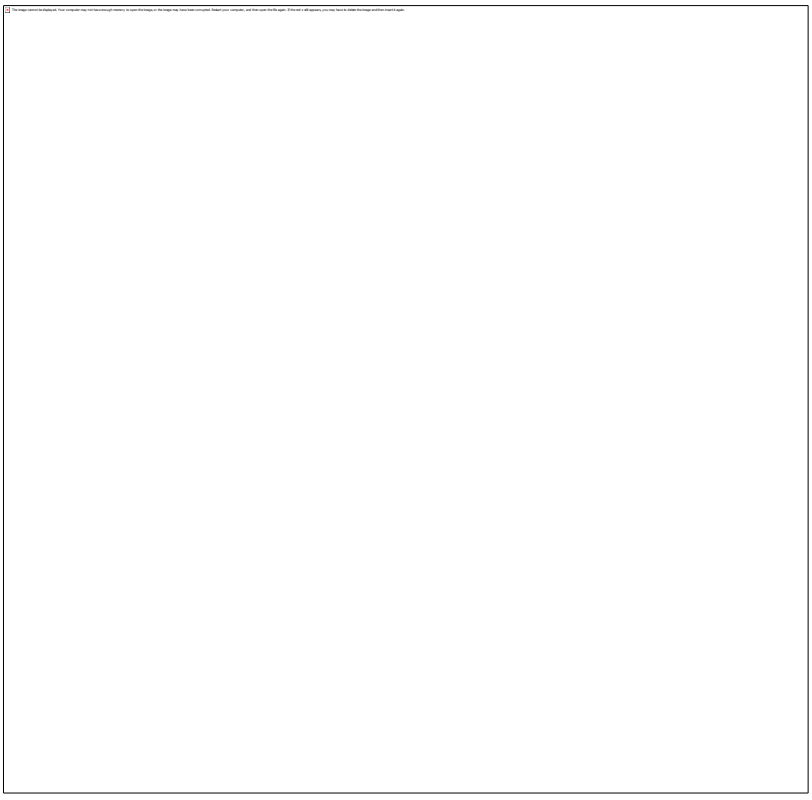


Figure2.1: Distribution of ectomycorrhizal trees in tropical Africa (from Bâ *et al*.,2011): (*1)* Rainforests in the Guinea-Congo region, (*2)*Open forests in the Sudano-Zambezian region, (*3)*

Savanna woodlands in the Sudano-Zambezian region*(Yellow)*Sahara desert in the North and Kalahari/Namib deserts in the South.

Ectomycorrhizal fungi display a large range of morphological characteristics. Thoen and Bâ (1989) observed that when based on colour of mantle, the yellow ectomycorrhizal fungi commonly associated with *Uapacaguineensis* belonged to genus *Austrogautiera*. Beige ectomycorrhizal fungi were usually *Lactarius gymnocarpus*, while pink ectomycorrhizal fungi were typically *Amanita rubescens*. Thoen and Bâ (1989) further noted that brown bristly ectomycorrhizal fungi were usually of*Coltricia cinnamomea* and whitish ectomycorrhizal fungi with a sclerodermic smell were of *Scleroderma verrucosum*.

African tropical forests are still under-sampled relative to temperate forests(Bâ *et al*., 2011). Thus, further surveys are neededto confirm the situation in Africa. This is in view of the conclusions made by Tedersoo and Nara (2010) that tropical forests have fewer ectomycorrhizal fungi than temperate forests. Studies in West Africa (Thoen and Bâ 1989; Thoen and Ducousso 1989; Rivière *et al*.,2007) showed that the number of harvested ectomycorrhizal fungi was remarkably high, with typical genera like *Russula*, *Lactarius*, *Amanita*, *Boletus*, *Cantharellus* and *Scleroderma* forming ectomycorrhizal associations just like in temperate and other tropical regionsof the world (Trappe,1962; Watling and Lee,1995; Yokota *et al*.,1996; Sirikantaramas*et al*.,2003; Tedersoo *et al*.,2007, 2011; Peay*et al*.,2010).

Ectomycorrhizal fungi fruiting regimes (sporocarp production) are largely unknown. In West Africa, some fungal species fruit after the first significant rainfall, others in the middle or at the end of the rainy season (Thoen and Bâ,1989). For example, work by Thoen and Bâ (1989) showed that *Scleroderma* sp. fruited during the rainy season, while *Coltricia cinnamomea* fruited

only at the end of the rainy season (Thoen and Bâ,1989; Sanon *et al*., 1997). Sporocarp production could therefore depend on several factors, including accumulated rainfall, diversity of host trees, forest types, stand age and overall climatic conditions among many other factors (Fleming, 1985; Lilleskov and Bruns, 2003). Fungal species diversity and composition also depends on several factors. The abundance of species collected from different sites inWest Africa was linked to forest types (whether rainforests or open forests), number of host tree species and climatic characteristics (total rainfall,rainfall distribution and duration of the rainy season) (Thoen and Bâ, 1989; Thoen and Ducousso, 1989; Sanon *et al*., 1997; Rivière*et al*., 2007). Generally, rainforests have higher fungal diversity than open forests and gallery forests. Thelephoroid fungi seem to invest more in vegetative growth than in sexual reproduction (Redecker, *et al*., 2001). In contrast, Amanitaceae, one of the most represented families in sampled sporocarps across parts of Africa, is usually almost absent on roots of host plants. Species of this family appear to invest much more in sexual reproduction than in vegetative reproduction (Redecker, *et al*., 2001). This kind of association, however, ceases to produce the expected mutual benefits between the host and the fungi. Hence,it is of no apparent advantage.

In terms of host ranges, ectomycorrhizal fungi display different putative host ranges. For example,*Russulaannulata* has a broad host range, while *Xerocomushypoxanthus*was only reported on *Uapacaguineensis*(Bâ *et al*., 2011). Several ectomycorrhizal fungi have a large distribution in tropical Africa (Bâ *et al*., 2011). For example, *Scleroderma dictyosporum* and *Scleroderma verrucosum* were recorded from all phytogeographical regions and forest types across Africa, regardless of the level of rainfall (Bâ *et al*., 2011).

2.2. Diversity and abundance of ectomycorrhizal fungi associations under forest disturbance

Anthropogenic disturbance and extent, together with disturbance regimes, have serious impacts on soil properties. Studies in West and Central Africa showed that forest disturbance that results from commercial logging may reduce or completely eliminate ectomycorrhizal fungi (Onguene and Kuyper, 2002; Asbjornsen and Montaguini, 1994; Egerton-Warburton and Allen, 2000; Helgason*et al*., 1998; Johnson, 1993; Thompson, 1987). Tree recruitment is consequently affected by the dwindling numbers of indigenous mycorrhizal fungi. Seedling survival and establishment can be seriously affected, thus having cascading implications on the structure of the forest ecosystem. Studies byJimenez-Esquilin *et al.* (2007)also demonstrated the drastic effects of land clearance and burning for crop cultivation, fallowing several years after the cropping period and permanent plantations in mono-cropping on the quantity and quality of indigenous mycorrhizal populations. Personal observations led to the conclusion that the practice of slash and burn also has an impact on the soil 'spore bank' just as it affects seed banks. Therefore, evolutionarily adapted spores or vegetative propagules are able to thrive after disturbance (Jimenez-Esquilin *et al*., 2007).

2.3. Ectomycorrhizal fungi in the mid-Zambezi Valley

Information on ectomycorrhizal fungi of the mid-Zambezi Valley is deficient. Studies carried out so far focused on improving agricultural yield (Baudron *et al*., 2008), effects of farming activities on plant and arthropod diversity (Tambara *et al*., 2012a and b), impact of tsetse eradication on human population trends (Chizarura, 2003), human-wildlife conflict in the mid-Zambezi area (Biodiversity Project, 2002) and work targeted on emblem animal and bird species (Biodiversity Project, 2002).

Studies focused on Southern Africa to date have targeted mostly South African and Zambian woodlands. These studies particularly examined conditions in miombo woodlands which are known to harbour several ectomycorrhizal fungi genera. Indications are that ectomycorrhizal fungi diversity is lower in tropical areas than in temperate regions (Tedersoo and Nara, 2010). As already noted, however, tropical Africa is still largely under-sampled (Bâ*et al*., 2011). Most of the ectomycorrhizal fungi recorded in southern Africawere identified from fruiting bodies. This does not provide a complete picture of their diversity, considering that the fruiting of fungi is controlled by several physical and chemical factors. In addition, a number of ectomycorrhizal fungi produce inconspicuous (hypogeous) fruiting bodies or reproduce vegetatively, having no fruiting bodies at all. Work done by Bâ*et al.* (2011) recordeda single ectomycorrhizal fungi species, *Scleroderma verrucosum*,in Zimbabwe. The same species also occurs in South Africa. Results from this study did not provide a complete review of ectomycorrhizae in Zimbabwe, considering also the different climatic conditions, soil types and vegetation characteristics of different localities in the country, let alone such other factors as agricultural practicesthat tend to vary spatially. These diverse conditions are likely to accommodate unique ECM fungi. Bâ*et al.* (2011) also listed *Coltricia cinnamomea* and *Gyroporusmicrosporus* as additional southern African ectomycorrhizal species to those already recorded in South Africa and Zambia.

Ramachela and Theron (2010) reported that ectomycorrhizal fungi protected roots of *Uapacakirkiana* seedlings against root pathogens. This study was, however, limited as it didnot specifically identify the ectomycorrhizal species or establish their diversity. Frost (1996)noted that the early death of some *Brachystegia* and *Julbernardia* seedlings in miombo woodlands was linked to seedlings' failure to establish associations with mycorrhizae by the time they shed their

cotyledons. This is an important aspect in tree recruitment, especially after human disturbance. This supposition needs further investigation in respect of *Brachystegia* and *Julbernardia* seedlings. Certain tree species dominate tropical forests and woodlands. Questionsarise as to what determines this dominance. This question still remains largely unanswered. Högberg and Nylund (1981) suggested that the widespread occurrence of ectomycorrhizae in miombo species' roots could enable them to exploit porous, infertile soils more efficiently than groups that lacked ECMs. This could explain the success of miombo woodlands in drier savanna landscapes. Högberg (1986) further attributed ectomycorrhizae to direct uptake of phosphorous from organic matter in phosphorous–deficient soils. He recommended further work in an attempt to answer the following questions (Högberg, 1992):

1. What is the significance of ectomycorrhizae?
2. Why are miombo woodlands dominated by species with ecto- rather than endomycorrhizae?
3. What is the contribution of ECMs to the mineral nutrition of the host plants?
4. Why are the dominant Caesalpinioideae in miombo woodlands ectomycorrhizal, but those on equally nutrient- poor Kalahari sands endomycorrhizal?
5. What are the costs to the plants of supporting these mycorrhizae, and what are the concomitant benefits?

Most of these questions still remain unresolved.

Other work in Zimbabwe focused on macrofungi and arbuscular mycorrhizal fungi. Sharp (2011) did extensive work on macrofungi, attempting specifically to create an inventory of edible and non-edible mushrooms. Her work focused on miombo woodlands, and contributed greatly to knowledge on fungi in Zimbabwe. Other work focused on problem fungal species, especially

those of commercial plantations. Plantations in the Eastern Highlands, an area with climatic conditions completely different to the study area, are affected by root rot disease caused by *Armillaria* species. (Wingfield *et al*., 2009). Unlike ectomycorrhizal fungi, these fungal species are purely saprotrophicand often result in the death of the host trees. Other work focused on arbuscular mycorrhizae, with work by Lekberg *et al*. (2008) focusing on effects of agricultural management practices on arbuscular mycorrhizal fungi abundance on smallholder farms. Their findings concluded that phosphorus fertilisation, fallowing and tilling did not significantly decrease arbuscular mycorrhizal fungi abundance.

2.4. Genetic fingerprinting in ectomycorrhizal fungi

There is no universally accepted DNA barcode for fungi. This is a serious limitation to ecological studies (Schoch*et al*., 2012). A DNA barcode is a taxonomic method that uses a short genetic marker taken from a standardised portion of an organism's DNA to identify it as belonging to a particular species through reference to DNA sequences in a library or database (Hebert *et al*., 2003a). Several techniques are adopted in the identification of fungal species(Wingfield *et al*., 2009). These include DNA based molecular techniques (Smith and Anderson, 1989), isozyme and protein analysis (Morrison *et al*., 2003), immunological assays (Burdsall*et al*., 1990) and morphological characterisation (Watling *et al*., 1982; Agerer, 1987-2006). While morphological characterisation is easy, its major shortcome is the lack of fruiting bodies and rhizomorphs in most ectomycorrhizal fungi. Isozyme and protein analysis techniques and immunological assays provide reproducible, reliable results, but their major limitation is that they are time consuming (Wingfield *et al*., 2009).

DNA based methods, in combination with morphological identification, are still the most favoured techniques in identifying ectomycorrhizal fungi species and understanding intra - or

inter-specific variation within fungal communities (Wingfield *et al*., 2009; Schoch*et al*., 2012). Studies show that interspecific variation is often greater than intraspecific variation (Hebert *et al*., 2003a; Hebert *et al*., 2003b). Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) is a simple procedure by which specific genomic DNA fragments can be amplified with the aid of oligonucleotide primers of random sequences (Junghans*et al*., 1998). Such molecular markers are in use in taxonomy (Lanfranco*et al*., 1993) or genetic mapping (Doudrick*et al*., 1995) of ectomycorrhizal fungi. Specific knowledge of the DNA sequence for the targeted genome is not required, as the primer will bind at any site in the sequence (Kumar andGurusubramanian, 2011). The method is thus popular in comparing DNA in organisms that have not been fully genetically characterised.

A number of target regions are used for molecular identification, characterisation and overall DNA diagnostics of ectomycorrhizal fungi using PCR techniques. The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in fungal phylogenetics (Schoch*et al*., 2012) (Figure 2.2). The highly conserved regions of the gene allows it to be sequenced rapidly directly from rRNA or DNA amplified by PCR with universal primers (Bosquet*et al*., 1990; White *et al*., 1990). The internal transcribed spacer (ITS) is a region containing two non-coding regions nested within the rDNA repeat between the highly conserved small subunit 5.8S and the large subunit rRNA genes (Gardes and Bruns, 1993) (Figure 2.2). This region is used for molecular identification of fungi (Gardes and Bruns, 1993). The ITS region is also easy to amplify in small samples and has been successfully used in dilute or highly degraded samples (Gardes and Bruns, 1993; Lee and Taylor, 1992). However, current ITS primers were developed to amplify a broad range of organisms, including plants, fungi, animals and protists (White *et al*., 1990). Since in most natural situations, plant DNA is more abundant than fungal DNA, this ITS region is less

favourable (Gardes and Bruns, 1993). Work by Wingfield *et al*. (2009) on Zimbabwean *Armillaria* species used the intergenic spacer (IGS) 1 region for molecular characterisation and identification (Figure 2.2). ThisIGS-1 region is located between the 26S and 5S rRNA genes (Sugita *et al*., 2002) andis commonly used in DNA diagnostics on *Armillaria* species in British Columbia (White *et al*., 1990).

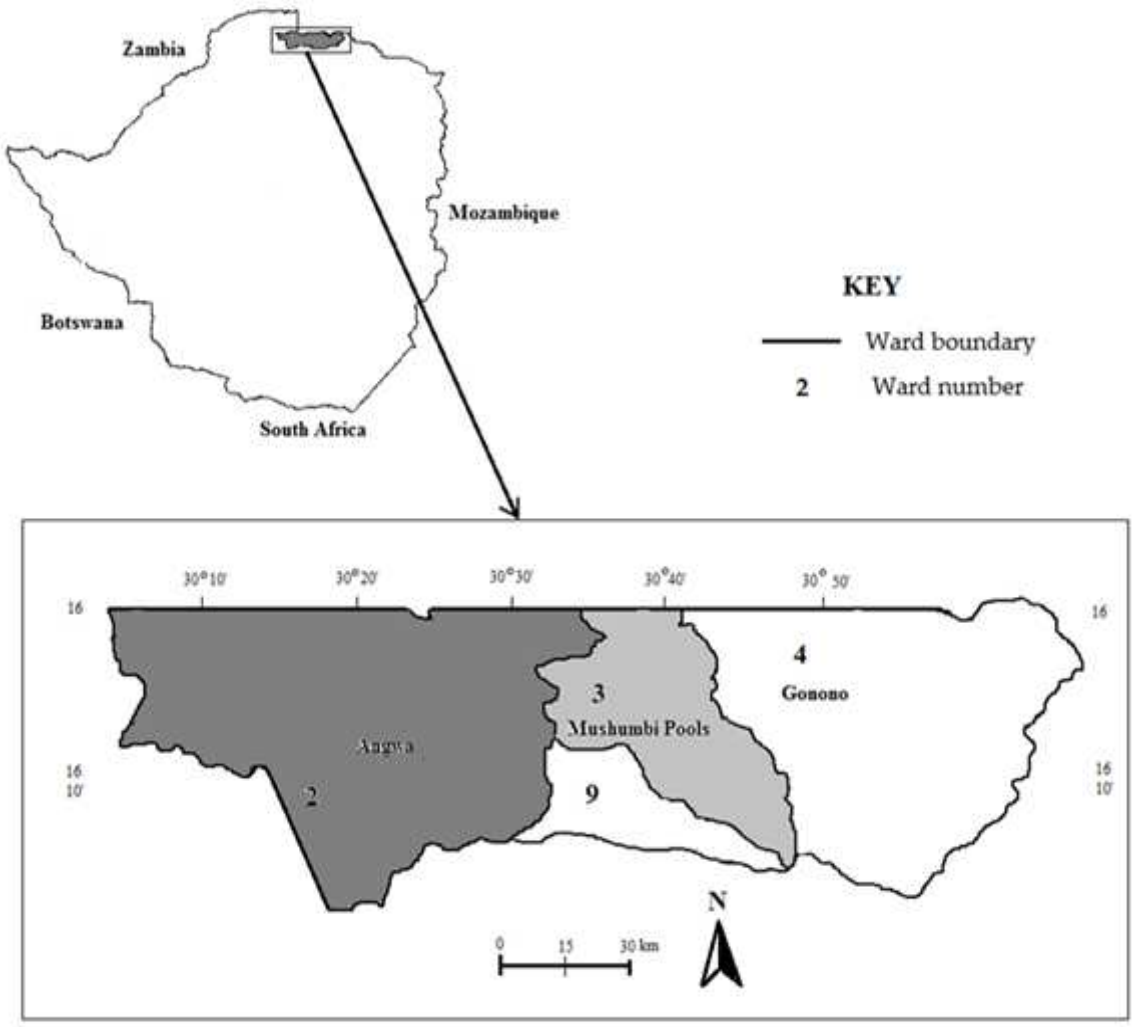
Figure 2.2: Organisation of fungal ribosomal RNA (rRNA) genes (from Liu, 2011).

DNA based molecular techniques which combine PCR with analysis of restrictionfragment length polymorphisms (RFLP) are relatively fast and reliable in ectomycorrhizal fungi identification (Gomes *et al*., 2002). The PCR-RFLP technique, which combines methods to detect polymorphisms in DNA regions amplified by specific oligonucleotide primers and restrictedwith different endonucleases, is successfully used in the analysis of regions of ribosomal DNA ofectomycorrhizal fungi (Gardes*et al*., 1991; Henrion*et al*., 1994; Bentley *et al*., 1995; Glen *et al*., 2001;Wingfield *et al*., 2009). The technique can be used in discriminating between closely related species within fungal communities (Gardes and Bruns, 1991).

CHAPTER 3: MATERIALS AND METHODS

3.1. Study area

The present studywas conducted in the Mushumbi and mainly Angwa communal areas of Mbire Rural District Council, mid-Zambezi Valley, Zimbabwe(Figure 3.1). The mid-Zambezi Valley extends between longitudes 30° and 31° and latitudes 15° 30 and 16° 20. It is 40 km wide and has an average altitude of 400 m. The area borders an escarpment (Mavuradonha Mountains) to the south, with a maximum height of 1400 m above sea level and stretches northwards to the



Zambezi River.

Figure 3.1: Study area covering Ward 2 and 3 of Mbire District, mid-Zambezi valley.

3.1.1. Climate and hydrology

Zimbabwe is divided into agro-ecological regions based on rainfall, temperature and soil capability (Vincent and Thomas, 1961). The mid-Zambezi area falls within a semi-arid region which is characterised by a dry tropical climate. It has a mean annual temperature of 25°C and minimum and maximum temperatures of 10°C and 40°C, respectively, with lowest temperatures occurring in the months of June-July and highest temperatures between October-November (Vincent and Thomas, 1961). The wet season (November to March) receives rainfall amounts that range between 350 mm and 650 mm, within a 36 day period (Vincent and Thomas, 1961). The long, dry season (April to October) completes the year. The hydrology of the area is complex, characterised by former floodplains of the Zambezi River, with two major rivers draining the area: Angwa River in Ward 2 and Manyame River in Ward 3 (Biodiversity Project, 2002).

3.1.2. Soils

Underlying geology of the study area consists mainly of Dande sandstone (Biodiversity Project, 2002). Soils are generally sandy, often locally shallow, rich in sodium, but lacking in organic matter (Mvuriye *et al.*, 2001). Agricultural activities are primarily carried out on two soil types, called *Mutapo*and *Bandate* in the local Shona language(Mvuriye *et al*., 2001). *Mutapo* soils are eutrophic and associated with sodic/saline areas. These are heavy soils, with high moisture holding capacity, and a depth reaching up to 3 metres (Mvuriye *et al*., 2001). *Bandate* soils are moderately heavy and make huge dust clouds when ploughed. They have high moisture retention capacity. Fertility rates in the latter are high, and the soils give good yields of cotton, maize, millet and sorghum (particularly during drier years) (Baudron *et al*., 2011). A detailed

description of soil characteristics of the two soil types was provided by Baudron *et al*. (2012) (Table 3.1).

Table 3.1: Soil characteristics of the two dominant soil types, *Mutapo*and *Bandate*(± standard error)Source: Baudron *et al*. (2012).

Soil type*	Depth (cm)	pH KCL	SOC (g/kg)	N (mg/kg)	P (cmol/kg)	K (cmol/kg)	Clay %	Silt %	Sand %
Bandate	0-10	6.2 ± 0.5	10 ± 3.2	0.8 ± 0.3	17.3 ± 13.4	0.68 ± 0.3	13.6 ± 4.0	18.2 ± 11.2	68.2 ± 14.6
	10-20	5.9 ± 0.6	8.7 ± 2.8	0.7 ± 0.2	11.7 ± 11.8	0.54 ± 0.3	15.5 ± 5.3	18.0 ± 10.8	66.4 ± 15.3
Mutapo	0-10	6.1 ± 0.5	7.7 ± 3.0	0.6 ± 0.2	11.8 ± 8.2	0.80 ± 0.3	14.9 ± 3.0	17.5 ± 5.5	67.7 ± 7.5
	10-20	5.9 ± 0.5	7.4 ± 2.6	0.6 ± 0.2	8.26 ± 5.7	0.69 ± 0.3	16.5 ± 3.9	17.9 ± 5.6	65.6 ± 8.5

*Local names

3.1.3. Flora and fauna

The mid-Zambezi area hosts some 700 plant taxa(Timberlake*et al*., 1993; Biodiversity Project, 2002). The natural vegetation is deciduous, dry savanna, and is dominated by *Colophospermum mopane,* with associations of *Acacia nilotica*, *Adansonia digitata*, *Combretum eleagnoides, Diospyros kirkii*, *Kirkia acuminata*, *Sclerocarya birrea*, *Terminalia brachystemma*, *T. sericea*, *T.stuhlmanni* and *Ziziphus mucronata* (Timberlake *et al*., 1993; Fritz *et al*., 2003; Gaidet *et al*., 2003). The area still hosts an important diversity of mammals, several of which are emblems of big African game (Fritz*et al*., 2003; CIRAD, 2004), with more than 40 species of large mammals and 200 bird species.

3.2. Sampling

Sampling was carried out along a 40 km transect belt cutting across the mid-Zambezi Valley. Sampling sites were randomly selected from fallows of different age groups (1-14 years), with two fallows in each age group. In total, 10 pairs of fallow fields were sampled. A single plot measuring 20m x 50m was randomly demarcated at about the centre of each selected fallow and sampled for the tree species. Tree species were identified *in situ*. Specimens were collected from species that could not be identified andthese were later identified at the National Herbarium in Harare. Tree stumps were ignored. Four soil cores were randomly collected from the vicinity of each tree species using a cylindrical steel corer of 4.5 cm diameter down to a depth of 20 cm. Samples from the same tree species were bulked into a composite sample and encased in a polyvinyl chloride (PVC) casing. Rootlets were also collected from each tree species, taking precaution to trace the fine roots to the plant. Precaution was also taken not to mix roots from different plants. Seedlings were uprooted completely, taking special care to lift the young plant with its surrounding soil. Rootlets were later carefully lifted from soil cores using forceps, placed in petri dishes and carefully washed with tap water while held by the forceps. Special care was taken not to disturb the ectomycorrhizal colonization. In order to maximise sampling efficiency, sampling was carried out twice in the same plots, during the months of January – February 2013 and 2014. This coincides with the peak growing season.

3.2.1. Sampling of mycorrhizae and sporocarps

Sporocarps of epigeous ectomycorrhizal fungi were collected within the vicinity of woody plants. Each fungal species was identified from microscopic examination of dried specimens and fresh fruit bodies based on photographs and descriptions provided in Onguene and Kuyper

(2002).Collection of sporocarps and soil samples was conducted during the same period as collection of rootlets.

3.2.2. Morphological classification of ectomycorrhizal fungi

Colonisation by ectomycorrhizal fungi was confirmed on each rootlet by closely viewing under a hand lens. Ectomycorrhizal colonisation was further confirmed by microscopic examination at x40 of whole mounts of root tips to determine the presence of a mantle and a Hartig net. Whole mounts of root tips were examined according to the following: presence of Woronin bodies or clamp connections at the septa (Agerer, 1987-2006)to determine ascomycete or basidiomycete affinity; mantle colour; organisation of the mantle hyphae and categorisation of the mantle according to tissue types (Agerer, 1987-2006); development of extraradicalhyphae; presence or absence of mycelial strands or rhizomorphs; presence of cystidia; pigmentation and ornamentation; and presence of crystals or exudates on hyphae and mantle(Agerer, 1987-2006).

3.3. DNA extraction

About 50 - 100 mg of mycelia from infested rootlets was carefully collected using a sterile pair of forceps and identified microscopically. DNA from mycelia was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA samples were stored at –20°C until needed.Extracted DNA was used for genetic characterisation of the fungal samples using the Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) and Restriction Fragment Length Polymorphism analysis of PCR-Amplified Fragments (PCR-RFLP) techniques.

3.3.1. Genetic fingerprinting

The fungal DNA obtained was used in two PCR experiments. The first experiment was intended for the detection of polymorphismsin the ectomycorrhizal fungi using the RAPD-PCR technique. A reaction volume of 25 µl was used for the RAPD-PCR using primer OPA 2 (5'-TGCCGAGCTG-3') according to Cenis (1992). The reaction volumes of 25 µl contained:1 µl of fungal genomic DNA, 1 µl of primer OPA 2, 2 µl of 25 mM dNTPs, 10 mM Tris-Cl (pH of 8.3), 50 mMKCl, 1.5 µl of 25 mM $MgCl_2$, 1µl of *Taq* DNA polymerase and the volume adjusted with water. The PCR profile was 94°C initial melt for 1 min, followed by 94°C for 1 minute, 50°C annealing for 1 min, 68°C extension for 2 minutes for 35 cycles, followed by 72°C final extension for 5 minutes and storage at 4°C. The amplification products were separated by electrophoresis in a 1.5% agarose gel immersed in Tris acetate-EDTA (40mM Tris, 20mM acetate, and 1mM EDTA, pH 8.0) buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera.

The second experiment was intended for the amplification of the 18S rRNA gene and detection of RFLPs. In this experiment the reaction buffer and cycling conditions used were as aforementioned. However, fungal generic primers were used:forward primer 5'-ACCCGCTGAACTTAAGC-3' and reverse primer 5'-TACTACCACCAAGATCT-3' according to Cenis (1992). The PCR amplicon was then digested with four restriction enzymes which were *Rsa*I, *Hinf*I, *Alu*I and *Hae*III. The products of the restriction digest with the restriction enzymes*Rsa*I, *Hinf*I and *Alu*Iwere analysed by gel electrophoresis on 1.5% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gels were visualised using a UV transilluminator and photographed using a digital camera. Digestions of the amplicons using restriction enzymes *Hinf*I, *Alu*I and *Hae*III were analysed by gel electrophoresis

on 5% polyacrylamide gel in Tris-borate-EDTA buffer and stained with ethidium bromide (0.1µg/ml). The gels were visualised using a UV transilluminator and photographed using a digital camera.

3.4. Genetic similarity assessment

The RAPD and RFLP fingerprint patterns were scored for presence (+) or absence (-) of bands and the number of bands present for each fungal sample. The proportion of bands that were shared between any two screened isolates was averaged over the total number of bands and used as the measure of similarity. The data were analysed using Nei and Li coefficients (Nei and Li, 1979) using the following formula:

Similarity coefficient of Nei and Li = 2a/ (b + c); where, a = number of similar bands in both isolates, b = total number of bands in the first isolate and c = total number of bands in the second isolate.

The similarity values for each primer were used for construction of a binary matrix for the ten ectomycorrhizal fungi isolates. The average of similarity values between each two isolates was calculated and included in a single similarity matrix. This matrix was also used for construction of a dendrogram according to UPGMA method (Michener and Sokal, 1957) using the online dendrograms construction utility, DendroUPGMA (http://genomes.urv.cat/UPGMA) (Garcia-Vallvé*et al*., 1999).

3.5. Data analysis

Since the data were non-parametric, a Spearman Rank correlation test at p=0.05 was used to test the relation between fallow age and mycorrhizal status of the host species. A generalised linear

model (GLM) with binomial response and link logit was used to test the relationship between mycorrhizal status, fallow age and host tree species. This was to determine whether either specific host species or fallow age explained the associations observed in the area. A Raup - Crick similarity plot was used to establish linkages in fungal symbioses between any pair of host species and the fallow age, since the data were recorded in presence/absence format.

CHAPTER 4. RESULTS

4.1.Establishing the ectomycorrhizal fungiassociated with fallowing

Plant communities within the fallow lands mainly included some thirteen mycorrhizal tree species from six families (Table 4.1). The six tree families were Combretaceae (*Combretum eleagnoides, C. mossambicense*), Ebenaceae (*Diospyros quiloensis*), Fabaceae (*Acacia tortilis, A. nigrescens, Afzelia quanzensis, Colophospermum mopane, Dichrostachys cinerea, Faidherbia albidaand Philenopteraviolacea*), Simaroubaceae (*Kirkia acuminata*), Rhamnaceae (*Ziziphus mauritiana*) and Tiliaceae (*Grewia monticola*).Ectomycorrhizal associates were sampled from the thirteen tree species.

Table 4.1: List of tree species in the fallow lands examined for presence of ectomycorrhizal fungi.

Species	**Local use**
Acacia nigrescens (Fabaceae)	Fodder, fence construction.
Acacia tortilis (Fabaceae)	Fodder, fence construction.
Afzelia quanzensis (Fabaceae)	Carvings, furniture, building.
Colophospermum mopane (Fabaceae)	Traditional medicine, firewood.
Combretum eleagnoides (Combretaceae)	Traditional medicine, firewood.
Combretum mossambicense (Combretaceae)	Traditional medicine, firewood.
Dichrostachys cinerea (Fabaceae)	Fodder, fencing.
Diospyros quiloensis (Ebenaceae)	Furniture, edible fruits.
Faidherbia albida (Fabaceae)	Fence construction, fodder.
Grewia monticola (Tiliaceae)	Edible fruits.
Kirkia acuminata (Simaroubaceae)	Domestic utensils.
Philenopteraviolacea(Fabaceae)	Fodder, tools, carvings.
Ziziphus mauritiana(Rhamnaceae)	Edible fruits.

Ten ectomycorrhizal species were isolated from the fallow lands (Plates 1 – 10). Five of them were readily identified from literature sources to species level using Agerer (1987-2002), Ingleby *et al*. (1990) and Onguene and Kuyper (2002). The identity of the other five could not be established and were thus designated A-E.

The illustration below (Figure 4.1) shows the structure of ectomycorrhizal fungi as they appeared on two plant (host) rootlets.

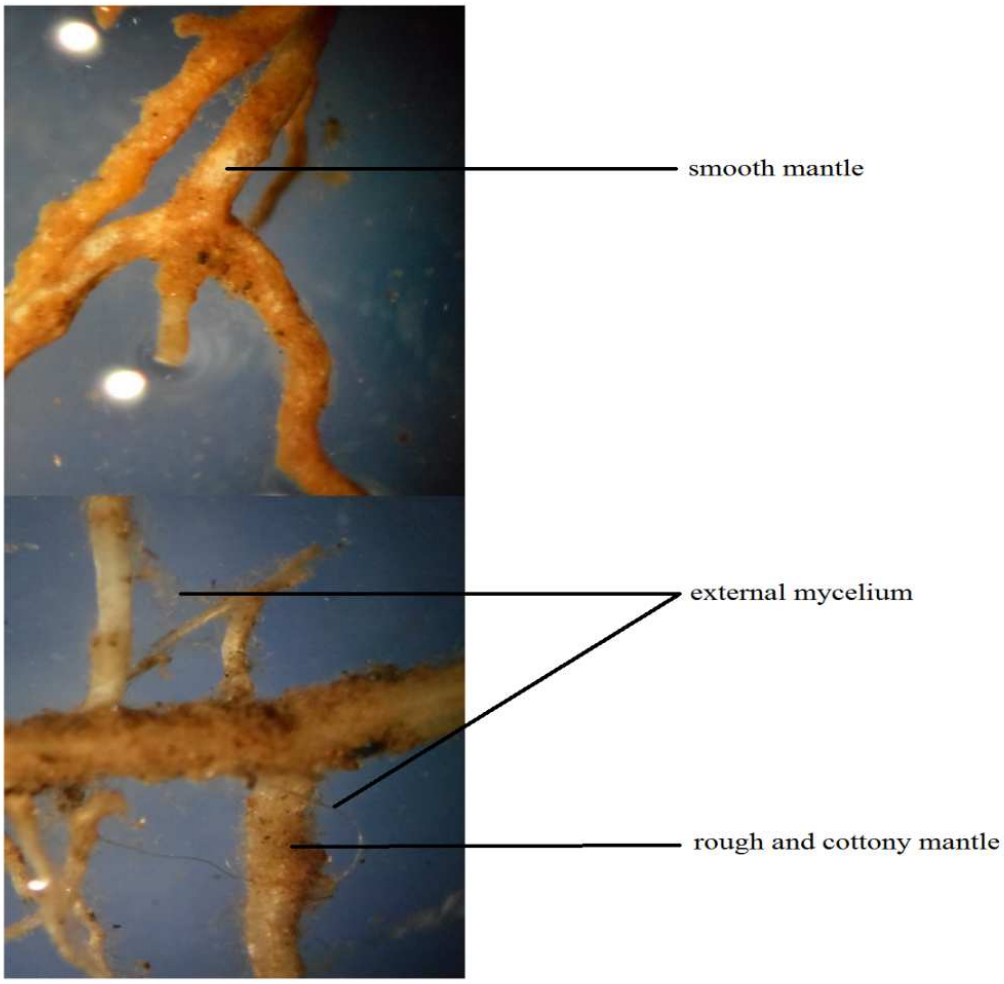


Figure 4.1: Two ectomycorrhizal species, *Thelephora terrestris* (above) on *Acacia tortilis* roots and *Lactarius gymnocarpus* (below) on *Kirkia acuminata* roots, showing different morphological characteristics used in identification.

Plate 1: *Lactarius gymnocarpus* ectomycorrhizal fungi colonising *Kirkia acuminata* rootlets sampled from a 10 year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: hairy, stringy and slightly beaded.
- Mantle colour – beige.

Morphology of unramified ends

- Shape - slightly bent.
- Colour – beige.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle dots presence – absent.
- Emanating hyphae – present and infrequent.

Morphology of rhizomorphs

- Colour – concolourous to mantle.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – pseudoparenchymatous.
- Mantle type – hyphae rather irregularly arranged and of no special pattern discernible (Type B).
- Septa clamps presence – absent.

Plate 2: *Boletus* sp. ectomycorrhizal fungi colonising *Philenopteraviolacea* rootlets sampled from a five year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: infrequent and ensheathed.
- Mantle colour – yellowish brown/brown and shiny.

Morphology of unramified ends

- Shape - sinuous.
- Colour – yellowish brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle dots presence – absent.
- Emanating hyphae – present and infrequent.

Morphology of rhizomorphs

- Colour – concolourous to mantle.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – plectenchymatous.
- Mantle type – ring-like arrangement of hyphal bundles (Type A) and sometimes hyphae rather irregularly arranged and of no special pattern discernible (Type B).
- Septa clamps presence – absent.

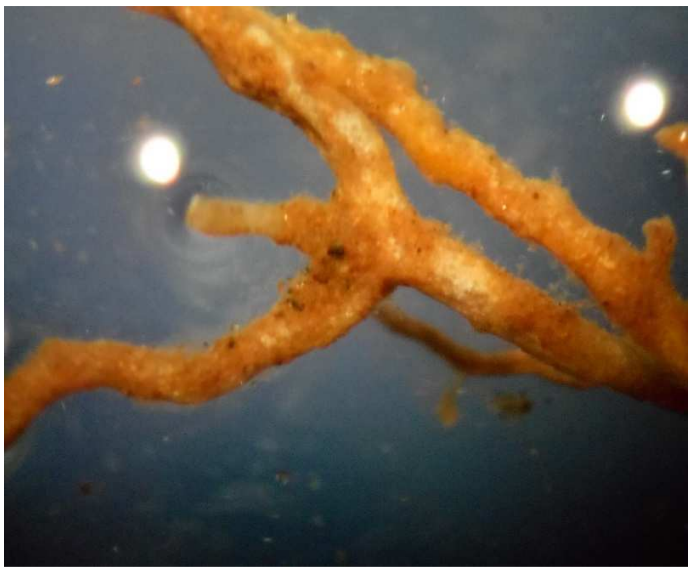


Plate 3: *Thelephora terrestris* ectomycorrhizal fungi colonising *Acacia tortilis* rootlets sampled from a five year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: lightly cottony.
- Mantle colour – rusty brown.

Morphology of unramified ends

- Shape - sinuous.
- Colour – brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle dots presence – absent.
- Mantle surface habit - shiny
- Emanating hyphae – present and infrequent.

Morphology of rhizomorphs

- Colour – concolourous to mantle.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – plectenchymatous.
- Mantle type – hyphae arranged net-like (Type D).
- Septa clamps presence – present and abundant.

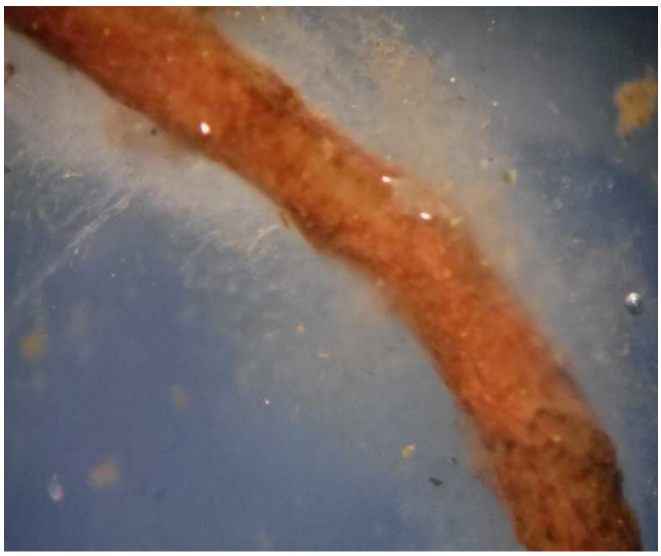


Plate 4: *Lactarius* sp. ectomycorrhizal fungi colonising *Acacia tortilis* rootlets sampled from a seven year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: frequent and cottony.
- Mantle colour – brown.

Morphology of unramified ends

- Shape – slightly bent and straight.
- Colour – brown with whitish tips.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle dots presence – absent.
- Emanating hyphae – present and frequent.

Morphology of rhizomorphs

- Colour – whitish brown.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – pseudoparenchymatous.
- Mantle type – epidermoid cells bearing a hyphal net (Type Q).
- Septa clamps presence – absent.

Plate 5: *Amphinema byssoides* ectomycorrhizal fungi colonising *Ziziphus mauritiana* rootlets sampled from a ten year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: abundant and cottony.
- Mantle colour – brown.

Morphology of unramified ends

- Shape – bent.
- Colour – yellowish brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle dots presence – absent.
- Emanating hyphae – present and abundant.

Morphology of rhizomorphs

- Colour – whitish.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – plectenchymatous.
- Mantle type – hyphae rather irregularly arranged and no special pattern discernible (Type B).
- Septa clamps presence – present and abundant.

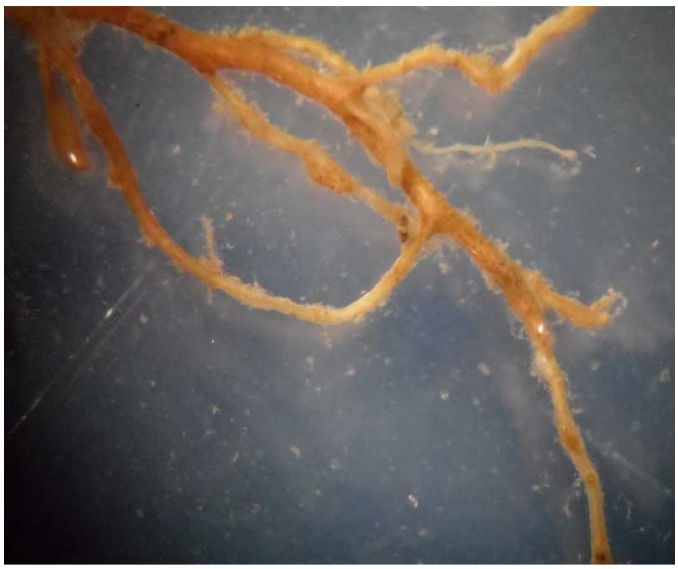


Plate 6: Species A ectomycorrhizal fungi colonising *Acacia nigrescens* rootlets sampled from a one year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: infrequent and stringy.
- Mantle colour – cream.

Morphology of unramified ends

- Shape – bent.
- Colour – ochre/yellowish.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle surface habit – rough.
- Mantle dots presence – absent.
- Emanating hyphae – present and infrequent.

Morphology of rhizomorphs

- Colour – ochre/yellowish brown.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – plectenchymatous.
- Mantle type – hyphae rather irregularly arranged and no special pattern discernible (Type B).
- Septa clamps presence – present and infrequent.

Plate 7: Species B ectomycorrhizal fungi colonising *Acacia tortilis* rootlets sampled from a nine year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – present: abundant.
- Mantle colour – whitish.

Morphology of unramified ends

- Shape – bent with tapering tip.
- Colour – yellowish white.
- Mantle surface visibility- indistinct.
- Mantle transparency - not transparent.
- Mantle surface habit – loosely woolly.
- Mantle dots presence – absent.
- Emanating hyphae – present and abundant.

Morphology of rhizomorphs

- Colour – yellowish white.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – plectenchymatous.
- Mantle type – ring-like arrangement of hyphal bundles (Type B).
- Septa clamps presence – present and abundant.

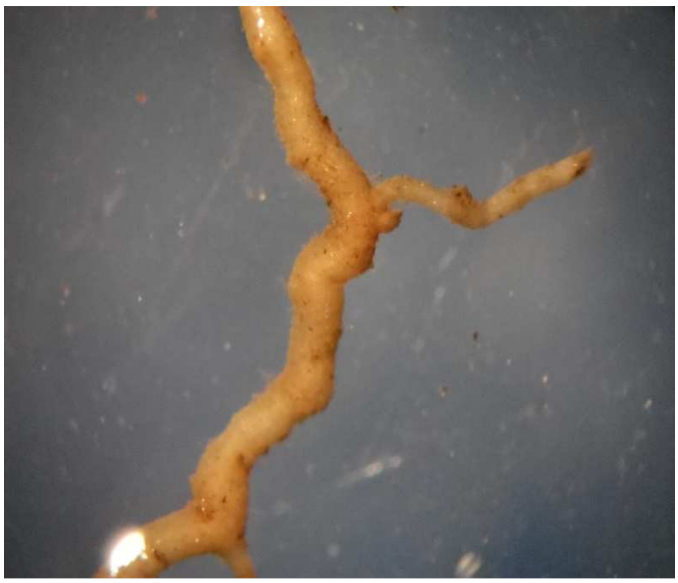


Plate 8: Species C ectomycorrhizal fungi colonising *Philenopteraviolacea* rootlets sampled from a three year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – absent/infrequent.
- Mantle colour – cream brown.

Morphology of unramified ends

- Shape – sinuous/bent.
- Colour – yellowish brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle surface habit – smooth and slightly warty.
- Mantle dots presence – absent.
- Emanating hyphae – absent.

Morphology of rhizomorphs

- Colour – concolourous to mantle.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Organisation – pseudoparenchymatous.
- Mantle type – angular cells and moulds of flattened cells (Type O).
- Septa clamps presence – absent.

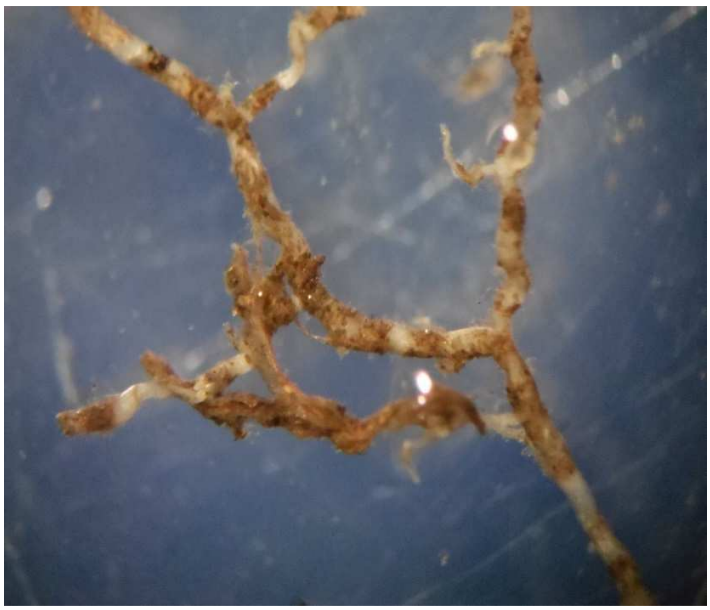


Plate 9: Species D ectomycorrhizal fungi colonising *Grewia monticola* rootlets sampled from a four year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – infrequent.
- Mantle colour – dark brown.

Morphology of unramified ends

- Shape – bent and slightly tortuous.
- Colour – dark brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle surface habit – not smooth (warty/grainy).
- Mantle dots presence – absent.
- Emanating hyphae – infrequent.

Morphology of rhizomorphs

- Colour – concolourous to mantle.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Completeness- patchy.
- Organisation – plectenchymatous.
- Mantle type – hyphae arranged net-like (Type D).
- Septa clamps presence – absent.

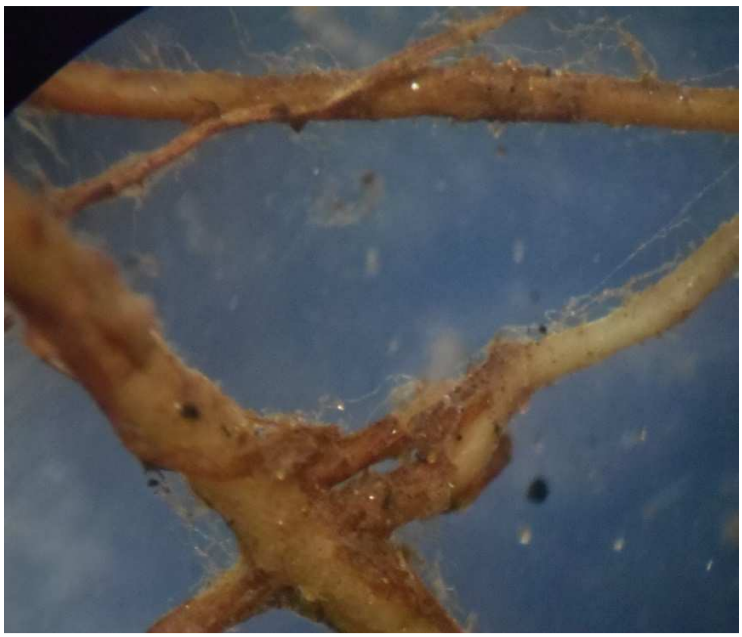


Plate 10: Species E ectomycorrhizal fungi colonising *Acacia tortilis* rootlets sampled from a five year old fallow.

Morphology of the mycorrhizal system

- Rhizomorphs – abundant.
- Mantle colour – brown.

Morphology of unramified ends

- Shape – tortuous.
- Colour – yellowish brown.
- Mantle surface visibility- present.
- Mantle transparency - not transparent.
- Mantle surface habit – not smooth (densely stringy).
- Mantle dots presence – absent.
- Emanating hyphae – abundant.

Morphology of rhizomorphs

- Colour – concolourous to mantle.
- Margin habit – hairy.

Morphology of sclerotia

- Presence – absent.

Anatomical features of the mantle

- Completeness- patchy.
- Organisation – plectenchymatous.
- Mantle type – ring-like arrangement of hyphal bundles (Type A) and occasional patches of roundish cells on the mantle (Type F).
- Septa clamps presence – absent.

The frequency of each of the ectomycorrhizal fungi observed in the fallow lands is shown in Table 4.2 below. Frequency was calculated as the number of fallow plots in which a species was detected and relative frequency was calculated as the total number of occurrences of a species divided by the total number of occurrences of all species. Figure 4.2 illustrates the relative frequencies of the ectomycorrhizal fungi.

Table 4.2: Ectomycorrhizal fungi species observed in fallows of all ages in the study area (+/- denotes presence or absence of fungal species, respectively).

Fungal species	**Fallow yrs:**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**14**	**Frequency**
Lactarius gymnocarpus		-	+	+	-	-	+	-	-	+	+	5
Lactarius species		-	+	-	+	+	-	+	-	+	-	5
Thelephora terrestris		-	-	+	-	-	-	-	-	-	-	1
Amphinema byssoides		-	-	+	-	-	-	-	-	+	-	2
Boletus species		-	+	-	+	+	-	-	-	-	+	4
Species A		+	-	-	-	-	-	-	-	-	-	1
Species B		-	+	-	+	-	-	-	+	-	-	3
Species C		-	-	+	-	-	-	-	-	-	-	1
Species D		-	-	-	+	-	-	-	-	-	-	1
Species E		-	-	-	-	+	-	-	-	-	-	1
No. of ECM/fallow		**1**	**4**	**4**	**4**	**3**	**1**	**1**	**1**	**3**	**2**	**24**

Ectomycorrhizal species of genus *Lactarius* (*L. gymnocarpus* and *Lactarius* sp.) dominated the fallow lands, occurring in five of the ten fallow ages. Species of genus *Boletus* also commonly occurred, and wererecorded in four of the ten fallow ages. Most of the ectomycorrhizal species only occurred within a single fallow age. This was especially true of the unidentified species A-E. The two *Lactarius* species (*L. gymnocarpus* and *Lactarius* sp.) recorded the highest relative frequency, followed by *Boletus* sp. and Species B.

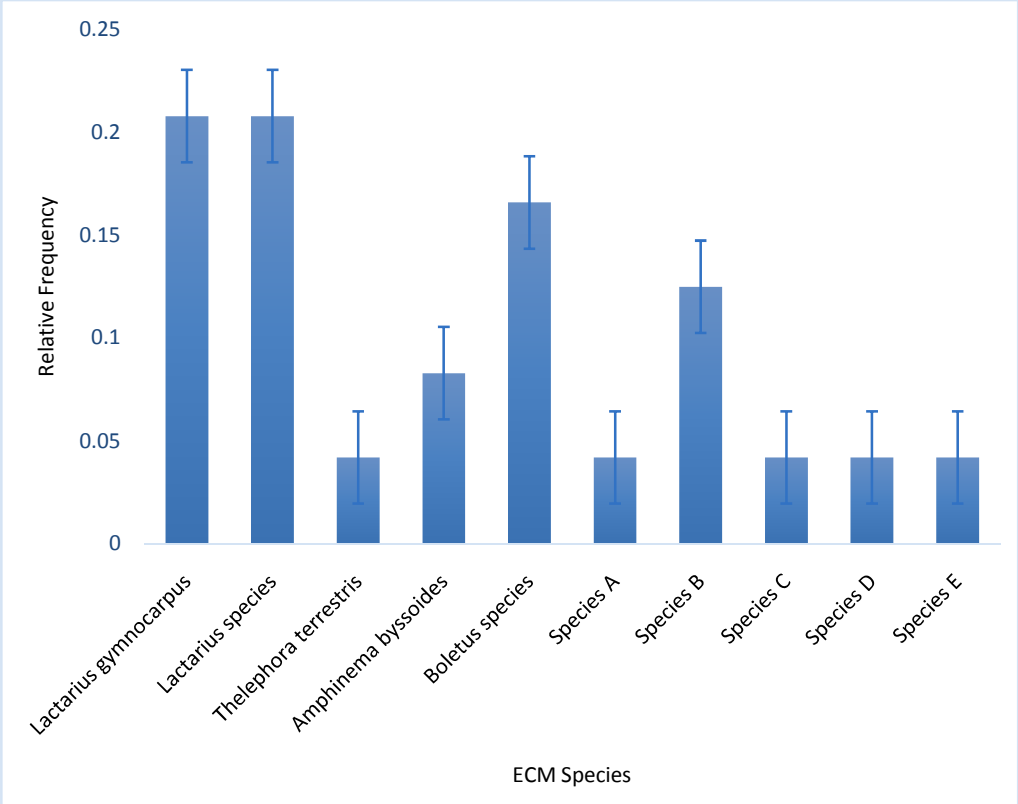


Figure 4.2: Ectomycorrhizal fungi (ECM) species relative frequencies in fallow lands of the mid-Zambezi area.

4.2.Comparing fungal species richness among fallows of different ages as a way of determining fungal community changes associated with succession

Disturbance regimes and environmental factors differed among the sampled fallows as the fallows were spatially separated throughout the study area. Family Fabaceae was the most dominant tree taxon in fallows, with *Acacia tortilis* subsp. *spirocarpa* occurring in all fallows, except in one-year old fallows(Table 4.3). Members of theFabaceae (subfamilies Caesalpinioideae, Papilionoideae and Mimosoideae) were commonly associated with ectomycorrhizae as shown in Table 4.3.At Fallow Age 1, only one mycorrhizaltree species,*Acacia nigrescens*, frequentlyoccurred in abandoned fields. This is a pioneer tree species

as noted by Tambara *et al*. (2012a). The tree species was only associated with one of the unidentified ectomycorrhizal species designated Species A. At Fallow Age 2, six tree species co-dominated the plant community, with four of them forming mycorrhizal associations. The four tree species harboured the following ectomycorrhizal species: *Boletus* sp. (associated with*Philenopteraviolacea)*, *Lactarius* sp. (associated with *Combretum* species), *Lactarius gymnocarpus*(associated with *Acacia tortilis*) and Species B (associated with *Combretum mossambicense*). Fallow Age 3 had only three mycorrhizal tree species. This fallow age recordedectomycorrhizal fungi that had not been observed in younger fallows. These were *Thelephora terrestris* (associated with *A. tortilis*), *Amphinema byssoides* (associated with *Combretummossambicense*) and an unidentified Species C (associated with *L. capassa*). At Fallow Age 4, all the tree species had mycorrhizal associations, except *Combretum eleagnoides*. There were also two unidentified fungal species observed in this fallow age group (Species B and D). Species B was recorded on *A. tortilis* instead of *C. mossambicense* as observed in younger fallow ages. Notably, Species D (associated with *Grewia monticola*) and *Boletus* sp. (associated with *L. capassa*) were recorded for the first time in this fallow age group. Four mycorrhizal tree species were recorded at Fallow Age 5. These were fewer than those recorded in Fallow Age 4. Two of the tree species (*Combretum eleagnoides* and *Faidherbia albida*) had no ectomycorrhizal associations. Fungal Species E (associated with *A. tortilis*) was recorded at Fallow Age 5. A few ectomycorrhizal species were recorded at Fallow Ages 6, 7 and 9 and at Fallow Age 10, despite the high number of mycorrhizal trees at these fallow ages. Fallow Ages 11 to 13 were unrepresented in the study area. Only two ectomycorrhizal fungi, *Lactarius gymnocarpus* and *Boletus* sp., which both associated with *Acacia tortilis,*were recorded at Fallow Age 14. No ectomycorrhizae were found in the other three species despite their being mycorrhizal tree species.

Table 4.3: Mycorrhizal status of some tree species in fallows of different ages.

Fallow age (years)	Tree species	Mycorrhizal Status*	ECM species
1	*Acacia nigrescens*	+	**Species A**
2	*Acacia tortilis*	+	*Lactarius gymnocarpus*
	Dichrostachys cinerea	-	-
	Ziziphus mauritiana	-	-
	Philenopteraviolacea	+	*Boletus* sp.
	Combretum mossambicense	+	**Species B**
	Combretum sp.	+	*Lactarius* sp.
3	*Acacia tortilis*	+	*Lactarius gymnocarpus, Thelephora terrestris*
	Philenopteraviolacea	+	**Species C**
	Combretum mossambicense	+	*Amphinema byssoides*
4	*Philenopteraviolacea*	+	*Boletus* sp.
	Combretum mossambicense	+	*Lactarius* sp.
	Acacia tortilis	+	**Species B**
	Combretum eleagnoides	-	-
	Grewia monticola	+	**Species D**
5	*Combretum eleagnoides*	-	-
	Philenopteraviolacea	+	*Lactarius* sp., *Boletus* sp.
	Acacia tortilis	+	**Species E**
	Faidherbia albida	-	-
6	*Acacia tortilis*	+	*Lactarius gymnocarpus*
	Combretum eleagnoides	-	-
7	*Acacia tortilis*	+	*Lactarius* sp.
9	*Acacia tortilis*	+	**Species B**
	Diospyros quiloensis	-	-
10	*Combretum eleagnoides*	-	-
	Dichrostachys cinerea	-	-
	Kirkia acuminata	+	*Lactarius gymnocarpus*
	Acacia tortilis	+	*Lactarius* sp.
	Philenopteraviolacea	+	*Lactarius* sp.
	Combretum mossambicense	+	*Amphinema byssoides*
	Ziziphus mauritiana	+	*Amphinema byssoides*
14	*Acacia tortilis*	+	*Lactarius gymnocarpus, Boletus* sp.
	Philenopteraviolacea	-	-
	Combretum mossambicense	-	-
	Afzelia quanzensis	-	-

*+/- denotes presence or absence of mycorrhizal colonisation, respectively.

Figure 4.3provides the number of dominant tree species recorded in relation to number of ectomycorrhizal fungi recorded in each fallow age. The number of ectomycorrhizal fungi species seemed to loosely correlate with number of dominant tree species reported for each fallow age. However, a sharp increase in tree species only resulted in a gradual increase in fungal species.Although fluctuations were noted in mycorrhizal tree species numbers with increasing fallow ages, the same was not the case with ectomycorrhizal fungi species. Fallow Ages 2 to 4 and 6 to 9 recorded an equal number of ectomycorrhizal fungi species (four species and one species, respectively) despite the differences in mycorrhizal tree species recorded for the different fallow ages.

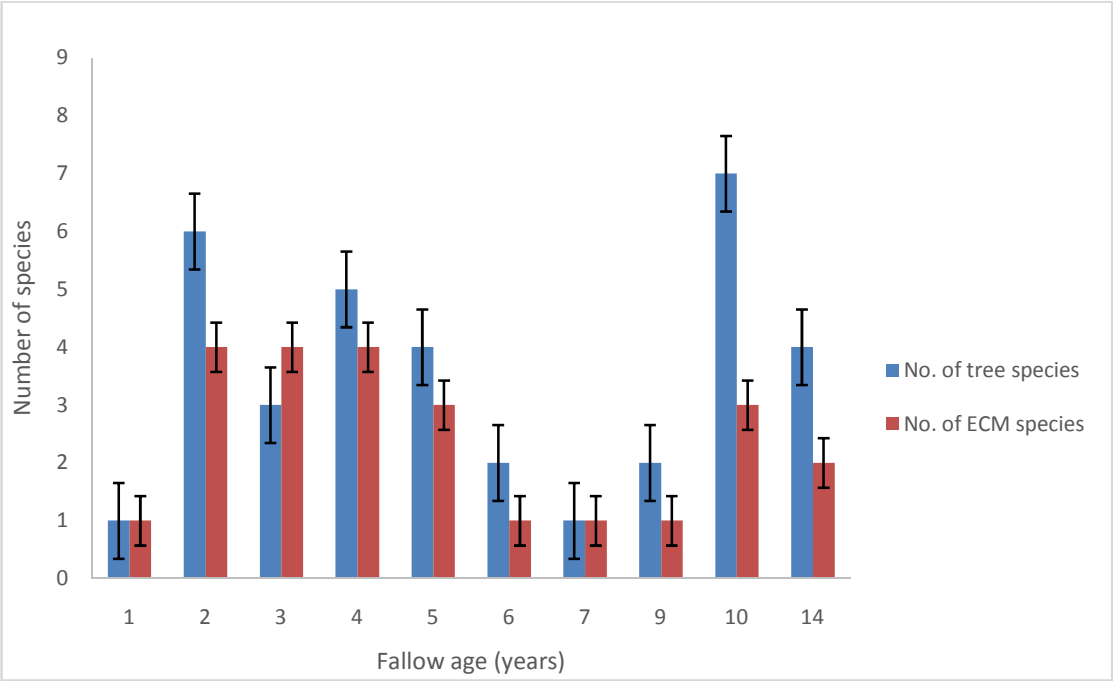


Figure 4.3: The relationship between number of ECM species and dominant tree species in fallows of increasing ages.

With each successional stage, as defined by fallow ages, ectomycorrhizal fungi gradually decreased in species number (Fig 4.4 below), with only one and two ectomycorrhizal fungi species recorded in older fallows (9 to 14 years), despite the increase in potential host tree species in older fallows (10 years) (Fig 4.5 overleaf). Figure 4.5 also shows that dominant mycorrhizal tree species generally increased with increasing fallow age as would be expected.

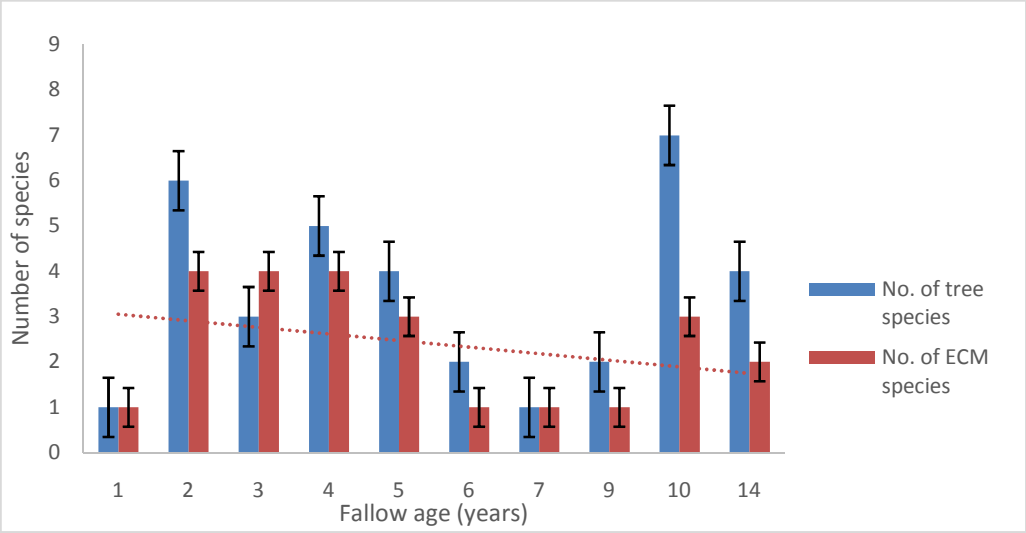


Figure 4.4: Trend of number of ECM species with increase in fallow age.

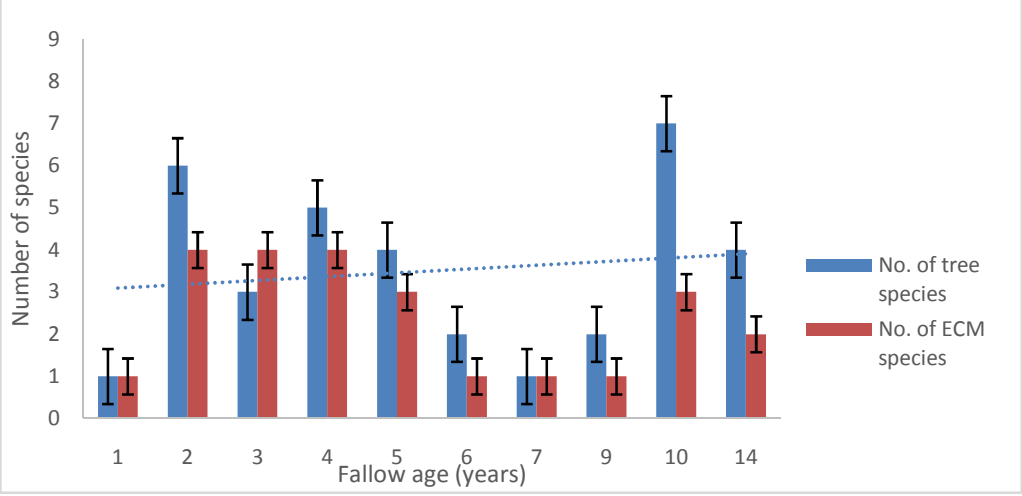


Figure 4.5: Trend of number of dominant tree species with increase in fallow age.

4.3.Establishing the genetic characteristics of dominant ectomycorrhizal fungi across fallows and host species

4.3.1.DNA Extraction

In order to establish intra- and inter-specific genetic variation in ectomycorrhizal species, only ectomycorrhizal fungi whose identity was confirmed were included in the study. Samples of *Lactarius gymnocarpus*, *Lactarius* sp., *Boletus* sp., *Amphinema byssoides* and *Thelephora terrestris* from fallows of different ages were studied. These species were found in one to three samples in the various fallow categories, depending on their availability in the area. Results of molecular analyses on ten samples of the five identified ectomycorrhizal fungi species are provided in figures and tables shown below.

The scale and purity of the DNA extracted by the ZR Fungal/Bacterial DNA MiniPrep™Kit was satisfactory (Figure 4.6). These results were not critical tothe quality of the PCR reactions and did not compromise the quality of the bands obtained in the study. Thus, the extracted DNA was used for further experiments.

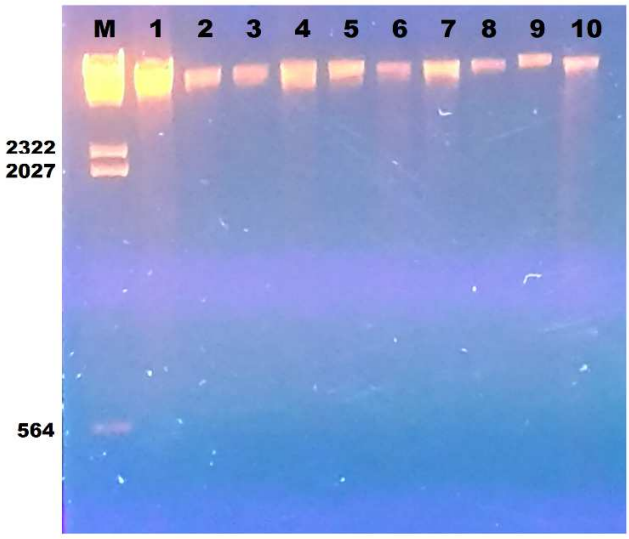


Figure 4.6: Agarose gel showing ten isolated fungal samples. Ten µl of the isolated fungal DNA mixed with 2 µl of 6X loading dye and electrophoresed through 1% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is the *Hind*III marker. Lanes 1-10 are the DNA samples from ten different fungal isolates.

4.3.2.PCR Amplification

The fungal genomic DNA was successfully amplified in the ten ectomycorrhizal fungi isolates (Figure 4.7) as evidenced by the high intensity of the bands.The illustration shows that the 18S ribosomal RNA (rRNA) gene was amplified in all ten fungal samples. The expected approximately 1.6 kb fragment was observed in all the ten samples with a notable difference in size of the fragments in Lanes 2, 3 and 8 when compared with fragments in Lanes 4-7 and 9-11. While fragments in Lanes 2, 3 and 8 were of the expected size, those in Lanes 4-7 and 9-11 were slightly smaller at approximately 1.5 kb. This difference in size of the amplicons suggests polymorphism in the 18S rRNA gene that was explained further by digestion with restriction enzymes.

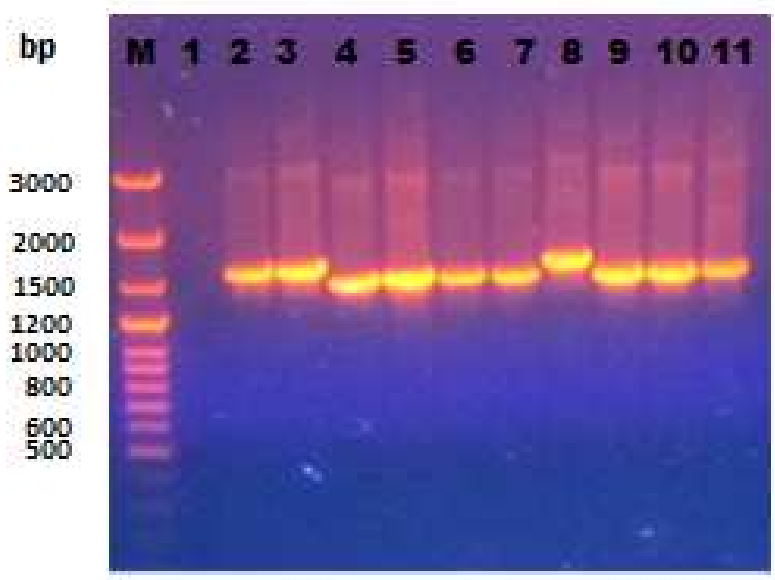


Figure 4.7: Agarose gel showing *Taq* DNA polymerase activity. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 1% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M isthe 100 bp Ladder Plus. Lane 1 is the negative control. Lanes 2- 11 are genomic DNA samples from 10 different fungal isolates amplified using fungal generic primers.

Table 4.4 (overleaf)provides descriptions of the ten fungal isolates sampled and analysed. Samples were taken from fallows of ages varying from 2 - 10 years based on the criteria of available fallow ages in the study area. The most dominant host species was *Acacia tortilis*subsp. *spirocarpa*which associated with all ectomycorrhizal fungi species sampled except *Boletus* sp. The most common ectomycorrhizal fungi taxonsampled was genus *Lactarius*, with *Lactarius gymnocarpus* and *Lactarius* sp. observed. Other ectomycorrhizal fungi species observed included *Boletus* sp., *Amphinema byssoides* and *Thelephora terrestris*.

Table 4.4: Isolated ectomycorrhizal fungi species and their hosts and fallow ages sampled.

Lane no.	Fungal sp.	Host sp.	Fallow age	Dendrogram Code
1	-	-	-	-
2	*Lactarius gymnocarpus*	*Acacia tortilis*	2	a1
3	*Boletus* sp.	*Philenopteraviolacea*	2	a2
4	*Boletus* sp.	*Philenopteraviolacea*	5	a3
5	*Lactarius gymnocarpus*	*Kirkia acuminata*	10	a4
6	*Thelephora terrestris*	*Acacia tortilis*	3	a5
7	*Lactarius* sp.	*Combretum mossambicense*	4	a6
8	*Lactarius* sp.	*Acacia tortilis*	7	a7
9	*Amphinema byssoides*	*Combretum mossambicense*	10	a8
10	*Amphinema byssoides*	*Ziziphus mauritiana*	10	a9
11	*Lactarius gymnocarpus*	*Acacia tortilis*	6	a10

4.3.3.DNA fingerprinting

RAPDs

The results of the RAPD reaction using primer OPA 2 carried out on all ten fungal samplesare shown in Figure 4.8. The reaction showed a distinct banding pattern and complemented some of the initial suggestions of polymorphisms. The banding pattern in Lanes 9 and 10 showed a similar profile suggesting a similarity of the two fungal samples, while that of the remaining eight samples showed differences. Lanes 2, 5 and 11 showed two clear and distinct fragments of different sizes, indicating differences in these samples. Similarly, samples in Lanes 3, 6, 7 and 8 showed a single fragment of a different size each, again indicating clear differences among the samples. Only the sample in Lane 4 showed four bands.

Table 4.5 overleaf shows the approximate band sizes from the RAPDs reaction. These results were further used to calculate genetic similarities among thefungal samples. Genetic similarity based on RAPDs calculated according to Nei and Li (1979) showed similarity ranging from 0to 1(Table 4.6). Most of the ectomycorrhizal fungi, however,revealed minimal or absence of similarity, ranging mostly from 0 –0.333. Highest similarity was recorded between Lanes 9 and 10 (100%). Lane 5 showed 0% similarity with the rest of the isolates. The dendrogram on Figure 4.9illustrates the clustering of the ten ectomycorrhizal fungi isolates according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Each arm of the dendrogram corresponds to the scaled genetic distance of the different isolates.The Cophenetic Correlation Coefficient (CP) can be defined as the linear correlation coefficient between the cophenetic distances obtained from the tree, and the original distances used to construct the tree. Thus, it is a measure of how faithfully the tree represents the dissimilarities among observations.

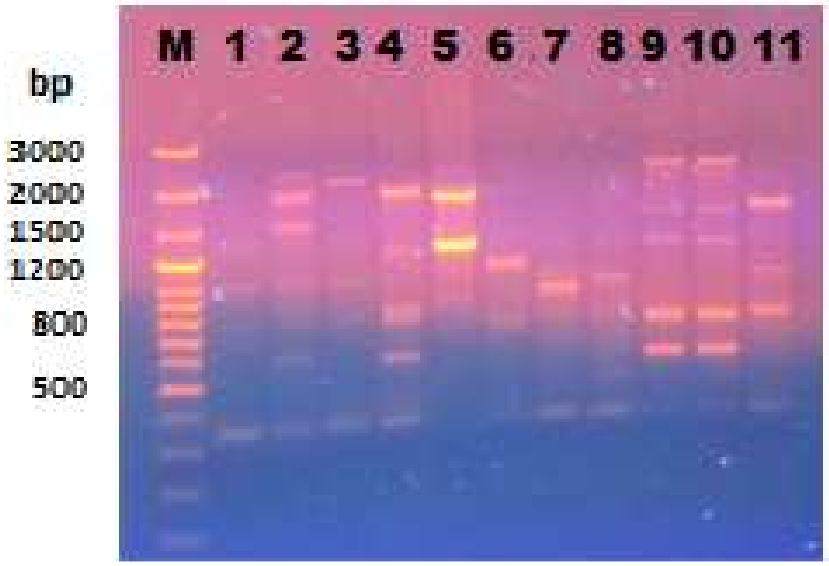


Figure 4.8: Agarose gel showing *Taq* DNA polymerase activity of RAPD reactions using primer OPA 2. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 1.5% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 100 bp Ladder Plus. Lane 1 is the PCR mixture with no enzyme. Lane 2- 11 are genomic DNAs from 10 different fungal isolates amplified using fungal OPA 2 primer.

Table 4.5: Band sizes of the ten ectomycorrhizal fungi samples calculated from the RAPDs results in Figure 4.8.

	Lane									
Band Size (bp)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
2600	-	-	-	-	-	-	-	+	+	-
2500	+	-	-	-	-	-	-	-	-	-
2300	-	+	-	-	-	-	-	-	-	-
2100	-	-	+	-	-	-	-	-	-	-
2000	+	-	-	+	-	-	-	-	-	-
1900	-	-	-	-	-	-	-	-	-	+
1600	-	-	-	-	-	-	-	+	+	-
1500	+	-	-	-	-	-	-	-	-	-
1350	-	-	-	+	-	-	-	+	+	-
1200	-	-	+	-	-	-	-	-	-	-
1100	-	-	-	-	+	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	-	+
900	-	-	-	-	-	-	+	-	-	-
700	-	-	-	-	-	+	-	-	-	-
650	-	-	+	-	-	-	-	-	-	+
500	-	-	-	-	-	-	-	+	+	-
450	-	-	+	-	-	-	-	+	+	-
420	-	-	-	-	-	+	+	-	-	+
400	-	-	+	-	-	-	-	-	-	-
370	-	+	-	-	-	-	-	-	-	-
350	+	-	-	-	-	-	-	-	-	-

Table 4.6:RAPDs similarity matrix of the ten ectomycorrhizal fungi samplescomputed with Jaccard coefficient.

*ECM Species	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
a1	1	0	0	0.25	0	0	0	0.143	0.143	0.167
a2		1	0	0	0	0	0	0	0	0
a3			1	0	0	0	0	0.111	0.111	0.125
a4				1	0	0	0	0.167	0.167	0
a5					1	0	0	0	0	0
a6						1	0.333	0	0	0.2
a7							1	0	0	0.2
a8								1	1	0
a9									1	0
a10										1

*Ectomycorrhizal fungi species names, host species names and fallow ages are outlined in Table 4.4 above.

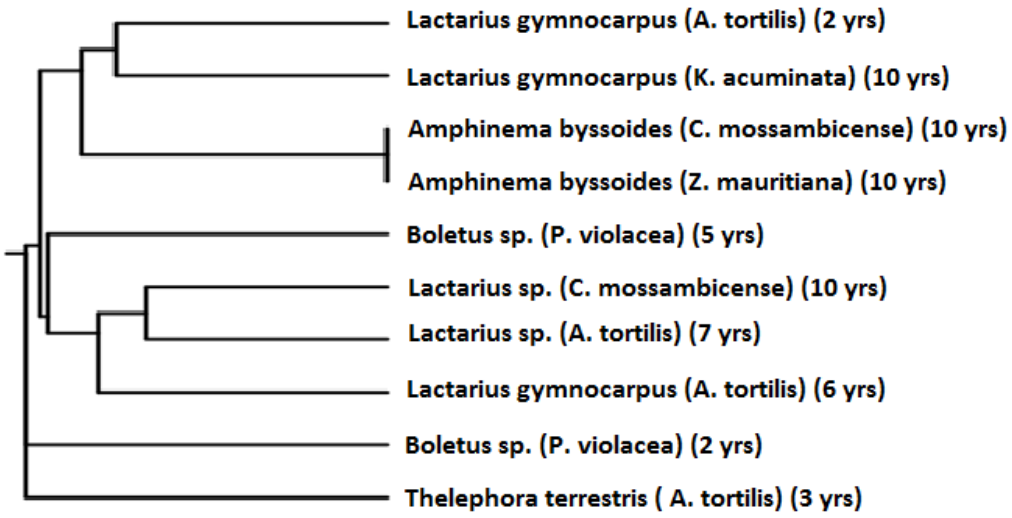


Figure 4.9:RAPDs dendrogram of ten ectomycorrhizal fungi using primer OPA 2 constructed according to the UPGMA method, using DendroUPGMA. Legend: Ectomycorrhizal fungus species (tree host) (age of fallow).
Cophenetic Correlation Coefficient (CP) = 0.654

PCR-RFLPs

PCR-RFLP digestions were carried out using four restriction enzymes,*Rsa*I, *Hin*fI, *Alu*I and *Hae*III, with the aim of detecting a wider range of polymorphisms, and also to further confirm the polymorphisms observed in the RAPDs.

Figure 4.10 shows the results of the digest using the enzyme *Rsa*I. The digestion of fungal samples in Lanes 2 and 3 produced two slightly similar fragments. The RAPDs data, on the other hand, indicateddifferences among the sampled fungi. Fungal samples in Lanes 4, 6, 7, 8 and 11 produced a single band each albeit of a different size, indicating that the five samples were different. Fungal samples in Lanes 9 and 10 produced two bands of the same size. When analysedtogether with the RAPD profile, the data suggest that the two samples areidentical and different from the rest of the fungal samples.

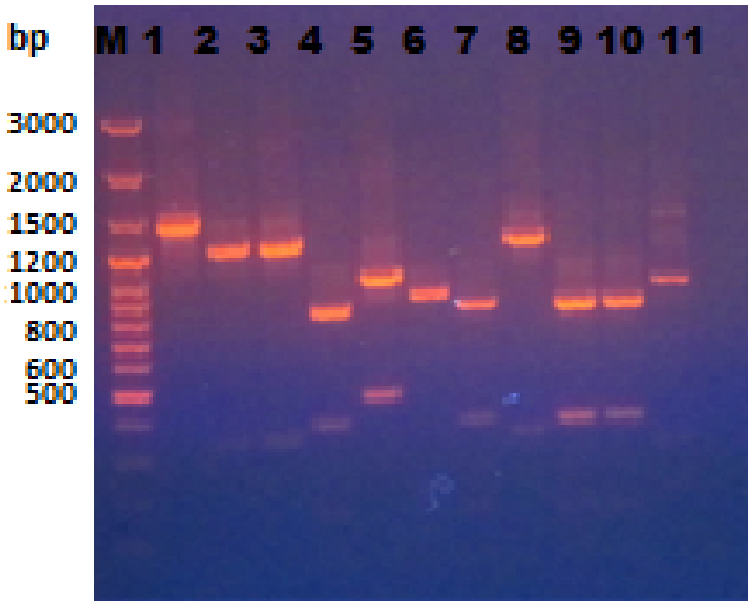


Figure 4.10: Agarose gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Rsa*I. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 1.5% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 100 bp Ladder Plus. Lane 1 is the undigested PCR fragment. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.

Table 4.7: Band sizes of the ten ectomycorrhizal fungi samples calculated from the PCR-RFLP digest using *Rsa*I results shownin Figure 4.10.

	Lane									
Band size(bp)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1350	-	-	-	-	-	-	+	-	-	-
1300	+	+	-	-	-	-	-	-	-	-
1020	-	-	-	+	-	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	-	+
950	-	-	-	-	+	-	-	-	-	-
900	-	-	-	-	-	+	-	+	+	-
850	-	-	+	-	-	-	-	-	-	-
500	-	-	-	+	-	-	-	-	-	-
400	-	-	+	-	-	+	-	+	+	-

Table 4.8:*Rsa*I digest similarity matrix of the ten ectomycorrhizal fungi samples computed with the Jaccard coefficient.

*ECM Species	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
a1	1	1	0	0	0	0	0	0	0	0
a2		1	0	0	0	0	0	0	0	0
a3			1	0	0	0.333	0	0.333	0.333	0
a4				1	0	0	0	0	0	0
a5					1	0	0	0	0	0
a6						1	0	1	1	0
a7							1	0	0	0
a8								1	1	0
a9									1	0
a10										1

* Ectomycorrhizal fungi species names, host species names and fallow ages are outlined in Table 4.4 above.

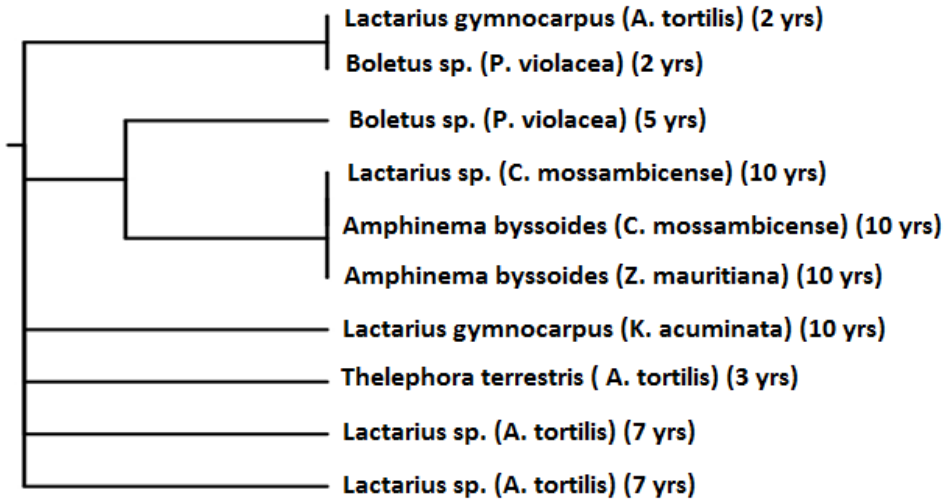


Figure 4.11:*Rsa*I digest dendrogram of ten ectomycorrhizal fungi, constructed according to the UPGMA method, using DendroUPGMA. Legend: Ectomycorrhizal fungus species (tree host) (age of fallow).
Cophenetic Correlation Coefficient (CP) = 0.999

The other three digests using*Hin*fI, *Alu*I and *Hae*III had poor band resolution on agarose gel. Therefore, they were cast on polyacrylamide gel to obtain a better resolution of the resulting fragments for accurate similarity assessments of the fungal samples. The high resolving power of polyacrylamide gel showed several more bands in all fungal samples.The gels illustrated in

Figures4.12, 4.14 and 4.16 show results of the three digestions. The polyacrylamide gel figures of *Hinf*I and *Alu*I digestions are shown in Appendix.

Results obtained after digesting amplified DNA with restriction enzyme *Hinf*I are shown in Figure 4.12. Lanes 2 and 6 revealed four similar bands contrary to previous results that confirmed differences. Notably, Lanes 9 and 10 showed similar banding patterns. All other lanes showed distinctly different banding patterns. In Figure 4.14(*Alu*I digest), Lanes 3, 4, 6, 9 and 10 illustrate similar fragments. All the other lanes illustrated unique banding patterns. Figure 4.13 which illustratesthe *Hae*III digestion, had Lanes 2, 3, 4, 6, 8, 9 and 10 all showing several common bands. Lanes 3, 4 and 6 and Lanes 9 and 10 had similar banding patterns. All the other lanes showed different banding patterns. Similarities in bands observed inLanes 9 and 10 were consistent in all the results obtained. The consequent band patterns, (shown in Tables 4.9, 4.11 and 4.13) genetic similarity calculations (shown in Tables 4.10, 4.12 and 4.14) and dendrograms (shown in Figures 4.13, 4.15 and 4.17) of the three restriction enzymes *Hinf*I, *Alu*I and *Hae*III respectively are shown below.

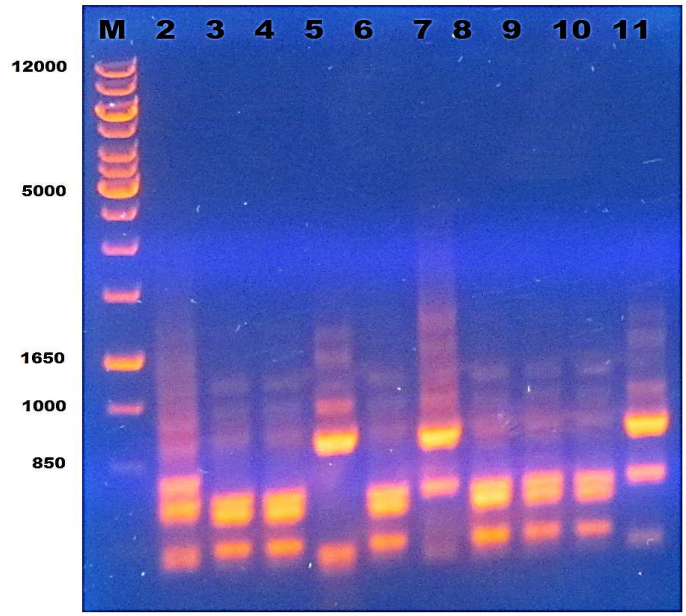


Figure 4.12: Agarose gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Hinf*I. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 1.5% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 100 bp Ladder Plus. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.

Table 4.9: Band sizes of the ten ectomycorrhizal fungi samples calculated from the PCR-RFLP digest using *Hinf*I results shown in Figure 4.12.

	Lane									
Band size(bp)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
700	-	-	-	-	-	-	+	-	-	-
630	+	-	-	+	-	+	+	-	-	-
	+	+	+	+	+	-	-	-	-	-
	+	+	+	-	+	-	-	+	+	-
	-	-	-	-	-	-	-	+	+	-
500	+	-	-	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-	-	-
	+	+	+	-	+	-	+	-	-	-
	-	-	-	-	-	-	+	+	+	-
	-	-	-	-	-	+	-	-	-	-
	-	+	+	+	+	+	-	-	-	-
	+	-	-	-	-	-	-	+	+	+
	+	+	+	+	+	+	+	-	-	-
	-	-	-	-	-	-	-	+	+	+
	-	-	+	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-	-	-

Table 4.10: *Hinf*I digest similarity matrix of the ten ectomycorrhizal fungi samples computed with the Jaccard coefficient.

*ECM Species	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
a1	1	0.364	0.364	0.3	0.4	0.182	0.273	0.167	0.167	0.1
a2		1	1	0.429	0.833	0.25	0.222	0.1	0.1	0
a3			1	0.429	0.833	0.25	0.222	0.1	0.1	0
a4				1	0.5	0.6	0.286	0	0	0
a5					1	0.286	0.25	0.111	0.111	0
a6						1	0.286	0	0	0
a7							1	0.111	0.111	0
a8								1	1	0.4
a9									1	0.4
a10										1

*Ectomycorrhizal fungi species names, host species names and fallow ages are outlined in Table 4.4 above.

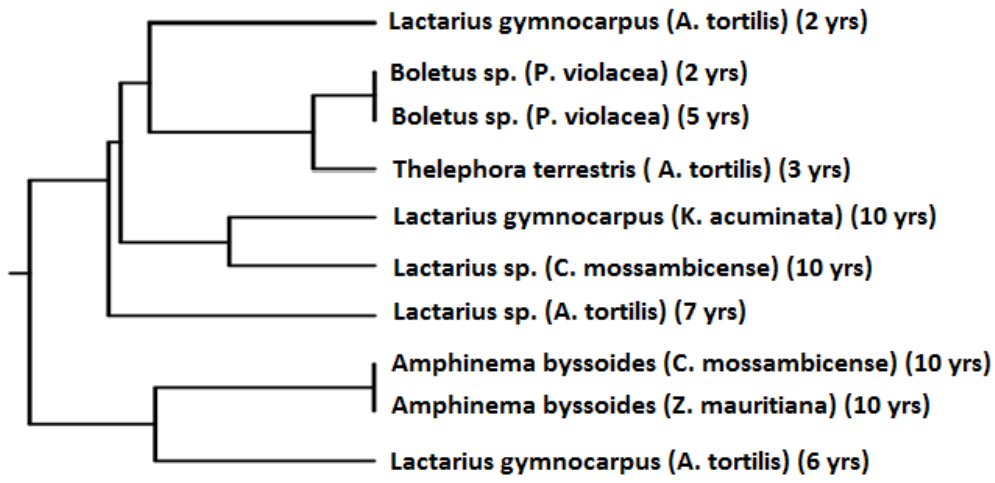


Figure 4.13: *Hinf*I digest dendrogram of ten ectomycorrhizal fungi, constructed according to the UPGMA method, using DendroUPGMA. Legend: Ectomycorrhizal fungus species (tree host) (age of fallow).
Cophenetic Correlation Coefficient (CP) = 0.883

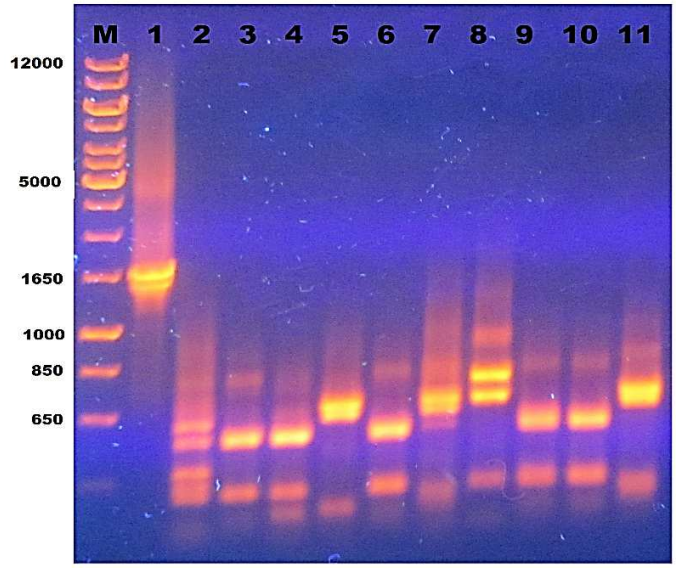


Figure 4.14: Agarose gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Alu*I. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 1.5% agarose gel in Tris acetate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 100 bp Ladder Plus. Lane 1 is the undigested PCR fragment. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.

Table 4.11: Band sizes of the ten ectomycorrhizal fungi samples calculated from the PCR-RFLP digest using *Alu*I results shown in Figure 4.14.

	Lane									
Band size(bp)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
650	+	-	-	-	-	-	+	-	-	-
640	-	-	-	+	-	-	-	-	-	-
600	-	-	-	-	-	+	-	-	-	+
	+	+	+	-	+	-	-	+	+	-
500	-	-	-	-	-	-	-	-	-	+
	+	+	+	-	-	+	-	+	+	-
	-	-	-	-	+	-	-	-	-	-
	+	-	-	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-	-	-
	-	+	+	-	-	-	+	-	-	-
	-	-	-	-	+	-	-	+	+	+
	-	-	-	+	+	+	+	+	+	+
	-	-	-	+	-	-	-	-	-	+
	+	+	+	-	-	+	+	-	-	+
	-	-	-	-	+	-	-	+	+	-
	-	-	-	-	-	-	+	-	-	-
	+	-	-	+	-	-	-	-	-	-

Table 4.12:*Alu*I digest similarity matrix of the ten ectomycorrhizal fungi samples computed with the Jaccard coefficient.

*ECM Species	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
a1	1	0.375	0.375	0.1	0.182	0.222	0.2	0.2	0.2	0.083
a2		1	1	0	0.111	0.333	0.286	0.286	0.286	0.111
a3			1	0	0.111	0.333	0.286	0.286	0.286	0.111
a4				1	0.25	0.143	0.125	0.125	0.125	0.25
a5					1	0.111	0.1	0.571	0.571	0.2
a6						1	0.286	0.286	0.286	0.429
a7							1	0.111	0.111	0.222
a8								1	1	0.222
a9									1	0.222
a10										1

*Ectomycorrhizal fungi species names, host species names and fallow ages are outlined in Table 4.4 above.

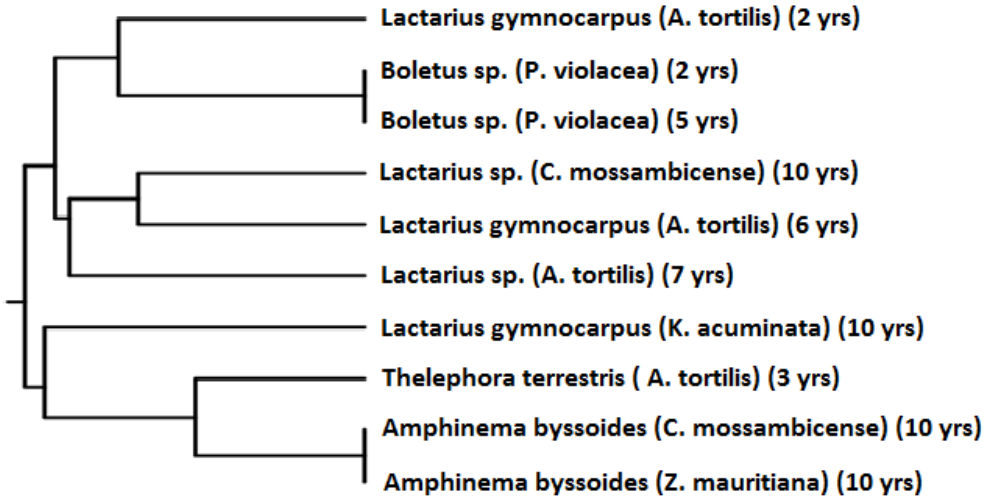


Figure 4.15:*Alu*I digest dendrogram of ten ectomycorrhizal fungi, constructed according to the UPGMA method, using DendroUPGMA. Legend: Ectomycorrhizal fungus species (tree host) (age of fallow).
Cophenetic Correlation Coefficient (CP) = 0.686

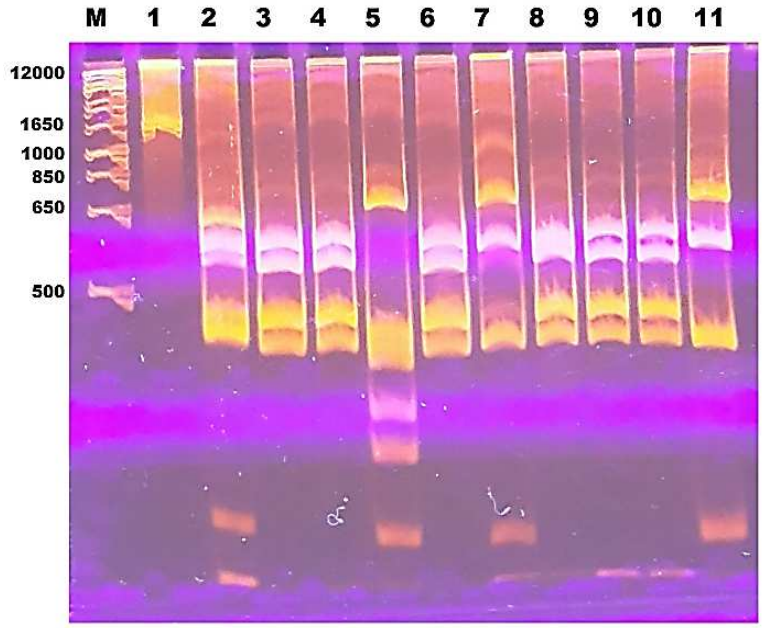


Figure 4.16: Polyacrylamide gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Hae*III. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 5 % polyacrylamide gel in Tris borate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 1kb Ladder Plus. Lane 1 is the undigested PCR fragment. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.

Table 4.13: Band sizes of the ten ectomycorrhizal fungi samples calculated from the PCR-RFLP digest using *Hae*III results shown in Figure 4.16.

	Lane									
Band Size (bp)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
700	-	-	-	+	-	+	-	-	-	+
650	+	-	-	-	-	-	-	-	-	-
590	+	+	+	-	+	+	+	+	+	+
550	+	+	+	-	+	-	+	+	+	-
500	+	+	+	-	+	-	+	+	+	-
	-	+	+	+	+	+	+	+	+	+
	-	-	-	+	-	-	-	-	-	-
	-	-	-	+	-	-	-	-	-	-
	-	-	-	+	-	-	-	-	-	-
	+	-	-	-	-	-	-	-	-	-
	-	-	-	+	-	+	-	-	-	+
	+	-	-	-	-	+	-	+	+	-

Table 4.14:*Hae*III digest similarity matrix of the ten ectomycorrhizal fungi samples computed with the Jaccard coefficient.

*ECM Species	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
a1	1	0.429	0.429	0	0.429	0.222	0.429	0.571	0.571	0.111
a2		1	1	0.111	1	0.286	1	0.8	0.8	0.333
a3			1	0.111	1	0.286	1	0.8	0.8	0.333
a4				1	0.111	0.375	0.111	0.1	0.1	0.429
a5					1	0.286	1	0.8	0.8	0.333
a6						1	0.286	0.429	0.429	0.8
a7							1	0.8	0.8	0.333
a8								1	1	0.286
a9									1	0.286
a10										1

*Ectomycorrhizal fungi species names, host species names and fallow ages are outlined in Table 4.4 above.

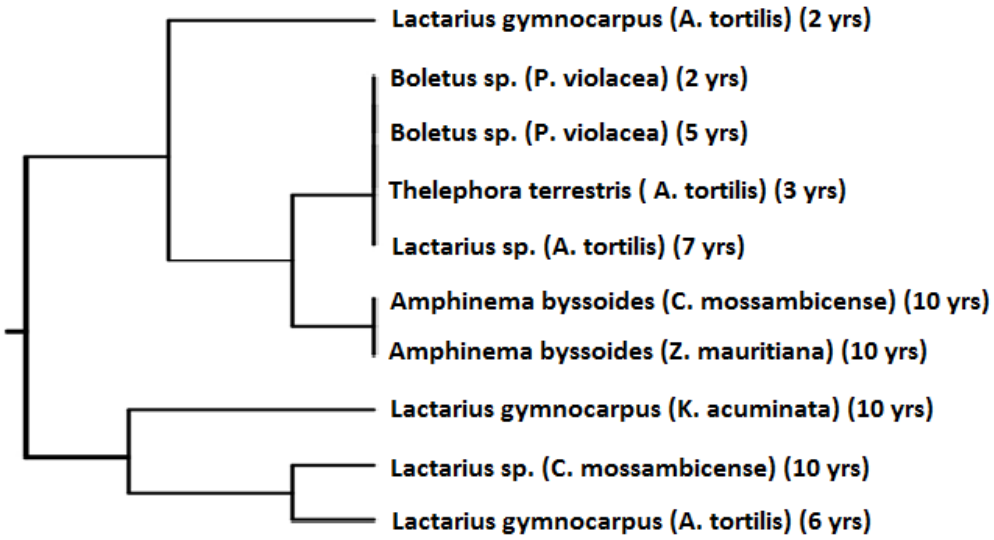


Figure 4.17:*Hae*III digest dendrogram of the ten ectomycorrhizal fungi, constructed according to the UPGMA method, using DendroUPGMA. Legend: Ectomycorrhizal fungus species (tree host) (age of fallow).
Cophenetic Correlation Coefficient (CP) = 0.896

4.4.Establishing whether fallow age or host specificity determines mycorrhizal status of tree species

There was no significant correlation between fallow age and ectomycorrhizal fungi for any of the host tree species ($p < 0.05$: 0.002). The generalised linear model with binomial response and link logit showed no significant relationship ($p > 0.05$: 0.079) amongectomycorrhizal fungi species in any tree species and fallow age. However, the Raup - Crick similarity index indicated that there was an interaction between fallow age and mycorrhizal status of tree species, as opposed to host specificity (Figure 4.18).

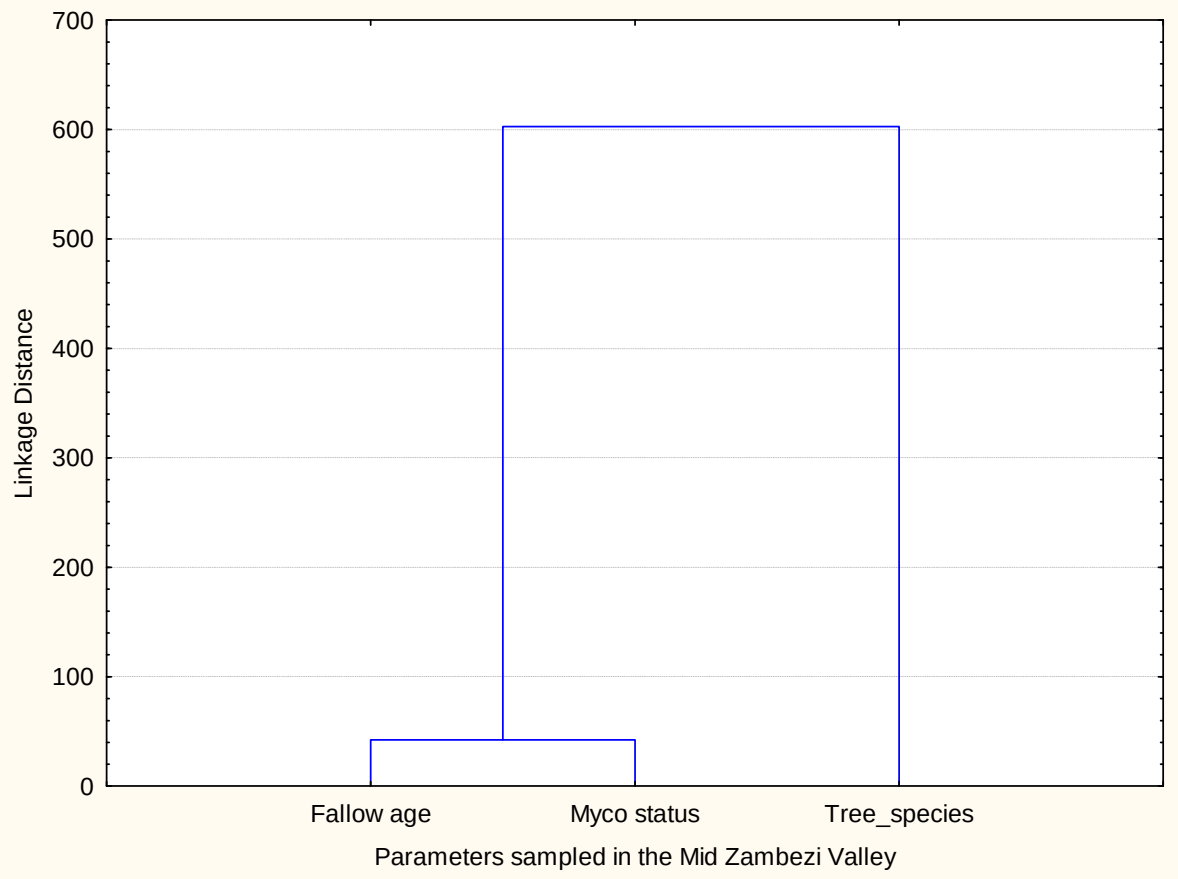


Figure 4.18: Raup - Crick similarity cluster plot using selected parameters for fallows sampled in the mid-Zambezi valley.

CHAPTER 5. DISCUSSION

5.1.Establishing fungal flora associated with fallowing

The increased demand for land in the mid-Zambezi area has led to a shortening of fallowing periods.Only fallows from ages 1 year to 14 years were identified and considered in the present study. Fallows ofages 8, 11, 12 and 13 years were, however, not represented in the study.Savanna communities only reach their climax stage after several decades. Thus, the fourteen year old fallows do not in any way represent the climax stage of plant community succession. Hence, the sampled fallows only provide an indication of the initial stagesof ectomycorrhizal community succession. Natural woodlands in Ward 2 were dominated by *Colophospermum mopane.* Associated with this species were *Combretum apiculatum, Diospyros kirkii* and *Crossopteryxfebrifuga. Julbernardia globiflora* and *Pteleopsis anisoptera* occurred on steep, inaccessible slopes that are unsuitable for farming. Riparian communities that are mostly disturbed by agricultural activities occur as narrow bands fringing major watercourses. Farming activities are primarily targeted at riverine fringes, an illegal practice in Zimbabwe known as stream bank cultivation that takes advantage of the high soil moisture in these zones. Fallows are of a different species composition.

Dry woodlandsin Ward 2 that include such species as *Xylia torreana*, *Pteleopsis myrtifolia*, *Entandrophragma caudatum, Pterocarpus lucens, Kirkia acuminata, Diospyros quiloensis, Markhania zanzibarica, Commiphora ugogensis,* several *Acacia* species and *Combretum* species are mostly unutilised for farming activities. The fungal diversity of these dry woodlandsis still largely unexplored because of the remoteness of the areas and for the fact that no farming activities occur in these woodlands. A comprehensive listing of the fungal flora in these areas awaits separate studies.

Ward 3 (Mushumbi area), is the most populated. Pressure for land in this area is high, especially in riparian zones. Abandoned fields within dry forests are difficult to distinguish as they have merged with original vegetation. Farmers in the area practise intensive farming and low scale animal husbandry. Fallow age in this area is significantly reduced. This area is characterised by high *Colophospermum mopane* formations referred to as 'cathedral mopane' (Biodiversity Project, 2002) and alsoincludes *Acacia* and *Combretum* species. Pressure for land in this area has impacted on ectomycorrhizal fungi hosts, hence possibly affecting ectomycorrhizal fungi composition and diversity.

5.1.1.Plant use and host selection

Tree species have multiple uses in the area (Table 4.1). Thus, tree felling is selective. Of importance are trees that provide traditional medicines and edible fruits. Other trees of varying importance are those used in construction, furniture and tool making and firewood. Several species of the genus *Combretum, C. mopane* and*Diospyros* speciesare of medicinal use in various ailments ranging from diarrhoea, skin problems, common colds to snake bites or even aphrodisiacs (Biodiversity Project, 2002). Depending on severity or endemism of a disease in a locality, these plants assume varying importance and are therefore spared during land clearing. Plant species producing edible fruits are also of local importance, especially *Ziziphus mauritiana*. This species, together with fruit species such as *Sclerocarya birrea*and *Adansonia digitata*are also spared during felling.Several species of *Acacia*, *C. mopane* and *Dichrostachys cinerea* are trees frequently felled for construction, with varying uses from poles, beams and posts (*C. mopane*) and pickets or thorny fences (*Acacia* species and *D. cinerea*). Others are used

for furniture with preference for *Diospyros kirkii*. Land clearing for cropping purposes is usually non-selective, but larger trees that provide shade or trees with edible fruits tend to be excluded.

Tree species composition, as outlined above, is important in shaping and maintaining fungal species diversity in the area sincemycorrhizal inoculum is maintained in the spared trees. The mycorrhizal fungi associations observed in subsequent fallowing periods largely rely on residual mycorrhizae as selective weeding/clearing of less preferred woody plants often occurs. This, therefore, is a discriminatory process,as onlylocally important plant species are protected. Selective clearing was thereforean important driver in shaping ectomycorrhizal fungicommunity composition in this study.

5.1.2.Ectomycorrhizal fungi associated with fallowing

The two *Lactarius* species,*Lactarius gymnocarpus* and *Lactarius* sp., occurred in almost half of the fallow ages sampled (Table 4.2), sometimes in fallows of different ages. These findings confirm the presence of this fungal genus in most ecosystems of tropical Africa as reported by Verbeken and Buyck (2002). The authors have noted that genus *Lactarius* is ubiquitous in Zambezian woodlands.*Boletus* sp.also frequently occurred in the sampled fallows.Residual trees, therefore, act as refugia for ectomycorrhizae in agricultural fields. *Boletus* sp. had a frequent association with *Philenopteraviolacea,* a species commonly spared during land clearing. Some of the ectomycorrhizal fungi species occurred singly in the sampled fallows. These may represent opportunistic species that thrive after disturbance. Such species tend to disappear in older fallows as part of community succession. As noted above, tree species composition reflects both some degree of community succession and effects of selective felling. Thus, the mycorrhizal fungal associations partly reflect residual mycorrhizae (as selective felling of less preferred woody plants often occurs) and tree regeneration (representing tree succession stages).

Similar observations have been reported by Tambara *et al*. (2012a, b) on tree species and arthropod species succession. Relative frequencies of recordedectomycorrhizal speciesrevealed the presence of genus *Lactarius*in most of the fallow lands. *Lactarius* species can be facultative saprotrophs. Thus, theydo not entirely depend on mycorrhizal associations (Verbeken and Buyck, 2002). The success of the second most frequent fungal species, *Boletus* sp., is of special interest however. The species is host specific. It was not expected to occur this frequently in tropical African environmentsthat are commonly dominated by generalist taxa (see Munyaziza and Kuyper, 1995; Buyck *et al*, 1996). However, as the species host tree (*Philenopteraviolacea)* is locally important, it acts as arefugium. The presence of *Boletus* sp., therefore, is associated with anthropogenic selection.

5.2.Fungal species richness as a means of determining fungal community succession

The dominance of only a few mycorrhizal host species (especially *A. nigrescens*) soon after abandonment of an agricultural field accounts for the low number of ectomycorrhizal species at Fallow Age 1 (one ECM).Under such conditions, there are few tree seedlings and sprouts. Propagules of ectomycorrhizal fungi are likely to remain in vegetative or dormant state for a period of time, awaiting the occurrence of more conducive conditions. Experimental studies have shown that some ectomycorrhizal fungi establish associations more rapidly on tree seedlings from airborne spores and other vegetative propagules, while others stay dormant for longer periods (Fox, 1986; Ingold, 1971). Emergence of suitable hosts and more conducive conditions, e.g. favourable successional chrono-sequences, optimal nitrogen content and microenvironmentsare likely to support the emergence of several other ECM species as observed at Fallow Age 2. Opportunistic ectomycorrhizal fungi start appearing at this fallow age. These ECMs could have had lowreserves in the surrounding woodland. Ecosystem disturbance due to

land clearing would have resulted in their sudden emergence, at least at the early pioneer stage.Such early seral fungi as *Thelephora terrestris*were recorded in young fallows that are at early successional stage,taking advantage of the increasing number of hosts. The diversity of ectomycorrhizal fungi in this study appears to be partly influenced by presence or absence of favourable hosts. Reports from previous studies, e.g. by Thoen and Bâ (1989),Härkönen*et al.*(1993),Munyaziza and Kuyper (1995) andBuyck *et al. (*1996), however, appear to suggest otherwise. Though ectomycorrhizal fungi seem to loosely follow the trend of tree species with increase in fallow age, older fallows showed a decreasing number of ectomycorrhizal fungi in spite of the increasing potential host species.Some mycorrhizal fungi are slow at establishing in new sites, but only successfully colonize after several years and eventually become dominant on the root system (Fox, 1986).Community succession in ectomycorrhizal fungi takes several decades to reach its climax state, with studies by Twieg*et al.* (2007) showing that ectomycorrhizal fungi climax stage is reached at ages of more than sixty five years. As stated earlier, though this study showed that fallow age and host species partly influence the mycorrhizal status of tree species, such other factors as soil physicochemical characteristics and microclimate may shape community succession in ectomycorrhizal fungi community.

5.2.1.Factors affecting ectomycorrhizal fungi community succession

Tambara *et al.* (2012a) have demonstrated that woody species in fallow lands of the mid-Zambezi area do change over time in accordance with fallows age. Tambara *et al.* (2012b) have also observed that changes in woody species diversity affect arthropod species diversity. Increased arthropod diversity enhances rapid nutrient cycling, thus accelerated community succession. The studied fallows are, however, spatially separated and transcending across ecologically different micro-environments. Thus, they are also under the influence of varied

factors unique to each fallow.It is therefore complex to fully understand the various mechanisms behind ectomycorrhizal fungi succession in the study area.

Several factors are important in determining fungal community succession. Tree community succession occursmore slowly. Herbaceous plant succession occurs faster than woody plant succession. Changes in arthropod diversity are more pronounced and rapid (Tambara *et al*, 2012b). Changes in below-ground activities are even faster and can be affected by several other minor changes that may be difficult to detect in organisms above ground. Such factors as microclimate, soil nutrient status, moisture, type of leaf litter and presence or absence of resource competitors influence the successional changes in ectomycorrhizal fungi (Chai *et al*., 2013). These contributory factors are likely to vary from one fallow to another, even among fallows of the same age. Thus, although the present study showed that fallow age and available host species partly influence mycorrhizal fungi composition and diversity, it should be appreciated that several other factors determine fungal community succession in the study area.

Ectomycorrhizal fungal community succession involves short distance dispersal of fungal propagules to new roots, long-range dispersal and persistence of the ectomycorrhizae in the ecosystemand their interactions with soil and environmental conditions(Brundrett, 1991). Hepper (1985) and Brundrett and Kendrick (1990)have noted the importance of presence of infective propagules when root growth activity occurs, as roots have a limited period of susceptibility. Thus, rapid colonisation of the root system is required for an effective association (Abbott and Robson, 1984; Bowen, 1987). This observation is true for *Lactarius gymnocarpus*in this study whichappeared in young fallows and persisted in all fallows of different ages.

Spore abundance and dispersal also contribute to community succession patterns in ectomycorrhizal fungi.Fast dispersing fungi rapidly colonise a disturbed site. This may be true particularly for *Thelephora terrestris*thatis known for its ability to disperse by spores and quicklycolonize a plant host (Colpaert, 1999).

Seasonal variation in spore numbers of ectomycorrhizal fungi can occur (Ebbers *et al*., 1987; Dhillion*et al*., 1988; Gemma and Koske, 1988; Gemma *et al*., 1989; Giovannetti, 1985; Louis and Lim, 1987). Spores are usually less abundant during periods of ectomycorrhizal formation and become more numerous during periods of root senescence (Brundrett, 1991). Peak periods of spore production are generally thought to coincide with periods of fungal resource remobilization from senescing roots (Gemma *et al*., 1989; Hayman, 1970; Sutton and Barron, 1972). This hypothesis is supported by observations showing that spore production is greatest when root activity is interrupted by a long dry season or land clearing (Janos, 1980; Mosse and Bowen, 1968; Redhead, 1977). Theindications in the present study showa marked peak in ectomycorrhizal fungi diversity at Fallow Age 2, after a one year lag following clearing of land for agricultural purposes.

Production of external hyphae varies between species of ectomycorrhizal fungi. It also can be influenced by soil properties and is,therefore,an important determinant of mutualistic efficacy(Abbot and Robson, 1985; Graham *et al*., 1982; Gueye*et al*., 1987).Ectomycorrhizal fungi hyphae respond to soil heterogeneity (Brundrett, 1991). Thus, the varied soil characteristics of the fallows in this study may also contribute to fungal composition and diversity as observed in the stands. Harvey *et al*. (1976) have noted that most of forest ectomycorrhizal fungi in their study occurred within organic soil fractions where litter, woody debris and charcoal

decomposition was occurring. Thus, older stands provide a more conducive environment for fungal proliferation due to the availability of leaf litter and woody debris. It has been shown that hyphae of mycorrhizal fungi preferentially occupy soil organic material (Mosse, 1959; St John *et al*., 1983; Warner, 1984) where they produce fine, highly branched, septate hyphae that may have an absorptive function (Mosse, 1959; Nicolson, 1959). As stated earlier, however, different ectomycorrhizal fungi have different capabilities to produce extensive mycelia. Thus, those that are able to proliferate more quicklywill have a competitive advantage. To also confirm that woody species respond slower compared to fungi, it has been shown that roots respond to spatial and temporal variations in soil nutrient supply, but are less efficient at this than are mycorrhizal hyphae (Brundrett, 1991).

Some mycorrhizal fungi apparently can utilise organic or insoluble nutrient sources that are normally thought to be unavailable to plants (Brundrett, 1991). Similar experiments have shown rapid transport of carbon, nitrogen, phosphorus and water by hyphal networks of ectomycorrhizal fungi (Finlay and Read, 1986; Francis *et al*., 1986; Haystead*et al*., 1988; Read *et al*., 1985; Ritz and Newman, 1986). Ectomycorrhizal fungi thus respond earlier and faster to changes in the ecosystem than woody species. Ectomycorrhizal fungi from the genus *Lactarius*, which are facultative saprotrophs, are therefore likely to utilise nutrients more efficiently than the other species observed. Thus, the taxon can easily proliferate and persist in older stands.

5.2.2.Hostassociations of ectomycorrhizal fungi in fallows of different ages

The two *Lactarius* species (*Lactarius gymnocarpus* and *Lactarius* sp.) indicated host generality as they were associated with roots of *Acacia tortilis*, *Philenopteraviolacea, Combretum mossambicense* and *Kirkia acuminata*(Table 4.4). Most *Lactarius* species, including *L.*

gymnocarpus, are reported to be facultative saprotrophs (Verbeken and Buyck, 2002). Studies elsewhere in tropical Africa support this observation (see Thoen and Bâ, 1989; Härkönen*et al.*, 1993; Munyaziza and Kuyper, 1995; Buyck *et al.*, 1996), pointing to their ubiquity in Zambezian woodlands. Thus, more generalist relationships can be inferred in a number of tropical African ectomycorrhizae. *Amphinema byssoides*was associated only with two tree species, *Ziziphus mauritiana* (Rhamnaceae) and *Combretum mossambicense* (Combretaceae). Though the nutritional benefits of having such an association are obvious, the real reason for this uniquely isolated association is not known. Lack of more favourable hosts may be a reason to explain this association. This could be an important observation in the study area, since very few studies in ectomycorrhizal fungi associations have been carried out in Zimbabwe. Studies by Bâ *et al*. (2011) have reported only one ectomycorrhizal fungus in Zimbabwe, *Scleroderma verrucosum*. Thepresent study, therefore, raises more questions and calls for further work in this field within the context of Zimbabwe.

Generalist ectomycorrhizal fungi have an evolutionary advantage over specific ectomycorrhizal fungi, especially in this semi-arid region where rainfall patterns are spasmodic and anthropogenic disturbances are frequent. Several other fungal species, however, showed significant levels of specificity, with *Boletus* sp. only associating with *Philenopteraviolacea* in fallows of different ages. This type of specificity is less common among African host species and any removal of such a host is likely to impact negatively on the symbiont. Successof this *Boletus* sp. can, however, be attributed to its host species *Philenopteraviolacea* which is selected and usually spared for its traditional importance. This emphasises the significance of anthropogenic activities in shaping the ectomycorrhizal fungi flora of the area.*Thelephora terrestris* was observed only once,in association with *Acacia tortilis*. Since this fungus was only observed at

Fallow Age 2, the species could be opportunistic and is probably eliminated in older fallows. Of the unidentified ectomycorrhizal fungi species, Species B formed associations with both *A. tortilis* and *C. mossambicense*. Other unidentified species occurred singly, with Species A associating with *A. nigrescens*, Species C with *L. capassa*, Species D with *Grewia monticola* and Species E with *A. tortilis*. Specificity might be a consequence of these ectomycorrhizal fungi disappearing in other stands as favourable hosts also disappeared. Some of the unidentified fungi observed could have been endomycorrhizal or arbuscular mycorrhizal fungi that require further anatomical work for positive identification. The present study was, however, dedicated to ectomycorrhizal fungi associations. The dominance of *Acacia tortilis* seedlings in young fallows and consequently its frequent occurrence in fallows of all ages could be attributed to its successful association with ectomycorrhizae, which offers it a competitive advantage over other tree species (Tsamba *et al.*, 2015). This trend is supported by observations by Frost (1996) in miombo woodland ecosystems, who suggested that early death of some *Brachystegia* and *Julbernardia* seedlings may be attributed to seedlings' failure to establish any mycorrhizal associations before they shed their cotyledons.

5.2.3.Fungal succession in fallows of different ages

Ectomycorrhizal fungi may be early or late seral fungi, depending on the stage of community succession in a previously cleared piece of land (Deacon and Fleming, 1992). Multi-seral fungi also occur in all stand ages following land clearance and subsequent regeneration. This categorisation may explain the presence or absence of some ectomycorrhizal fungi in the sampled fallows. Most of the fungal species observed only occurred in young fallows and disappeared in older fallows.For example, *Thelephora terrestris* only appeared in young, two-year old fallows and disappeared in older stands. This ectomycorrhizal fungi species is known

for its ability to disperse and quicklycolonise a plant host from spores (Colpaert, 1999). The species may be designated as an early seral species. Other early seral species include Species A, C, D and E, appearingin fallows younger than 5 years. Early seral fungi are opportunistic, thriving only for a short period before more competitive species take over. The rest of the ectomycorrhizal fungi found in this study appeared across fallows of all ages. Species of *Lactarius* (*Lactarius gymnocarpus* and *Lactarius* sp.) represent such a group. These findings support those of Bâ*et al.* (2011), thus confirming the ubiquity of the genus. *Boletus* sp. also appeared in fallows across all ages, including two-year old fallows and the 14-year old fallows (oldest sampled). The genus represents a multi seral fungal group (Bâ *et al*., 2011). The unidentified ectomycorrhizal fungi, Species B also appeared in stands across all ages. *Amphinema byssoides* was observed at Fallow Ages3 and 10 only. The seral status of the species becomes difficult to explain. The species, however, more frequently occurred in older fallows. Fallows in the present study (14 years or younger) represent early successional stages of a savanna ecosystem because the study area is subjected to short clearance-fallowing-clearance cycles that result from increased demand for agricultural land. This tends to disrupt normal community succession. Results of the study, therefore, cannot fully help to describe the pattern of community succession among ectomycorrhizal fungi of the area. This also explains why late seral designates were difficult to identify due to the disruption of the normal community succession. A longer fallowing and recovery period is likely to provide a more complete picture of community succession that identifies ectomycorrhizal fungi that become more successful at later stages of succession.

5.2.4.Soil Characteristics

Ectomycorrhizal fungi have been reported as having symbiotic relationships with certain groups of plants even among some of the very harsh environmental conditions, which include saline soils and sites with heavy metals (Egerton-Warburton and Griffin, 1995; Jourand *et al*., 2010; Colpaert *et al*., 2000; Colpaert *et al*., 2004; Krznaric *et al*., 2009). These characteristics highlight the ubiquitous nature of ectomycorrhizae. Soils of the mid-Zambezi valley are unlikely to impede ectomycorrhizal growth. The *Bandate* soil, with pH of 5-6, falls within the optimal range for ectomycorrhizal growth (Kagama and Yamanaka, 2014). These conditions are, therefore, not limiting to ectomycorrhizae growth. It can be assumed that host species availability is one of the key determinants of composition and diversity of ectomycorrhizae in the study area.

5.3.Establishing the genetic characteristics of ECM across fallows and hosts species

5.3.1.PCR Amplification

Thesuccessful rRNA amplification revealed minor size polymorphisms in the PCR amplification. The size polymorphisms are presumably dueto variations in the size and/or number of introns present in theamplified segments in these conserved genes.This difference was explained further by digestion with restriction enzymes and performing RAPDs reactions.

5.3.2.RAPDs fingerprinting

The RAPDs reaction showed a distinct banding pattern, and complemented some of the initial suggestions on polymorphisms. The results suggested the existence of both inter- and intra-specific genetic diversity among the five ectomycorrhizal fungi species sampled. *Amphinema byssoides* sampled from the hosts,*Combretum mossambicense* and *Ziziphus mauritiana* showed

similar genetic characteristics, with a similarity score of 1 (Table 4.6). These isolates were also included in the same cluster. These findings suggested that the two isolates could either belong to the same individual fungus or very closely related individuals of the species. Ectomycorrhizal fungi are known to form extensive networks within the soil that interconnect several plants. This allows for sharing of nutrients among the host plants (Smith and Read, 1997). This also facilitates "communication" of the host plants (Bais*et al*., 2004). The two *Lactarius gymnocarpus* isolates sampled from *Acacia tortilis* in a two year old fallow and *Kirkia acuminata* in a ten year old fallow showed a genetic similarity of 0.25. They also appeared in the same cluster. This suggested that different host plant species may play a role in the genetic evolution of ectomycorrhizal symbiont, an evolutionary characteristic associated with nutritional requirements. Studies by Kwiatkowski *et al*. (2012) have suggested co-evolution between host and symbiont. Akiyama *et al*. (2005) have confirmed this host manipulation on arbuscular mycorrhizal fungal activities. An isolate of *Lactarius gymnocarpus* from *Acacia tortilis*on a six-year old fallow had a genetic similarity of 0.167with an isolate from *Acacia tortilis* in a two-year old fallow. These findings to suggest the role of fallow age in selecting ectomycorrhizal fungi with specific genetic make-up. The two *Lactarius* sp. isolates from *Combretum mossambicense* (seven-year old fallow) and *Acacia tortilis* (eight-year old fallow) also showed 0.33 similarity. The isolate were also clustered together. This emphasises the host influence on genetic make-up of the ectomycorrhizal symbiont.

The effect of fallow age was more evident in two *Boletus* sp. that shared the same host species, *Philenopteraviolacea*. The two species were sampled from fallows of different ages, two-year and five-year old fallows. The two species had a genetic similarity of 0. This differenceexplains the fact that although the two *Boletus* sp. showed similar morpho-anatomical characteristics, they

certainly represent two separate distinct taxa. The single *Thelephora terrestris* isolate was characteristically different from any other ectomycorrhizal fungi isolate, showing 0 similarity with any other ectomycorrhizal fungi species sampled. These findings may be important in explaining the lower success rate in dominance of this fungal species compared to the rest of the ectomycorrhizal fungi species observed in the area.

The genetic diversity, especially in one species (as shown by *Lactarius gymnocarpus*) may be an important factor that enhancessymbiotic relationships with several host species thereby increasing its survival capacity as opposed to host specific ectomycorrhizal fungi. Thoen and Bâ (1989),Härkönen*et al.* (1993),Munyaziza and Kuyper (1995) andBuyck *et al.*(1996)have reported low host specificity among most African ectomycorrhizal fungi species,thus supporting the fact that the majority of ectomycorrhizal fungi utilise the most available host tree species.

5.3.3.PCR-RFLP

RFLPs were detected inthe PCR amplification products upon digestion with *Rsa*I.These generally indicated varying degrees of genetic diversity in the ectomycorrhizal fungi samples. Fingerprinting patterns underscored the genetic differences revealed by the RAPDs. However, the number of restriction fragments produced for most of the samplesdid not produce a reliable comparison of the relative genetic similarity of the samples.Ectomycorrhizal fungi samples that produced single bands, though of different sizes indicatedintra- and interspecific genetic variability. The *Amphinema byssoides* samples continued to show similar RFLP profiles further confirming results of the RAPDs.The additional digestions carried out using restriction enzymes *Hin*fI, *Alu*I and *Hae*III showed varying genetic profiling on the ten ectomycorrhizal samples, with *Hin*fI showing more polymorphisms. However, consistently similar patterns were evident in

the two *Amphinema byssoides* samples taken from two different host species. This further confirmed findings from the other results obtained, suggesting that the two samples were hyphae from the same specimen, forming an underground communication highway (Bais*et al*., 2004).The *Boletus* sp.samples showed similar banding patterns in *Hin*fI, *Hae*III and *Alu*I digests though from different fallow plots, showing the retained genetic similarities of these two samples, characteristic of different individuals sampled of one species (Henrion*et al.*, 1992). The effect of fallow age on fungal genetic characteristics was still evident, especially in*Lactarius gymnocarpus* samples which had different banding patterns. Also, *Lactarius* sp. sampled from *Combretum mossambicense* and *Acacia tortilis* showed different banding patterns suggesting effects of host species on genetic characteristics of the symbiont. These findings confirm observations from other literature inferring that hosts select and shape their associated symbionts (Schluter and Foster, 2012; Kiers *et al*., 2011). The solitary *Thelephora terrestris* sample showed similar RFLP profiles with *Boletus* sp. in *Hin*fI,*Alu*I and *Hae*III digestsbut however maintained its unique banding pattern with the *Rsa*I digest. This justifies the need to perform several digests with different restriction enzymes in order to make sound conclusions on genetic similarities.

Similarity tests using the Jaccard coefficient and clustering were performed on the PCR-RFLP digestion results. Upon analysing the *Rsa*I digest results, similarity ranged from 0 to 1,however, with little to no genetic similarity in the fungal samples, ranging mostly from 0 to 0.33.This meant that minor genetic variations were observed in the sampled fungal species from digestions with this restriction enzyme. The two species, *Lactarius gymnocarpus* sampled from *Acacia tortilis* in Fallow Age 2 and *Boletus* sp. sampled from *Philenopteraviolacea* in Fallow Age 2 showed a similarity score of 1. The reasons for this similarity, though of interest, are unknown. The two *Amphinema byssoides* samples showed consistent similarity and clustering as with all

other previous results, and as stated earlier, indicating that this could have been hyphae belonging to the same individual fungus.However, this could also explain low intraspecific genetic diversity in this species, resulting in very closely related individuals of the species. Noticeably, the *Lactarius* sp. sampled from *Combretum mossambicense* in Fallow Age 4which showed a similarity score of 1 with the two*A. byssoides* samples and were clustered together show that shared hostselection could be due to certain genetic characteristics of the fungi(Akiyama *et al*., 2005).However, due to limited restriction sites identified by the enzyme *Rsa*I, this result could be a chance encounter. The enzyme *Rsa*I expressed low genetic variability in the ectomycorrhizal fungi samples, as shown by the few branches in the dendrogram. With a cophenetic correlation coefficient (CP) value of 0.999, this dendrogram fitted well with the results of the *Rsa*I digest.

Upon analysis of the *Hinf*I digest, similarity score ranged from 0 to 1, showing varying degrees of genetic similarity in the fungal samples. The two *Boletus* sp. samples showed similar restriction patterns with a similarity score of 1 and were clustered together. They also were sampled from one host species, *L. capassa*, though in different fallows of two and five years. This is generally expected because restriction sites in samples of the same species normally occur at the same position thus digestion yields similar fingerprinting patterns (Henrion*et al*., 1992). A similar observation was also made in *A. byssoides* samples which were clustered together.The *T. terrestris* sample showed similarity of 0.833 with the two *Boletus* sp. samples thus also clustering together. The reason for this clustering could not be determined. Also, the *L. gymnocarpus* were not clustered together,showing someintraspecific genetic variability in this species. Undoubtedly, this is a characteristic that favours its host generality tendencies (Thoen and Bâ, 1989).

Analysis of the *Alu*I digest pattern showed similarity scores ranging from 0 to 1 with generally minor similarity observed, ranging mostly between 0 and 0.57. The *Lactarius gymnocarpus* species sampled in Fallow Age 10 was differentfrom all the sampled ectomycorrhizal fungi after digestion with restriction enzyme *Alu*I and it consequently associated with a unique host (*K. acuminata*). The two *Boletus* sp. samples had a similarity score of 1, as well as the two *A. byssoides* samples. They were also clustered together. These findings confirmed previous results from digestions with other restriction enzymes in this study. The genetic variability of the three *L. gymnocarpus* samples is further confirmed, supportingreports onintraspecific genetic polymorphismsof this fungal species (Bâ*et al*., 2011).

Digestion with *Hae*III also showed varying levels of genetic similarity, with scores ranging from 0 to 1. The two *Boletus* sp. samples, *Thelephora terrestris* from *A. tortilis* sampled in Fallow Age 3 and *Lactarius* sp. sampled from *A. tortilis* in Fallow Age 7all showed a similarity score of 1 upon analysis of the*Hae*III digestion and were clustered together. As has been earlier indicated, the clustering of samples from the same species is sometimes expected upon digestion.The two *Boletus* sp. samples also had a similarity score of 1. They also appeared in the same cluster. These findings further confirm the shared similarity of samples of the same species upon digestion with certain enzymes due to similarities in restriction sites (Henrion*et al.*, 1992). Remarkably, the *Lactarius* sp. sampled from *C. mossambicense* in Fallow Age 4 and *L. gymnocarpus* sampled from *A. tortilis* in Fallow Age 6 had a similarity score of 0.8 and appeared in the same cluster. The reasons for their clustering may be due to similar restriction sites in these two species.

5.4.Establishing whether fallow age or host specificity determines mycorrhizal status of mycorrhizal tree species

The Spearman rank correlation showed no significant correlation between fallow age and mycorrhizal status of host tree species. This seemed to suggest that specific host preference of the fungi as opposed to fallow age determines mycorrhizal status of a tree species. These findings are contradictory to what is reported elsewhere wheretropical ectomycorrhizal fungi are reported as being less specific (Thoen and Bâ, 1989; Onguene and Kuyper, 2002) thantheir temperate counterparts (Tedersoo and Nara, 2010; Smith and Read, 2008). The GLM showed that the mycorrhizal status of any given host tree species does not dependon the fallow age or specific host species, but to other factors not investigated in the present study. These factors may include appropriate host genotype, nitrogen, other soil nutrientsor microenvironment (Chai *et al*., 2013). The GLM support findings that suggest low or non-significant host specificity among fungal species.However, the Raup-Crick cluster analysis indicated that fallow age rather than host preference determines mycorrhizal status of any tree species. These findings suggest low host specificity, with prominent host tree species that included *Philenopteraviolacea*, *Acacia tortilis* and *Combretum mossambicense*sharing the same symbionts. Thus, fallow age played a prominent role in determining mycorrhizal status of host tree species. Thoen and Bâ (1989),Härkönen*et al.*(1993), Munyaziza and Kuyper (1995),and Buyck *et al.*(1996) all support this view, indicating that the majority of ectomycorrhizal fungi tend to utilise the most available host tree species, especially in tropical areas of Africa. This, however, could partly be a result of chance due to the randomised procedure adopted in the selection of sampling plots.

The present study confirmed the unreliability of epigeous sporocarps as indicators of mycorrhizal presence as most of the fungi either produced inconspicuous sporocarps or none at

all. A similar observation has been made by Tedersoo *et al*. (2007). Most of the mycorrhizal associations were identified from tree rootlets. In regions of poor soil nutrient status and low rainfall, mycorrhizal fungi reproduce vegetatively by means of hyphae. Hence, a low proportion of resources is channelled towards sexual reproduction (Lilleskov*et al*., 2002). In the present study, five ectomycorrhizal species were identified:*Lactarius gymnocarpus, Lactarius* sp., *Thelephora terrestris*, *Amphinema byssoides* and *Boletus* sp.The small number of ectomycorrhizal fungi observed in the present study is not unique in tropical African tree communities. Several studies have confirmed the low diversity of ectomycorrhizal fungi in African tropical regions despite the abundance of mycorrhizal tree species. Results of the present study indicate that the lower number of host tree speciesresulting from agricultural activity induced land clearing could have impacted on the diversity of ectomycorrhizal fungi. This, therefore, must be considered as one of the negative impacts of intensified cultivation.

CHAPTER 6.SYNTHESIS

This thesis provided baseline information on ectomycorrhizal fungi diversity in a semi-arid region in the mid-Zambezi area. This area of research, though of considerable importance, has been largely ignored. Most studies havefocused on above ground ecosystem processes, as emphasised throughout this thesis. The main objective was to examine how the practice of fallowing in the mid-Zambezi valley affects biodiversity at the detritivore trophic level. Its main thrust was on establishing ectomycorrhizal fungi diversity in fallows, the patterns of community succession in ectomycorrhizal fungi over a fallow chrono-sequence.

Farming has a direct impact on woody species, especially on their diversity (Tambara *et al*., 2012a). Tambara *et al.*(2012b) also demonstrated its effects on arthropod diversity. The present study showed that plant use results inselective retention of some host species. This selective retension of hosts impacts on fungal species composition and diversity (Tsamba *et al.*, 2015). Family Fabaceae which dominates fallows, is common in the study area, and includes many known ectomycorrhizal tree species (Bâ *et al*., 2011). Speculations from the study indicate a link between dominance of *Acacia tortilis* and its role as a major mycorrhizal host, thus inferring the role of mycorrhizal associations in the species success. This study supported results reported elsewhere that indicate the dominance of fungus genus *Lactarius* in tropical African woodlands. The few ectomycorrhizal fungal species observedcould partly result from an absence of favourable host species, or primarily from the semi-arid environment of the area which is unfavourable to ectomycorrhizal fungi. Adaptive species therefore thrive, in particular, generalist fungi, like species of genus *Lactarius*that are facultative saprotrophsand those that possess such dormancy mechanisms like spores or other propagules (Fox, 1986). *Boletus* species and its host *Philenopteraviolacea*demonstrated the impact of anthropogenic selective felling on species diversity, both on plant and fungi, with the fungi's survival predominantly determined by its host which is selected for despite the fungi's specificity tendencies. It is of significance to note that variation in ectomycorrhizal community has profound effects on an ecosystem, particularly its functioning and structure (van der Heijden*et al.*, 1998). The cascading effects of such activities go largely unnoticed since they are usually indirect and long term(van der Heijden*et al.*, 1998). There is, therefore, need to have a wholesome conservation strategy as opposed to species or taxon targeted conservation.

This study also showed that fallow age does have an effect on ectomycorrhizal fungi species, with some negligible degree of dependence on available host species. Younger fallows were dominated by opportunistic ectomycorrhizal fungi species. Variation in ability to disperse or persist in the soil and quickly colonise root tips through spores or other propagules are the likely determinants of initial ectomycorrhizal fungi community composition immediately following disturbance (Deacon and Fleming, 1992; Lilleskov and Bruns, 2003). With increased fallow age, these ectomycorrhizal fungi species are either completely replaced by other ectomycorrhizal fungi taxa or generally decline in abundance and become insignificant (Visser, 1995). This study showed a loose trend betweenectomycorrhizal species diversity and host tree species diversity. This observation suggests that ectomycorrhizal fungi community succession is an independent event from plant community succession in the mid-Zambezi area. This observation therefore points to other factors that were not investigated in the present study. LeDuc*et al.* (2013) havestated that mechanisms behind changes in ectomycorrhizal fungi communities and succession systems remain unclear. Though this study showed that the soil characteristics in the study area are unlikely to impede ectomycorrhizal growth, soil nitrogen availability has been singled out as one of the influentialnutrients in determining ectomycorrhizal fungi communities (Taylor *et al*., 2000; Lilleskov*et al*., 2002; Cox *et al*., 2010). It has been hypothesised that fluctuations in this nutrient may be a driver in ectomycorrhizal community composition and fungal species development in the different stands (Abuzinadah and Read, 1986; Finlay *et al*., 1992). This may be important, especially considering the dominance of the nitrogen fixing *Acacia* species in the area, which may be an important driver in determining ectomycorrhizal fungi species across all fallow stands. The results of thisstudy also highlight that multi-seral fungi have a higher chance of survival due to their early establishment which provides them with greater chance to proliferate, exploiting larger areas for nutrient uptake and early establishment

which results in associations with several tree species earlier than other fungal species. However, work by Twieg*et al.* (2007) showed ectomycorrhizal fungi community composition stabilising at 65 years. Thus, categories like early-seral, multi-seral and late-seral were used loosely in the present study.

The amplified rRNA gene, in combination with other DNA probes, has been used in the characterisation of genetic variation of field ectomycorrhizal fungi populations (Henrion*et al.*, 1992). RAPDs have been shown to be a more reliable technique for comparing genetic similarities than RFLPs (Garcia *et al.*, 2004). While RAPDs are simpler to perform in principle compared to RFLPs (Williams *et al.*, 1990; Caetano-Anolles*et al.*, 1991; Paran*et al.*, 1991; Welsh *et al.*, 1991), they are usually dominant rather than co-dominant markers (dos Santos *et al*, 1994). RAPD patterns are also affected by different concentrations of reactions and cycle conditions (Weeden, *et al.*, 1992). Thus, a combination of both methods was used in this study. Consistent results were shown in the *Amphinema byssoides* for both the RAPDs and all RFLPs using the different restriction enzymes. It has been shown that RFLPs show more polymorphisms depending on enzyme (Galal, 2009). As was expected, similar species tended to cluster together, e.g. *Boletus* sp., *Lactarius* sp. and *Amphinema byssoides*. This is because of similarities within the restriction sites in same species, as opposed to different species. Intraspecific genetic variation was more evident in *Lactarius gymnocarpus*. This species also showed generalist tendencies, hence its survival. Studies have shown that ectomycorrhizal fungi are able to select plant genotypes on seedlings that support high levels of ectomycorrhizal colonisation (Rosado *et al.*, 1994). However, the present study seemed to suggest that plants also play an important role in selecting ectomycorrhizal fungi with favourable genetic

characteristics for colonisation, a result shown by high genetic similarity among ectomycorrhizal fungi that share the same host species. Studies by Akiyama *et al.* (2005) support this observation.

The results from this study showed that fallow age influenced ectomycorrhizal fungi species diversity, in accordance with community succession. This shows the strong influence of natural community succession patterns in the area. Tree species played a minor and negligible role in determining ectomycorrhizal fungi in the area. The study also supported other studies which state that ectomycorrhizal fungi species in tropical Africa are generalists (Thoen and Bâ, 1989; Onguene and Kuyper, 2002) as opposed to temperate forests where most ectomycorrhizal fungi are host specific (Smith and Read, 2008).

6.1.Effects of fire on ectomycorrhizal fungi diversity

Fire has a direct and major selection pressure on ectomycorrhizal fungi (Dahlberg, 2002). Few ectomycorrhizal fungi have evolved adaptive measures to fires (Dahlberg, 2002). This is a major concern in the mid-Zambezi area where fires are common during land clearing. It is also not uncommon for fires to escape and burn uncontrollably, destroying vegetation. However, fires in the area are commonly of low intensity. Forest fires cause radical changes in saprotrophic fungi (Moser, 1949; Petersen, 1970; Holm, 1995; Rahko, 1997). They also cause drastic changes and sometimes complete elimination of fruiting ectomycorrhizal fungi (Petersen, 1971; Holm, 1995). When fire intensity is low, ectomycorrhizal fungi species composition remains mostly unchanged (Rahko, 1997; Dahlberg *et al.*, 2001).

Fire has been known to cause significant losses in ectomycorrhizal fungi biomass, especially in the first 20 cm of the soil surface, comprising of the litter layer and organic soil horizons

(Dahlberg, 2002). Mikola*et al.* (1964) have reported that ectomycorrhizal fungi protected by the upper soil layer survive the detrimental effects of heat and are therefore an important link to ectomycorrhizal fungi communities post-fires. Interestingly, certain fungal spore germination is stimulated by heat (El-Abyad and Webster, 1968; Jalaluddin, 1967), however, not in ectomycorrhizal fungi. In ectomycorrhizal fungi, addition of charcoal has been shown to improve germination by removing inhibitory compounds in some *Lactarius* species (Fries and Sun, 1992). This may be an important contributing factor in the dominance of this taxon in fallows of the study area. Fires also affect soils, which in turn affect plant and microbial communities, particularly mycorrhizal fungi. Slash pile burning, which is common in the study area, has similar injurious effects with bigger fires. Jimenez-Esquilin *et al.* (2007) have studied the effects of slash pile burning on soils. In their study, it has been shown that temperatures under each pile reach up to 300°C. Their findings have also shown that slash piles increase soil pH and extractable nitrogen and phosphorous while decreasing total carbon levels. They, therefore, have concluded that slash pile burn may negatively impact on forests at localised scales due to alteration in specific soil properties associated with plant and fungi re-establishment. This information may, therefore, be extrapolated to the current study, especially because slash and burn agriculture is common in the area. Fire may therefore be a major determinant of the ectomycorrhizal fungi community structure of the mid-Zambezi area, affecting local soil characteristics and properties important for both plant and fungal restoration.

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APPENDIX

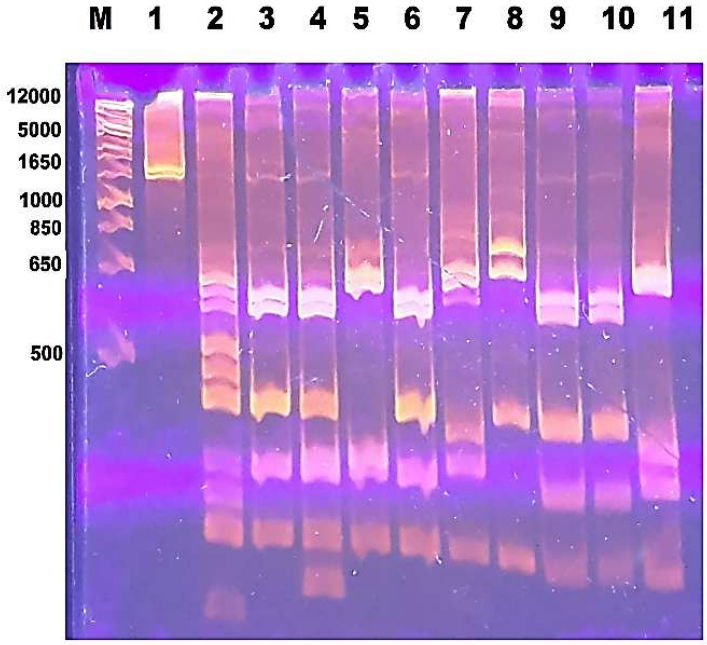

Figure A1: Polyacrylamide gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Hinf*I. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 5 % polyacrylamide gel in Tris borate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 1kb Ladder Plus. Lane 1 is the undigested PCR fragment. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.

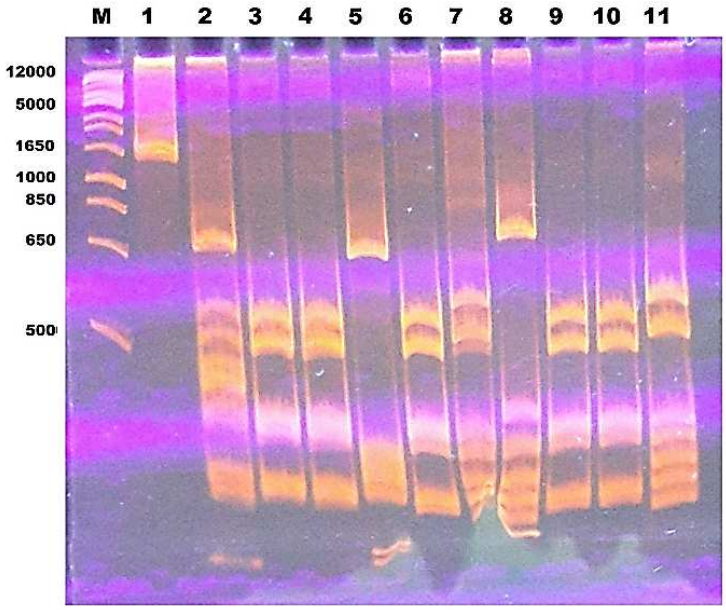

Figure A2: Polyacrylamide gel showing *Taq* DNA polymerase activity of PCR-RFLP digestions with the restriction enzyme *Alu*I. Fifteen µl of the PCR was mixed with 3 µl of 6X loading dye and electrophoresed through 5 % polyacrylamide gel in Tris borate-EDTA buffer and stained with ethidium bromide (0.1 µg/ml). The gel was visualised using a UV transilluminator and photographed using a digital camera. Lane M is in the 1kb Ladder Plus. Lane 1 is the undigested PCR fragment. Lane 2- 11 are genomic DNAs from 10 different fungal isolates digested with the restriction enzyme.