Ectomycorrhizal characterisation, species diversity and community dynamics in *Pinus patula* Schelcht. et Cham. plantations

by

Greer Leigh Hawley

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Supervisor: Dr J. Dames

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Declaration

I the undersigned hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signed…….. Date……..

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ABSTRACT

Ectomycorrhizal (ECM) associations are important elements of forest biomes, connecting and transferring nutrients through an intricate and complex system of hyphal networks, ensuring plants of the nutrients they require, in nutrient poor soil. ECM research and particularly investigations into the diversity of the fungal partners has not received much attention in South Africa, hindering the advance of research in this field. This has been attributed to the difficulty of identifying the mycobionts involved in the symbiosis. The objectives of this study were to examine the ECM fungal diversity associating with *Pinus patula*, in selected forest plantations in Mpumalanga, South Africa. Both morphological and molecular techniques were used to identify specimens of both sporocarp collections and ECM root tip morphotypes. Morphological analysis of the ECM root tips involved characterisation of root morphology such as colour, branching and texture, and anatomical analysis examined hyphal arrangement in the root mantle and rhizomorphs. Molecular analysis involved sequencing of the Internal Transcribed Spacer (ITS) region and comparative BLAST analysis. Twenty-four sporocarp species were identified from 13 genera, namely: *Amanita*, *Boletus*, *Clavulina*, *Inocybe*, *Lactarius*, *Rhizopogon*, *Russula*, *Scleroderma*, *Suillus*, *Tricholoma*, *Thelephora*, *Tomentella* and *Xerocomus*. ECM root tip analysis led to the characterisation of 7 wild-type morphotypes identified as an *Albatrellus* sp., 2 *Amanita* species, a *Rhizopogon* sp., *Thelephora terrestris*, a *Tomentella* sp. and *Scleroderma citrinum*. A secondary objective was to determine whether fertilisation treatments within the study sites were responsible for differences in fungal species community structure. No evidence of a change in species diversity or shift in species composition was encountered. It is envisaged that these comprehensive ECM descriptions will be used as reference material to stimulate continued research in this field in South Africa.

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1. INTRODUCTION

1.1 What are mycorrhizal associations?

Mycorrhizal associations are described as a mutual symbiosis occurring between a fungus and its host plant. This interaction occurs in and on the root tips, the interface of nutrient uptake in plants and involves a bi-directional exchange of nutrients (Smith and Read, 1997). Organic and inorganic nutrients in the soil are sourced through extensive hyphal networks (Kendrick, 1992) that dramatically increase the surface area for nutrient uptake. The nutrients that are absorbed and assimilated by the fungus are transferred to the plant host, in exchange for photosynthetically derived compounds (Smith and Read, 1997). These associations are common, with approximately 90% of all land plants bearing one or the other and sometimes more than one type of mycorrhizal association (Smith and Read, 1997).

There are several types of mycorrhizas that can be identified by recognition of their structural differences of formation (Smith and Read, 1997). The two most common mycorrhizal types are:

- Arbuscular mycorrhiza (AM), which are formed by fungal members of the orders Glomerales, Diversisporales, Paraglomerales and Archaeosporales (Phylum Glomeromycota), and are represented by the families: Glomeraceae, Gigasporaceae, Diversisporaceae, Acaulosporaceae, Paraglomeraceae, Geosiphonaceae and Archaesporaceae (Schüßler *et al.,* 2001). AM fungi associate with a wide range of plant groups such as angiosperms, gymnosperms, pteridophytes and mosses (Pocock and Duckett, 1985) many of which have been recorded as being AM (Smith and Read, 1997). AM fungal hyphae are aseptate, characterised by intracellular penetration of hyphal structures such as arbuscules, vesicles and intracellular coils, into plant cortical cells and may also develop intercellular hyphal networks that run along the length of roots, as well as intraradical and extraradical spores (Smith and Read, 1997).

- Ectomycorrhizas are characterised by a fungal mantle (sheath) surrounding the lateral roots of plants, an intercellular hyphal network around the epidermal and cortical cells forming a Hartig net and extra-radical growth of hyphae and rhizomorphs (Figure 1.1). Fungal members of the Basidiomycota dominate this type of mycorrhiza (45 genera) with members of Ascomycota occasionally forming ectomycorrhizas (18 genera), and Zygomycota very rarely forming ectomycorrhizas (members of the genus *Endogone*) (Carlile *et al.*, 2001, Brundrett *et al.*, 1996, Smith and Read, 1997). Genera such as *Amanita*, *Boletus*, *Cantharellus*, *Cortinarius*, *Inocybe, Laccaria*, *Lactarius, Rhizopogon*, *Russula*, *Suillus* and *Xerocomus* are common and well-recognised ectomycorrhizal (ECM) Basidiomycota genera (Cairney and Chambers, 1999). Although ECM fungi are represented by approximately 6000 species, development of ECM associations appear to be limited to a narrow range of plant host species (Kendrick, 1992). ECM fungi are considered to form more species-specific associations, while AM fungi are generalists. However, some ECM fungal species will associate with a wide range of hosts, for example, *Pisolithus* spp. that associate with 20 confirmed host plant genera (Cairney and Chambers, 1999), as opposed to members of the genus *Suillus* that associate exclusively with plant hosts within the family Pinaceae (Dahlberg and Finlay, 1999). ECM fungi generally, but not without exception, associate with perennial woody tree species belonging to plant genera that commonly include: *Abies*, *Betula*, *Fagus*, *Picea*, *Pinus*, *Populus*, *Quercus* and *Salix*, (Europe and America), and *Acacia, Eucalyptus*, *Nothofagus* in Australia (Brundrett *et al*., 1996), and members of Caesalpiniaceae and Dipterocarpaceae in Southern Africa (Högberg, 1986). Many of the plant species naturally found in the Northern Hemisphere are planted in managed forest plantations in the Southern Hemisphere, such as *Pinus*. Although ECM plant host diversity appears to be limited, plant hosts from 130 genera in 43 plant families have been recorded (Carlile *et al.*, 2001).

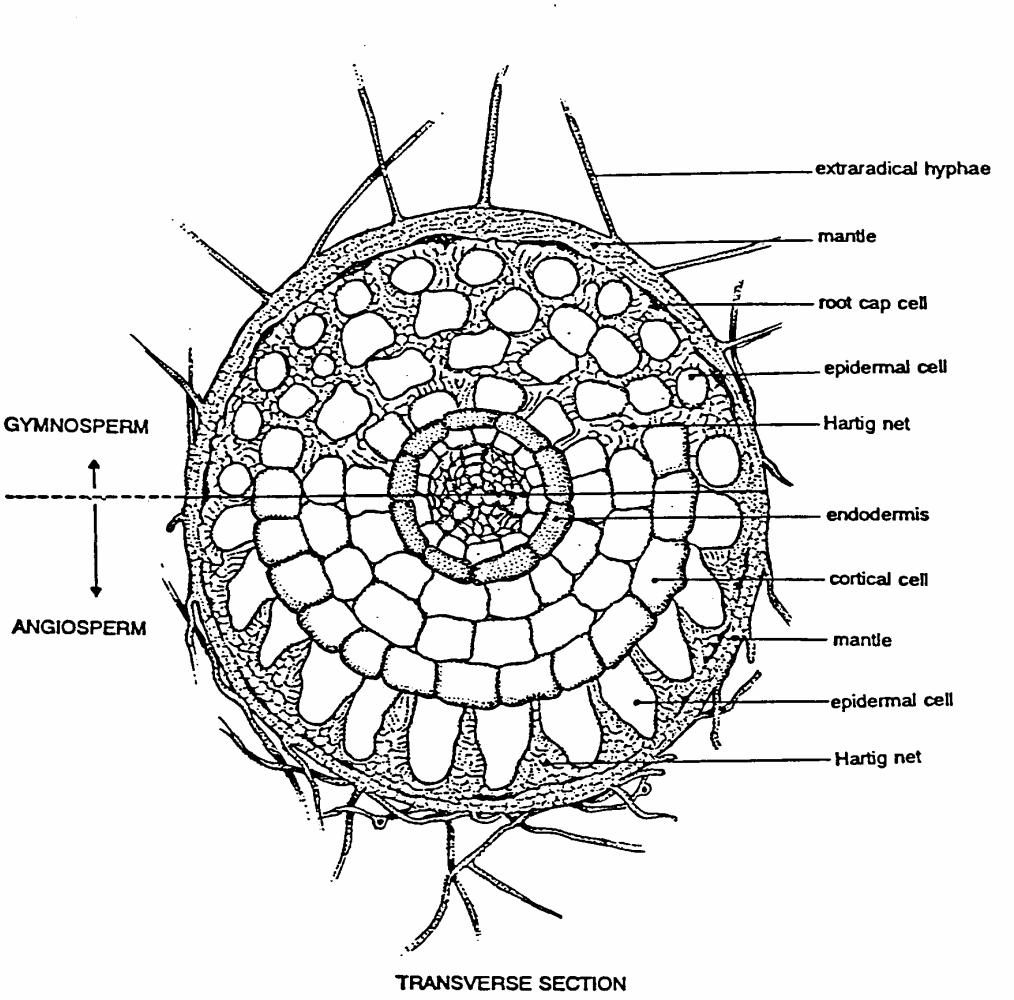


Figure 1.1 Section diagram of an ECM association including fungal and plant partners, demonstrating the key distinguishing features that characterise ECM (Brundrett *et al*., 1994).

There are three other associations that are worth mentioning briefly, namely ectendo-, ericoid- and orchid mycorrhizas. Ectendo- associations combine features of AM and ECM in the sense that there is a Hartig net, thin to no mantle and intra-cellular hyphae (Smith and Read, 1997). Ericoid and orchid mycorrhizas associate with hosts of members of Ericaceae and Orchidaceae, respectively. The prime focus of this study is on ECM associations, and further discussion will concentrate on ECM unless indicated otherwise.

1.2 Ectomycorrhizal life strategies

1.2.1 Colonisation

ECM fungi exist in the rhizosphere as emanating hyphae, extra-radical hyphae and aggregations of hyphae called rhizomorphs, where the primary function is nutrient acquisition and translocation. For the colonisation of new lateral plant roots to occur, propagules such as viable spores (oidia) and/or hyphae in the rhizosphere must be present. Lateral roots may also become colonised by a fungus already associated with the plant up-root by developing acropetally with the new growth (Smith and Read, 1997). Colonisation of a host root depends on recognition both by the fungus and the host plant, compatibility of the two organisms and the colonisation potential of the fungus (Smith and Read, 1997).

Spores, as propagules, undergo germination, which is thought to be promoted by host plant root exudates. Spore germination of AM spores has been shown to be stimulated by flavonoids and polyamines produced in plant root exudates (Poulin *et al.*, 1993). Using single spore cultures of *Rhizopogon luteolus*, *Suillus luteus* and *Suillus granulatus*, Theodorou and Bowen (1987), demonstrated high percentage germination (54 ± 10%) in the presence of *Pinus radiata*, suggesting that ECM fungal spore germination was stimulated by plant host root exudates. The germination response was also found to be specific, as germination was not stimulated by *Eucalyptus globulus*, *Medicago truncatula*, *Trifolium subterraneum* or *Lolium perenne*. Ali and Jackson (1988) provided evidence for different requirements for spore germination. Their results showed germination rates of up to 96% of *Paxillus involutus*, associated with birch trees in untreated soil, but spores of *Laccaria laccata* and *Hebeloma crustuliniforme* were only stimulated, in the presence of birch, in a mineral salts medium, but not in soil. Miller *et al*., (1994) showed that spores can be effective propagules for at least a full growing season where spores of *Suillus brevipes*, *Suillus tomentosus*, *Lactarius scrobiculatus*, *Rhizopogon subcaerulescens* and *Rhizopogon rubescens* persisted in the litter layer from fall to spring.

Germination involves the production of a germ tube that elongates, shown by Ali and Jackson (1988) to grow towards plant host roots. Upon contact, plant-fungus recognition processes are initiated. Giollant *et al*., (1993) reported the presence of recognition binding sites on plant roots of spruce and pines for a lectin protein that was detected on the hyphal walls of *Lactarius* spp. Microfibrils, found linking *Pisolithus tinctorius* to *Eucalyptus urophylla* roots, induced by contact after recognition, contained evidence of glycoprotein secretion by fungal hyphae that have been found to act as an adhesive to the plant root (Lei *et al*., 1990).

On contact with compatible associates, a number of metabolic and structural changes occur within the cells of the plant and hyphae. Biochemical and mechanical changes are driven by regulation of gene expression to form a complex recognition system, necessary for the structural development of symbiosis. Changes in gene expression and metabolism of protein production are induced by symbiosis and involve the up/down regulation of polypeptides of both the fungus and the host plant (Hilbert and Martin, 1988). The study conducted by Hilbert and Martin, (1988) highlighted three important observations with regards to the soluble polypeptide expression in associated and non-associated *Eucalyptus globulus* roots with *Pisolithus tinctorius* mycelia. Firstly, a reduction of 50% (fungal) and 80% (plant) of the total polypeptides was recorded when the species were in mycorrhizal association compared to the expression of free-living mycelia and non-colonised *E. globulus* roots. Secondly, polypeptides that were expressed in association were already expressed in the free-living organisms. Thirdly, specific mycorrhizal associated polypeptides were generated. In this study, Hilbert and Martin (1988) identified 10 ectomycorrhizal specific polypeptides, and thereby introduced the term ectomycorrhizans. Similar changes in gene expression were demonstrated by Hilbert *et al*., (1991) observed that during early stages of colonisation of *Eucalyptus globulus* by *Pisolithus tinctorius*, seven symbiosis-related (SR) proteins were identifiable. Tagu and Martin (1996) who also studied the *Eucalyptus*/*Pisolithus* association introduced the term ectomycins, which include hydrophobins, which are cell wall proteins that are up-regulated during symbiotic development. Although the studies only

concentrate on a single set of species, it can be assumed that SR genes are homologous, and that this model can therefore be expanded into a general understanding of gene expression during fungal infection. However, when considering the specificity of some host plant-fungal associations, it is likely that a diversity of different SR proteins exist that can be associated with specific plant-fungus associations. Additionally, upon contact of the ECM fungus with the plant host, epidermal cells undergo physiological changes, from containing numerous endoplasmic reticula to being largely vacuolated (Smith and Read, 1997).

The structural formation of the association occurs once the fungus has made contact with the apex of an uncolonised lateral root. Primarily, the formation of a Hartig net occurs directly behind the apex of the root. On contact, the fungus grows around the root and acropetally to keep up with root elongation. Once the lateral root has ended elongation, a swelling, recognised as an ECM root tip, is formed (Smith and Read, 1997). The cause of swelling is hypothesized to be induced by an indole hypaphorine, inhibiting auxin activity, preventing root elongation and resulting in root swelling (Reboutier *et al*., 2002).

Emanating hyphae and rhizomorphs then develop from the mantle and extend out into pockets of nutrient rich soil, forming dense networks. These hyphae function as transporters of nutrients and potential propagules for the colonisation of new lateral roots.

1.2.2 Sporocarp formation and Sporulation

Sporocarp formation is the sexual reproductive stage of a fungus, the function of which is spore dispersal of the species. Sporocarps are the result of two monokaryon hyphae that meet to form dikaryon hyphae with a pair of sexually compatible nuclei. The cell is now technically diploid, but contains two haploid nuclei (Smith and Read, 1997). Occasionally, the hyphae may contain nuclei from more than two individuals and are then termed polypoid (Carlile *et al.,* 2001). Compatibility of strains and nuclear migration are controlled two by unlinked genetic factors, A and B. Factor A encodes transcription factors that

regulate the development of the dikaryotic mycelium. Factor B is thought to encode for pheromones and receptors that control interactions between the nuclei of the two strains (Smith and Read, 1997). Dikaryotic hyphae then either form ECM associations or sporocarps (Smith and Read, 1997). In case of the latter, the hyphae elongate up towards the soil surface. A specialised hyphal layer called the hymenium undergoes meiosis to produce spores (Carlile *et al.,* 2001). The hymenial hyphae may be exposed or enclosed and are known as basidia, asci or sporangia, according to the ontogeny of the spore (Carlile *et al.,* 2001) and are borne in corresponding sporocarps known as basioma and ascoma. The position of the hymenial layer may be exposed, forming along gills, within pores or along spines, or enclosed within the sporocarp. According to Hibbett *et al.*, (1997) gilled sporocarp morphology has evolved at least six times, and enclosed ball-like morphology, at least four times, based on the sequencing and phylogenetic analysis of nuclear and ribosomal DNA. Sporocarp morphology is therefore not monophyletic, i.e. descended from a single origin. An example of paraphyly is the enclosed sporocarp morphologies observed in the phylum Basidiomycota (genus *Scleroderma*) and in the phylum Ascomycota (genus *Tuber*).

The fruiting bodies of Basidiomycota ECM, known as sporocarps (or basidioma), are often presented with a stipe, pileus (cap), and lamellae (gills) or pores, although the variety of sporocarp morphology is large. Fruiting bodies develop only from the secondary mycelia, initially forming a primordium, after which, the hyphae undergo elongation, extending a stipe to a suitable position to release spores borne on the fertile hymenium (Kendrick, 1992). The hyphae in the secondary mycelium characteristically have clamp connections, which are necessary to maintain the dikaryotic state of the mycelium. The spores develop on basidia, which develop four protrusions, known as sterigmata, the tip of which swells. The nuclei undergo fusion and meiosis and the four resulting haploid nuclei move into the four sterigmata. The tip of the sterigmata is then separated by a cell wall from the basidium, forming a spore and is dispersed actively using a surface-tension catapult mechanism (Carlile *et al*., 2001).

1.3 Nutrient acquisition and assimilation

ECM fungi have been shown to absorb and exchange a number of nutrients with their host plants. Marschner and Dell (1994) summarised that phosphorus (P), ammonium (NH_4^+), nitrate (NO_3^-) and potassium (K) were involved in fungus-plant transfers, but probably the most important and the most widely investigated, is the uptake and transfer of P.

Extracellular enzymes released by fungal hyphae tips degrade organically bound compounds. Organic P compounds, often in the form inositol phosphate, are degraded by extracellular phosphatases and organic acids, such as oxalic acid, that release P from organic compounds (Marschner and Dell, 1994). The P is then assimilated as polyphosphate, which reduces the P concentration within the hyphae. Similarly, extracellular acid proteinases are involved in the breakdown of organic nitrogen (N) compounds (Finlay *et al.*, 1992) such as amino acids and proteins. The litter layer is a rich source of organic nutrients, which fungi break down, assimilate and transfer to the plant.

Complex carbon compounds, such as lignin and cellulose, present in soil are primarily found in the litter and organic layers. Melin (1925, in Smith and Read, 1997) found that ECM had a limited ability to degrade and use these complex carbon compounds and it was therefore assumed that ECM could only make use of simple carbohydrates released into the soil in the form of exudates from plants and micro-organisms. Cairney and Burke (1994) emphasise that, although evidence of extracellular endo- and exoglucanase has been documented from *Pisolithus tinctorius*, the level of cellulytic enzyme activity is much lower than some saprotrophic wood decomposers, e.g. *Trichoderma reesei*. Hydrolytic activity of carbon compounds, from complex starch and cellulose to more simple sugars, such as glucose and fructose, varies within and between species and it is thought that this enzyme activity is functional only at the colonisation stage, where plant cell wall softening is needed for hyphal penetration (Cairney and Burke, 1994).

Most ECM fungi can assimilate forms of complex inorganic P and N, such as phosphates, ammonium and nitrates. Oxalic acid produced by the fungus is a

strong solubiliser of mineral phosphates, for instance calcium phosphate that can then readily be assimilated (Zohlen and Tyler, 2004). Mineral nitrogen, in the form of ammonium and nitrate, is available for uptake in the soil. Martin *et al*., (1986) suggested that fungi utilise a combination of glutamate dehydrogenase and glutamine synthetase pathways to assimilate N. The N is then transported to the plant in the form of glutamine or glutamate. Finlay *et al*., (1988) used ^{15}N-labelled ammonium to follow the N uptake and assimilation by *Rhizopogon roseolus*, *Suillus bovinus*, *Pisolithus tinctorius* and *Paxillus involutus* associated with *Pinus sylvestris*. The authors found that glutamine/glutamic acid, alanine and aspartate/asparagines are important nitrogen sinks and that amino acids were used for N storage. Also, Finlay *et al*., (1989) supplied ^{15}N-labelled ammonium and nitrate to a *Paxillus involutus/Fagus sylvatica* symbiosis. N was incorporated into a wide range of free amino acids and the study, once again, identified Glx, Asx and Ala as important N sinks. All fungi are known to readily assimilate ammonium but studies show that nitrate is less readily mobilised. Utilising different nitrogen sources as substrates for three strains of *Cantharellus cibarius*, Rangel-Castro *et al*., (2002) experimentally determined that nitrate is assimilated less readily than ammonium and that there exists a variation between the strains in their ability to assimilate the different nitrogen sources. Similarly, Finlay *et al*., (1989) showed that although assimilation of nitrate was lower than ammonium, there was significant uptake by the fungus. ECM will more readily utilise inorganic than organic nutrients (Smith and Read, 1997). However, Dames (1996) found that 80% of all ECM root tips were present in the litter/organic layer. The ECM fungal abundance in, and associated preference, to these layers indicate that they are strong competitors for organic nutrient resources.

Van Breeman *et al*., (2000) found strong evidence indicating that ECM fungi, namely *Piloderma croceum*, *Cenococcum geophilum* and *Suillus bovinus*, were responsible for micro-penetrations into feldspar rock. It has been suggested that the extra-cellular low-molecular weight organic acids released by fungi are predominantly responsible for the weathering of rock. Van Breeman *et al*., (2000) hypothesised that although the fungal hyphae present

in the rock samples are potentially saprotrophic, it was highly unlikely as very low carbon resources are present in rock layers of soil. Blum *et al*., (2002) demonstrated the relevance of fungal weathering to plant nutrition as calcium, obtained from calcium phosphate (apatite), was used mostly by ECM trees, indicating a direct transfer of calcium from fungus to plant.

1.4 Nutrient transport

Within the hyphal networks, nutrients move from one region to another for purposes of growth and development. ECM mycelia translocate nutrients bi-directionally, to and from the associated plant host. Carbon compounds acquired from the plant root tip are transported to the hyphal tips where they are required for growth and hyphal extension into the soil. Nutrients assimilated by the fungus from the soil need to be translocated to the plant root. As there are no separate organs to accommodate flow to and from the mycorrhizas, bi-directional flow must occur in the same hyphae, or aggregations of parallel hyphae. Rhizomorphs are aggregations of hyphae, often consisting of specialised hyphae, the structures of which function as conduits for nutrient transfer (Cairney, 1992). Central hyphal columns often lack septa allowing nutrients an easy passage through the hyphae. In Basidiomycota ECM fungi, septate hyphae contain a central pore (dolipore) through which cytoplasmic streaming can occur (Smith and Read, 1997).

Ashford *et al*., (1975) and Cox *et al*., (1975) simultaneously and independently reported polyphosphate granules in small vacuoles. These organelles have been implicated as the 'vehicles' responsible for the translocation of phosphorus (Ashford *et al*., 1975). Cox *et al*., (1980) hypothesised that the polyphosphate vacuoles transported along hyphae by cytoplasmic streaming. Allaway and Ashford (2001) and Uetake *et al*., (2002), demonstrated motile vacuolar translocation in the ECM fungus, *Pisolithus tinctorius*, and AM fungus, *Gigaspora margarita*, respectively. Uetake *et al*., (2002), using the fluorescent probe, Oregon Green 488 carboxylic acid diacetate, identified different shaped vacuoles ranging from spherical to tubular. The authors noted that tubular vacuoles appeared to be motile as a result of cytoplasmic streaming, whereas the spherical vacuoles were static.

1.5 Nutrient and carbon transfer between ECM fungus and plant

The transfer of inorganic nutrients to the plant and photosynthetically derived carbon to the fungus is presumed to occur simultaneously along an interfacial apoplast (Smith and Read, 1997). Hartig net hyphae are highly branched to increase the surface area available for nutrient exchange and penetrate into the cortical cells in coniferous associations (Peterson and Bonfante, 1994). Since there is no intracellular penetration of the plant cortical cells, no continuous symplast exists. Nutrients must pass through the hyphal symplast and wall, into the apoplastic space, through the plant cell wall and into the cortical cell symplast, and vice versa (Peterson and Bonfante, 1994). The exact mechanisms by which the exchange occurs are not well understood. Simple carbohydrates, mainly in the form of sucrose (Bücking and Heyser, 2003), as well as glucose and fructose, are produced through photosynthesis in the shoot of the plant. Excess carbon is transported down the phloem tissue within vascular bundles. Plant roots traditionally function to absorb inorganic nutrients from the soil and act as storage organs for carbon. Carbon transferred to the cortical cells occurs via plasmodesmata found in pits that join these cells, and are not interrupted by fungal hyphae (Nylund, 1980). Smith *et al.*, (1994) suggest that carbohydrates from the plant, and inorganic nutrients from the fungus, are released in the apoplasm via passive efflux or diffusion. This is then followed by active uptake and assimilation of carbohydrates into the fungus and nutrients in the plant via enzyme controlled channels (ATP-ase) across the respective membrane (Smith *et al.*, 1994).

1.6 Ecological function

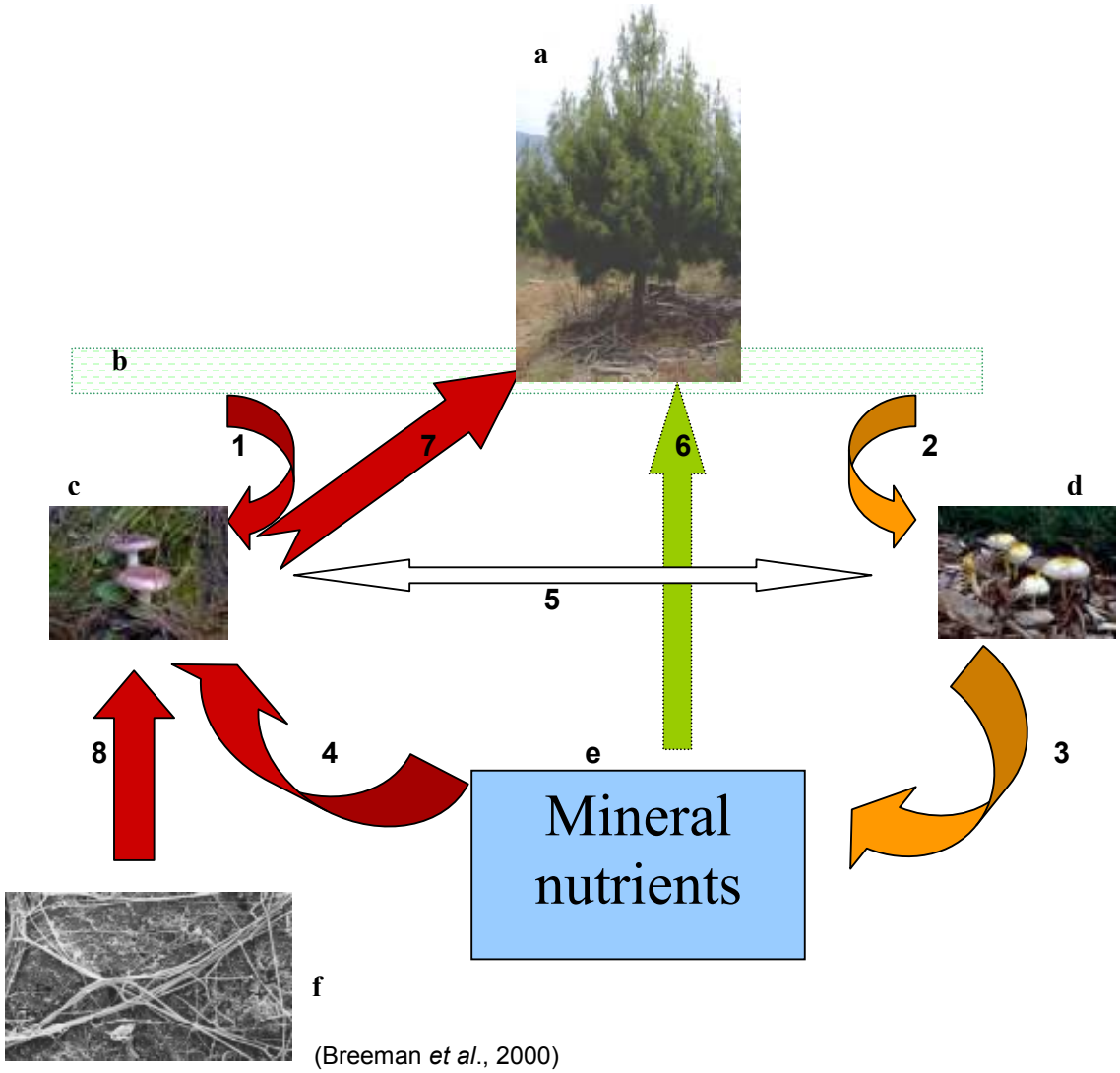
1.6.1 Nutrient cycling

Mycorrhizal fungi are major components of nutrient cycling in most, if not all, vegetation types. The cycling of nutrients is complex and may involve numerous organisms performing processes such as the decomposition of organic and complex inorganic compounds, the release and assimilation of inorganic minerals and the transport and transfer of these nutrients to host plants. ECM fungi play a primary role within this cycle (Fig 1.2), supplying nutrients to the host plant outside the depletion zone or mobilising otherwise

inaccessible complex/organic nutrient forms. Plants benefit from mycorrhizal associations by gaining direct access to degraded nutrients and bypassing other trophic levels, such as saprotrophic decomposition (Fig 1.2).

ECM fungi are known to assimilate nutrients from a range of different sources. Plant litter is a rich source of organic nutrients for which a number of soil organisms, including ECM fungi, compete. Within boreal and managed plantation systems, ECM fungi are particularly relevant in the decomposition and release of nutrients from the litter layer. A study by Dames *et al*., (1998) in South Africa demonstrated the over-accumulation of litter in high altitude *Pinus patula* stands. Further more, the results indicate a build-up of immobilised nutrients of N and P and the major cations K, Ca and Mg (Dames *et al*., 2002). It was concluded that litter accumulation was not a result of increased litter production, but rather attributed to low levels of decomposition by organisms such as ECM fungi. Litter/organic decomposition relies heavily on rhizosphere microbial activity and this process is compromised when soil conditions, such as pH, are not optimal for microbial growth.

Decomposition of organic material by saprotrophic micro-organisms creates a pool of mineral nutrients that can also be assimilated by ECM fungi. Rock weathering properties of ECM fungi provide direct access to nutrients, providing the ECM plants with a nutritional advantage. ECM host plants have a competitive advantage in terms of nutrient assimilation, as ECM fungi are able to by-pass the traditional nutrient cycle, absorbing nutrients directly from the litter layer without first having to be degraded by saprotrophic organisms (Fig 1.2).



(Breeman *et al*., 2000)

Figure 1.2 ECM and saprotrophic fungal nutrient cycling in forested biomes.

The host plant, *Pinus patula*, is represented (a). The rhizosphere is denoted by (b). ECM fungi and saprotrophic fungi are represented by (c) and (d), respectively. The pool of mineral nutrients available for uptake is represented by (e). Hyphae have been shown to mineralise rock material shown in (f) (picture taken from Van Breeman *et al*., 2000). Organic matter is present in the rhizosphere (b), which is derived from plant litter. Both saprotrophic and ECM fungi are able to decompose the organically bound nutrients (1 and 2). Saprotrophic fungi then release mineral nutrients into the nutrient pool (3) and

ECM fungi assimilate these nutrients (4). Nutrient transfer occurs between the two groups of fungi. Exudates of soil borne organisms and dead organic matter are the source of organic nutrients (5). Uncolonised plant roots are able to take up nutrients independently from the pool (6), but are dependent on ECM to gain access to nutrients outside depletion zones (7). Rock weathering mycorrhizas enhance plant access to minerals (8). The ECM fungi are able to provide direct accessibility (1-7) to nutrients for the host plant, without their having to first be released into the nutrient pool (e). The ability of ECM fungi to assimilate both organic and inorganic nutrients provides the plant with a more reliable nutrient source.

1.6.2 ECM interaction with soil micro-organisms

Mycorrhizas are thought to potentially have both positive and negative effects on other soil micro-organisms. Olsson *et al.*, (1996) found inhibitory effects of 20-50% on bacterial activity in the presence of ECM fungi such as *Paxillus involutus*, *Laccaria bicolor*, *Thelephora terrestris*, *Laccaria proxima*, *Suillus variegatus* and *Hebeloma crustuliniforme*. Bacterial activity was recorded by measuring the incorporation of thymidine. Reduced uptake is probably a result of fungal antibiotic production and competition for limited nutrient resources (Cairney and Meharg, 2002). Contrasting these results, Olsson and Wallander (1998) demonstrated stimulatory effects of *Suillus variegatus* on bacterial activity with amended soil containing biotite nutrient additions. The resultant stimulation/inhibition of bacterial activity as a direct consequence of the nutrient content of the soil supports the argument for potential competition for resources. Olsson and Wallander, (1998) also noted effects of ECM fungi on the community structure of the bacteria in close proximity to the host plant roots. To identify the bacteria found associated with ECM fungi, Bowen and Theodorou (1979) isolates from roots of *Pinus radiata* were cultured. These included three *Pseudomonas*-type and four *Bacillus* species. The study found a reduction of ECM colonisation of between 42-100% in the presence of these bacteria.

Alternatively, the co-existence of some associated bacteria, known as mycorrhizal ‘helper’ bacteria, is thought to be beneficial. The review by Garbaye (1994), suggests that mycorrhizal helper bacteria stimulate ECM

fungal germination and enhance ECM formation. Mycorrhizal helper bacteria are also thought to enhance the breakdown of organic nutrients by secreting digestive acids and oxalates, breaking down organic N and C in the soil (Garbaye, 1994). Bacteria have also been identified as possible components of mineral rock weathering associated with ECM (Van Breeman *et al*., 2000).

Saprotrophic and ECM fungi compete for organic nutrient resources in the litter layer. Gadgil and Gadgil (1975) highlighted the importance of the existence of both mycorrhizal and saprotrophic fungi in forest biomes. They demonstrated that there was a decrease in litter decomposition when both trophic groups were present and suggested that this was due to competitive antagonism. Leake *et al.*, (2001), showed antagonistic interaction in microcosms between *Phanerochaete velutina* and *Suillus bovinus* and correlated the interaction with a reduced carbon pulse from the plant to the growing region of the mycelium. This implies a resultant dysfunctional ECM association where normal nutrient exchange is not occurring (Cairney and Meharg, 2002). Lindahl *et al.*, (2001), showed competitive sequestration of phosphorus between a saprotroph, *Hypholoma fasciculare* and an ECM fungus, *Suillus variegatus* and concluded that the potential for either fungus to out-compete depended on the carbon source available to either competitor.

ECM fungi are also strong competitors with soil-borne root parasites. The antagonistic modes are both mechanical and biochemical. In the mechanical mode, ECM fungi compete for colonisation space in the root and create physical barriers, such as the mantle (Marx, 1973). The biochemical mode involves antifungal compounds released by ECM fungi, as found by Kope *et al.*, (1991) in *Pisolithus arrhizus* that suppress pathogenic growth and sporulation. Sen (2001) showed an inhibitory effect of *Suillus bovinus* in association with *Pinus sylvestris* against colonisation of pathogenic uninucleate *Rhizoctonia* species. The same inhibitory effects were not observed when the plant host was inoculated with *Paxillus involutus* and *Wilcoxina mikolae*, indicating differences between species in protecting host plants from pathogenic infection. Sen (2001), also found associated *Bacillus*

species with *S. bovinus*, suggesting a combined effort of both bacteria and ECM fungi to protect the plant host.

1.7 Factors affecting ECM fungal growth

1.7.1 Mycelial growth

Although seasonal moisture and temperature changes affect fungal growth, soil pH is the greater determining factor influencing fungal growth and fungal species diversity. Culture experiments on fungal ECM species show sensitivity to a pH of 7 (Hung and Trappe, 1983). Optimal pH ranges differ between and within species, but are generally acidophilic (pH 3-5). Although in culture, growth appears to be pH dependant; the same effect was not evident when *Piloderma croceum* was associated with plant hosts, as outlined in the study carried out by Erland *et al*., (1990). The study demonstrated that although the optimum pH growth range of *P*. *croceum* occurred at pH 4-5 in culture, an extensive hyphal network developed on peat, buffered at pH 7.3. Even though the mycelial network was not as dense as at pH 3.8, there was still significant growth. Therefore, *in vitro* growth experiments may not accurately reflect *in vivo* processes (Erland *et al*., 1990).

On an international level, mycorrhizal research has been undertaken for decades and has contributed to the understanding of physiological function and factors that affect fungal behaviour. Of particular interest are the effects of pollution, liming, acid rain and fertilisation of forests on the diversity of ECM fungi, as these factors change the soil nutrient composition (Finlay, 1995). Many studies produced contrasting community structure results in response to the above-mentioned disturbances. Studies addressing the possible effects of nitrogen deposition on the ECM community due to NO_3^- industrial emissions and NH_4^+ accumulation of animal stock waste have been conducted. Lilleskov *et al*., (2002) investigated an area comprising a gradient from high ammonia deposition to low deposition. The authors found that species richness decreased from 29 species in the low N to 7 species in the high N sites. The authors found not only a decrease in ECM fungal diversity, but also a shift in dominant species from *Piloderma byssinum*, *Amphinema byssoides*, Thelephoroid 3, *Tricholoma inamoenum* and *Lactarius theiogalus* in the low N

sites to *Paxillus involutus*, *Lactarius theiogalus*, Basidiomycete 1, *Tomentella sublilacina* and *Cenococcum geophilum* in the high N sites.

The enhanced nutrient concentrations in the soil resulting from intensive stock farming, industrial and vehicular emissions (acid rain) and managed plantations result in decreased pH and solubility of nitrogen and phosphorus compounds and increased solubility of aluminium (Arnolds, 1988).

In managed plantations there are number of disturbance factors that may affect ECM abundance and species composition. Repeated harvesting of forest residues was found, by Mahmood *et al.*, (1999) to decrease ECM fungal colonisation from 295 roots/metre root lengths to 181 ECM roots per metre root length. Clear-felling and slash burning not only change the litter dynamics, but have also been shown to remove some ECM fungal species (Bruns *et al*., 2002), at least from the upper soil layer. Further more, Bruns *et al*., (2002) suggested that fire removes certain ECM fungal species from the forest floor as the re-appearance of *Suillus pungens* that were collected in sites post-fire, were new genets and were not revived from mycelial stock.

The fertilisation of managed plantations to enhance plant growth includes the addition of nutrient supplements such as nitrogen (N), phosphorus (P) and potassium (K). Fertilisation and the effects they have on ECM fungal populations, within all plantation environments, have received much attention. These studies have concentrated on short-term effects, but lack sufficient information regarding long-term effects (Erland and Taylor, 2002). Erland and Söderström (1990) studied the effects of pH on ECM communities and reported an increase in root colonisation of up to 100% at pH 5, but colonisation decreased to 40% at pH 7. Chalot *et al*., (1995) reported that amino acid uptake, including alanine and aspartate of the ECM fungus, *Paxillus involutus*, is reduced at pH 4.5 to 6.4 and conclude that this may have consequences regarding the ability of the fungus to colonise host plants.

Nutrient addition of P has a direct effect on ECM fungi, inhibiting both mycelial growth and effective colonisation of host plant roots. Treseder (2004), using a

meta-analysis of a number of studies, recorded a 32% decline of mycorrhizal abundance resulting from P fertilisation in a range of biomes. Additionally, the application of N fertilisers may have a considerable short-term effect on both the species richness and abundance, although recent studies maintain that a shift, instead of a decrease in species composition, is evident. Fransson *et al*., (2000) studied the ECM root tip community in a Norway spruce forest fertilised with ammonium and nitrate. The results indicated that there was no overall effect of N fertiliser on the species richness or abundance, but that a change in the relative abundance of dominant species occurred.

There is considerable variation between different ECM fungal species in their ability to breakdown, assimilate and transfer nutrients to the host plant. Therefore, shifts in the species diversity could have direct consequences on host plant nutrition. The function of individual ECM fungal species in the field is largely unknown, hence the effect that shifts in ECM fungal communities have on plant nutrition is uncertain (Erland and Taylor, 2002).

1.7.2 Sporocarp formation

Factors that affect sporocarp production include a number of environmental factors such as nutrient availability, light, moisture and temperature. Natural gradients of rainfall correlated positively with ECM sporocarp biomass and species richness in a study conducted by O'Dell *et al.*, (1999). The production of large sporocarps, such as those produced by ECM fungi, requires a large nutrient input (Kendrick, 1992). Fungi that produce these sporocarps normally have a large mycelial network accessing nutrients, in particular carbon, needed for the production of the spores (Carlile *et al.*, 2001). ECM fungi primarily rely on host plant carbon products to meet the energy requirements for sporulation. The dependence of fungi on host-plant carbon to produce sporocarps was demonstrated by Högberg *et al.*, (2001). The study involved the tree girdling of *Pinus sylvestris*, inhibiting carbon allocation to the roots. This resulted in a decrease of species richness from 11 species in the control plots to one in the early girdled plots and a reduction in abundance from 252 sporocarps in the control plots to four in the early girdled plots.

Light, particularly in the blue and UV spectrum, may have an effect on some fungi (Kendrick, 1992), by influencing the development of phototropic sporocarps. Light may determine the position of the sporocarp and the release of the spores. Some fungi show no response to light and will sporulate in darkness (Carlile *et al*., 2001).

Added to these factors are those of nutrient additions to the soil through fertilisation treatments, acid rain pollution and liming (Smith and Read, 1997) that may have positive or negative effects on species abundance and richness. It is has been suggested that the response of sporocarp production to fertilisation techniques, particularly N, is more acute than that of ECM roots in terms of species diversity and abundance (Wallanda and Kottke, 1998). Lilleskov *et al.*, (2001) noted a large decrease in species diversity from 144 to 14 in response to increased N application and Pampolina *et al.*, (2002) recorded a 14% reduction in sporulation in response to high P application. Wallanda and Kottke (1998) summarised that N applications may have considerable effects, usually a reduction of sensitive species, on both species richness and abundance, although some generalist ECM fungal species may be unaffected.

1.8 Distribution

ECM fungi are most prevalent in temperate and boreal forest biomes, particularly on acidic soils, forming a belt across the Northern hemisphere, where plant diversity is relatively low (Smith and Read, 1997). Another belt across the tropical and sub-tropical regions also demonstrates a diversity of ECM. Research conducted by Högberg (1986), Härkönen *et al.*, (1993) and Thoen (1993), reviewed the diversity of ECM plant hosts in tropical Africa from Zambia, The Republic of Congo, Zaire (now the Democratic Republic of Congo), to Sudan. The temporal regions in the Northern hemisphere belt and the tropical forests have evolved their own set of associated ECM fungi, and are therefore endemic to these regions.

When considering the global forestry industry, it is apparent that plantations of exotic tree species have maintained a diversity of their associated mycorrhizal

fungi. ECM fungi naturally associated with *Eucalyptus* and *Pinus* in their natural range are found in South Africa and are associates originating predominantly from Europe, North America and Australia (Dunstan *et al*., 1998).

On a more localised scale ECM fungal species composition and diversity is driven by biotic factors such as host plant species composition and abiotic factors such as season and soil quality and composition (Smith and Read, 1997). Additionally, a theory of successional dynamics also suggests that stand age may also determine the composition of ECM fungal species present (Smith *et al*., 2002, Bigg, 2000, Jumpponen *et al*., 2002) and that community structures can be temporally distributed. Successional concepts can be applied to virtually all disturbed or newly acquired resource or ecological niche and may involve any living organism from plants to mammals (Begon *et al*., 1990). This concept includes the chronological succession of species from fast, aggressive colonisers to either a complete replacement or simply an addition of slower growing/developing species.

The ECM fungal successional model suggests that a few relatively non-host-specific, spore-dispersed fungi (early stage), rapidly colonise early root tips of young plants and that there is a gradual addition of more slowly propagating ECM fungal species (late stage) that colonise older roots (Lilleskov and Bruns, 2003). It has also been suggested that the nutrient requirements of early and late stage ECM fungi are different. Early stage fungi are partially saprotrophic and can subsidise their energy requirements from other carbon sources and are therefore less reliant on the plant for carbon. Late stage ECM fungal species appear to be more dependent on the host plant for a source of carbon, which may only be available when the plant is established (Smith and Read, 1997). This theory is supported by Jumpponen *et al*., (2002) who recorded ECM fungal species occurring at a receding glacial front in the North Cascades mountain range (Washington, USA). The authors found that only species such as *Cortinarius decipiens*, *C. tenebricus*, *Inocybe lacera* and *Laccaria* cf*. montana* were found in areas that had been deglaciated for less that 40 years, where-as an additional five species of *Cortinarius* sp., *Lactarius*

sp. and *Suillus* sp. occurred in older stands of between 70-100 years. Smith *et al*., (2002) also support successional progression of ECM fungal species by demonstrating that the presence of over a third of all the species recorded in the study could be explained by stand age.

On the other hand, a study conducted by Termorshuizen (1991) found differences in species composition between stands of *Pinus sylvestris* of the same age as well as between stands of differing ages. It was concluded that soil properties, mainly the humus content, were more important than stand age in determining species composition. Although successional progress of species has been shown, evidence to support that this is the primary force determining the species composition in a stand, is lacking. Additional factors, such as soil type and plant species composition (Termorshuizen, 1991, Smith and Read, 1997) have been shown to play such a major role that successional models cannot accurately predict a set of ECM fungal species, but may explain differences experienced in similar conditions.

In addition, stand age, defined as young or mature/established is relative and is used casually between studies. For example, Jumpponen *et al*., (2002) used vegetative guidelines to determine that a young site was between 30-60 years old, while an older site was between 70 and 100 years old. Smith *et al*., (2002) conducted stand-age studies on a young stand with canopy cover representing 30-35 years of age, a rotation age of 45-50 years and old sites of over 400 years old. Alternatively, Temorshuizen (1991) used the stand of 4-13 years to represent a young stand and 50-80 years to reflect an old stand. In South Africa, where tree growth is notably more rapid, harvest size is reached at 25-35 years old, and is considered a mature stand. Given the variation of ages to describe young and old stands, the arbitrary guidelines to determine stand age categories are not adequate. In addition, a more integrated understanding of all the factors affecting the presence or absence of species, and therefore the determination of ECM fungal species populations, is required to understand responses to a range of different environmental conditions.

1.9 Ectomycorrhizal identification

Species identification of the fungal partner in the ECM association is necessary in all aspects of interactive studies. Species units in community surveys form functional population sets, the identity of which is important for comparative and analytical purposes. ECM fungi were traditionally identified from their sporocarps. The morphology, at both the macro- and microscopic levels, have been well defined for many of the fungal families, and are used to categorise specimens into relative genera and species (Courtecuisse, 1999). Many ECM fungal community studies still utilise sporocarp surveys, but this method is now considered an inaccurate reflection of the ECM fungal community in its vegetative state at the root tip level (Dahlberg, 2001, Jonsson *et al*., 1999).

For this reason, a concerted effort has been applied to identifying ECM fungi from root tip material. Agerer (1987-2002) and Ingleby *et al*., (1990) characterised types of hyphal morphology observed on and in the mantle, which have consequently been used to identify particular fungal genera. All root tip morphological characterisation has been conducted from root material identified from sporocarps, through mycelial tracing between one and the other. While morphological descriptions provide a solid foundation for further development in this field and in spite of the thousands of ECM root characterisations conducted in the last two decades (De Roman *et al.*, 2005), the application of this method to large field collections of root material for identification purposes is not practical as fungal species identification (without the mycelial connection to an identified sporocarp) may not be conclusive or accurate.

Molecular techniques developed in the last two decades have been increasingly used to solve taxonomic discrepancies (Weiβ *et al*., 1998) as well as to define community compositions and population structure (Burke *et al*., 2005, Peter *et al*., 2001). The majority of studies involve the extraction of ribosomal DNA and the amplification of the Internal Transcribed Spacer (ITS) region, although other regions such as the large ribosomal subunit are also utilised. The DNA regions are then either sequenced and compared to other

sequences on website databases such as Genbank or UNITE, or used in other analyses such as restriction fragment length polymorphism (Peter *et al*., 2001, Jonsson *et al*., 1999) or terminal restriction fragment length polymorphism (Burke *et al*., 2005), that allow definitive to identification of the specimens.

1.10 Forestry in South Africa

The occurrence of natural ECM associations in the indigenous vegetation types from South Africa is virtually unexplored, but there are indications of ECM fungi from collections earlier in the 20th century, such as *Amanita* spp. and *Scleroderma* spp. in the Knysna and Tsitsikamma indigenous coastal forest (Doidge, 1950).

South Africa's ECM diversity is mainly associated with tree species used in the forestry industry, which has become one of the largest plant producers in South Africa. Three main genera are cultivated on an economic scale, namely the hardwood species of *Eucalyptus* and *Acacia*, and the softwood species of *Pinus*. South African plantations cover approximately 1.3 million hectares of land with approximately 52% of the plantations being *Pinus* spp. (Smit and Pitcher, 2003). *Pinus sylvestris* was initially introduced into South Africa, followed by *P. pinaster* and *P. pinea* towards the end of the 17th century (Poynton, 1980). The establishment of many of the *Pinus* species was hindered by the lack of suitable ECM fungi and it was only after humus and roots from native soils were imported that these species could successfully be established. *P. patula* Schelcht. et Cham., was first introduced to South Africa from Mexico in 1907. Some of the problems experienced by foresters in Mpumalanga were highlighted by Carlson (1994), where concentration of P was measured at an average of 1.9mg per kg or less in the soil, presenting nutritional limitations for ideal plant growth. Also, Dames (1996) identified a possible cause for concern for high soil acidity experienced in *Pinus* stands. The author found pH levels in litter and soil components ranging from pH 4.04 to as low as pH2.16. Additionally, the accumulation of large litter layers has been recorded in South Africa (Dames *et al*., 1998).

Although cases of exotic ECM fungi swapping to indigenous or native tree species (and vice-versa) have been reported in some Northern and Central African species e.g. *Pisolithus tinctorius* and *Scleroderma* sp. (Diédhiou *et al*., 2005, Martin *et al*., 1998) no indigenous ECM fungi have been found or recorded in South Africa. The miombo ECM community reaches only the most northern territories and does not extend down into this country. No reports of host swapping have been made in South Africa, but this field is undeveloped. The potential for host and fungal swapping to occur has not yet been investigated.

The Institute for Commercial Forestry Research (ICFR), is currently trying to achieve maximum sustainable production through optimum utilisation of site resources (ICFR Annual Research Report, 1999). It has been suggested that by adopting the following four strategies this objective may be fulfilled:

1. Adoption of management strategies and planning operations to ensure successful re-establishment and optimise early growth of out-planted samplings
2. Optimise nutrition through fertiliser additions of inorganic N and P
3. Creating and adopting vegetation management strategies
4. Control of pests and diseases by testing new pesticides and fungicides

ICFR also seeks to increase fire yield, disease (often fungal) and insect resistance and improve stem form and seed production. The methods considered in the Annual Reports (1999 and 2004) produced by the ICFR, is genetic selection and selective breeding of favourable traits within the genus, creating a set of superior characteristics that fulfil their economic purpose. Selective breeding for improved growth, cold tolerance and insect and disease resistance is carried out (ICFR report, 2004). Other strategies focus on fertilisers, pesticides, stand density, rotation length and under canopy burning. Mycorrhizas are not generally considered, even though they contribute enormously to nutrient cycling and uptake in commercial plantations (Dames, 1996). Mycorrhizal research could play an important role as it could contribute to an integrated management programme. As expansion of land allocated to forestry in South Africa is limited by legislation and

licensing requirements (National Water Act, 36 of 1998, Chamberlain *et al*., 2005), sustainable maximum yield land-use strategies must be adopted. This will involve a deeper understanding of the health and contribution of the rhizosphere and the organisms that sustain soil conditions. Other factors such as litter/organic material re-cycling, pest control agents, fertiliser applications and rotation length will need to be addressed in terms of sustaining re-usable agricultural land. At present no ECM inoculation is practiced in the nurseries or during out planting of forestry trees and is not recognised as an important factor within this industry.

1.11 ECM research in Africa

Very few authors have conducted research involving ECM fungi in Southern Africa in the last 25 years. Högberg and Alexander (1986) carried out research in Zambia, where 12 angiosperm tree species were recorded as ectomycorrhizal. The ECM status of tree species were derived by tracing the root from the base of tree to the fine feeder roots. The ECM feeder roots were then identified under the dissecting microscope. The host plant species identified were *Marquesia macroura*, *Monotes africanus*, *Uapaca kirkiana*, *U. nitida*, *U. sansibirica*, *Brachystegia floribunda*, *B. longifolia*, *B. spiciformis*, *B. utilis*, *Julbernardia globiflora*, *J. paniculata* and *Pericopsis angolensis*. Although the study includes ECM descriptions, the emphasis in their study was on the plant associates, with no identification of the fungal aspect of the ECM relationship.

According to Högberg, (1982) central-southern Africa is dominated by ECM plant species. This is not, however, the case in South Africa, where extensive natural forests do not exist. South African research has barely broached the mycorrhizal topic, although Allsopp and Stock (1993), Skinner (2001), and Hawley and Dames (2004), (Appendix 4) have classified the mycorrhizal status of a number of indigenous plant genera. The majority of the findings have recorded only arbuscular and ericoid mycorrhizal associations. In South Africa no indigenous ECM fungal population has yet been determined. Although a small area in the extreme northern parts of South Africa bordering Zimbabwe are characterised by miombo type vegetation, the extent of ECM

plants and fungi have yet to be clarified. Miombo woodlands have been claimed to host a variety of ECM fungi but the extent of knowledge of these associations and their ecological role in South Africa is virtually non-existent.

In managed forest plantations, Marais and Kotzé (1977) confirmed the presence of *Amanita muscaria* and *Boletus edulis*, associating with *Pinus patula* in South Africa. ECM roots were linked to sporocarps identified in the field by tracing rhizomorphs from the sporocarp to ECM roots. Similarly, ECM roots of *Lycoperdon umbrinum* were obtained, but rhizomorphs of *Tuber rapaeodorum* were not detected and therefore no ECM roots were identified.

Further research has been conducted by a few authors but is limited in terms of the contribution towards understanding the ECM associations and the communities that inhabit the managed plantations. Lundquist (1986, 1987a,b), published a good record of all the fungi associated with the genus *Pinus* in South Africa, however, the information regarding the trophic status of the fungal species was ignored. The species list was based on the fungal accessions from the National Collection of Fungi, which are biased against undetectable fungi below ground, or non-sporulating fungi. van der Westhuizen and Eicker (1987), noting the lack of mycorrhizal notation in the listed material, recorded 21 ECM fungi species in the order Basidiomycota. The species list was derived from sporocarp collections only, which are limited to describing the above ground ECM fungal diversity.

Van Greuning and van der Westhuizen (1990) recorded ECM features of *Thelephora terrestris*, which has since been recorded in resupinate form (Kõljalg *et al.*, 2000), and is commonly found in nurseries. Theron (1991), examined the effectiveness of *Pisolithus tinctorius*, *Rhizopogon luteolus*, *R. rubescens* and *R. vulgaris* as ECM associates on *Eucalyptus* and *Pinus* species. The study revealed a variation of effectiveness between the isolates in both the ability to form mycorrhizas as well as the benefit to plant nutrition in different soil conditions. Carlson, in 1992 (and again in 1994), observed a response of ECM fungal species to acidic precipitation. The analysis was carried out with seven unidentified morphotypic ECM root tip types and

concluded that there was no decrease in root colonisation, but a shift in abundance of these group types was detected. Relihan and Laing (1996), explored the incidence of mycorrhizal colonisation in nurseries of *Pinus* and *Eucalyptus* spp. and found low colonisation of *Eucalyptus* roots and an increasing colonisation of *Pinus* roots with age. Khalil *et al.*, (1999) went on to emphasise the importance and necessity of mycorrhizal inoculation when planting out seedlings into previously cultivated soils. The authors found poor establishment of *Pinus patula* outplanting associated with a low colonisation by only three fungal species of ECM. The study also concluded that previously cultivated soil does not support mycorrhizal development, unlike virgin soils.

Additionally, South Africa does not host an indigenous ECM fungal flora, therefore virgin soils would need to be inoculated to enhance outplanting survival. Given these results, one would assume that the forestry industry would incorporate mycorrhizal inoculations, but appear to have chosen to ignore these findings and no nursery inoculation is practiced. Studies conducted by Dames (1996) were the first in South Africa to combine an ecosystem study with the functionality of the ECM. The study showed that approximately 80% of the feeder roots of *Pinus patula* were ECM and occurred mainly in the litter layer. The contribution of the ECM to the nutrient cycle suggested a role in the decomposition of organic N and P compounds and complex inorganic P compounds.

Dames *et al*., (1999) were also the first in South Africa to conduct research on the characterisation of ECM roots based on morphology and anatomy as outlined by Agerer (1987-2002). Two morphotypes were identified as *Scleroderma citrinum* and *Boletus pinicola*. Until this thesis, no molecular studies have been conducted.

The research of ectomycorrhizas in South Africa is lacking both in terms of understanding which ECM fungal species are effective within plantations, as well as the functional community composition and their role within the nutrient cycle. Much of the research has been limited to sporocarp identification, with

very little below ground investigation. Difficulty in identifying ECM root tips has largely been responsible for the inadequate development of this field. Skills and experience are required for morphological and anatomical characterisation of ECM root tips and are vital for fungal identification purposes. With this element lacking in South Africa, diversity and population studies are difficult or near impossible. Although there is abundant information internationally a global acceptance of the role and function of the ECM symbiotic association, very little relevance has been attached to this functional symbiosis in South Africa. It is the objective of this project to identify, using a variety of methods, the ECM population within *Pinus* plantations in South Africa, and to utilise the information as a basis from which further research projects may develop in this country.

2. OBJECTIVES AND STUDY SITE DESCRIPTION

2.1 Motivation

In South Africa, there are very real obstacles that may hinder the growth and sustainability of forestry, such as limited land-use expansion, soil fertility and second rotational decline (Dames *et al*., 2002). These concerns demand attention, particularly pertaining to maintaining current resources and more importantly to soil productivity and fertility throughout numerous plantation rotations, while increasing production yields. It may be obvious to soil ecologists that microbial diversity and functionality are crucial when addressing these challenges, but given the dearth of information currently being assimilated regarding the understanding of these processes in South Africa, research addressing these issues is not being carried out. One of the main explanations for the lack of attention in this area is the enormous information gap concerning the microbial species involved with soil maintenance and the cycling of nutrients. The importance of mycorrhizal fungi, in particular, is poorly understood n South Africa. This study was therefore undertaken to increase ectomycorrhizal knowledge in South Africa by applying a multi-faceted approach to species identification and to provide a more comprehensive assessment of ECM fungal diversity in *Pinus patula* plantations. This work has never before been conducted in South Africa and provides a basis from which further studies may expand.

2.2 Objectives

Ectomycorrhizas form an integral element in the nutrient cycling of forest systems, which are pertinent to sustainable practices in managed plantations in South Africa. Research in this field is limited and only a few studies have been conducted to record the fungal species diversity. Identification techniques, both morphological and molecular, have so far not borne significant outcomes. For research in this field to continue, identification skills must be developed and knowledge of the established species diversity, both above and below ground, is required. The following objectives must be accomplished to fulfil this gap in the research.

The primary objectives of the research were firstly, to collect and identify sporocarps based on morphology from two regions within South Africa found associating with *Pinus* spp. to determine the overall above ground diversity of ECM fungi. Secondly, sporocarp data within plots fertilised with nitrogen and phosphorous were enumerated to determine whether the above ground diversity and abundance were affected by these applications. Thirdly, sporocarp data were used to determine climatic effects on sporocarp formation, which were enumerated from within the nutrient treated plots.

The fourth objective was to characterise the root tip morphology of dominant ECM fungi associated with *Pinus patula* and to identify any potential ECM fungal species, which have not been recorded from above ground collections. The aim of this objective was to create a platform for morphological characterisation of ECM root tips from which this field may develop locally.

The fifth objective was to confirm morphological identification of both the above and below ground fungal species using molecular techniques. The sixth objective was to use the molecular data to expand on the knowledge of below ground species diversity, which may in turn contribute to the understanding of overall ECM fungal species diversity in South Africa. The seventh objective was to use the molecular data to link above and below ground species and determine if there is a correlation between them.

A minor objective of this research was to determine, using morphotyping techniques, any potential effects on community structure, both in terms of diversity and relative abundance, of ECM fungal species in response to a) nutrient applications of nitrogen and phosphorous and b) climatic or successional changes.

Any information derived from these objective is new and relevant to South African forestry and indeed the industry throughout southern Africa. It provides new insight into a field otherwise virtually untouched in South Africa and creates a platform of skills necessary for this field of research to develop.

2.3 Study sites

2.3.1 Temperature, rainfall and geography

Study sites were situated around Sabie in the Mpumalanga Province of South Africa (Fig 2.1). The climate is subtropical, experiencing very mild winters and hot, humid summers. The area is dominated by intensive forestry. Rainfall figures averaged over a thirty-year period range from approximately 100-150mm in the summer months of November to March and 10-50mm in winter from May to August (Internet 1). The mean annual rainfall experienced was 735.6mm. Temperatures over a thirty-year period ranged from 18˚C (min) to 30˚C (max) in the summer months, and 6˚C to 27˚C in the winter months.

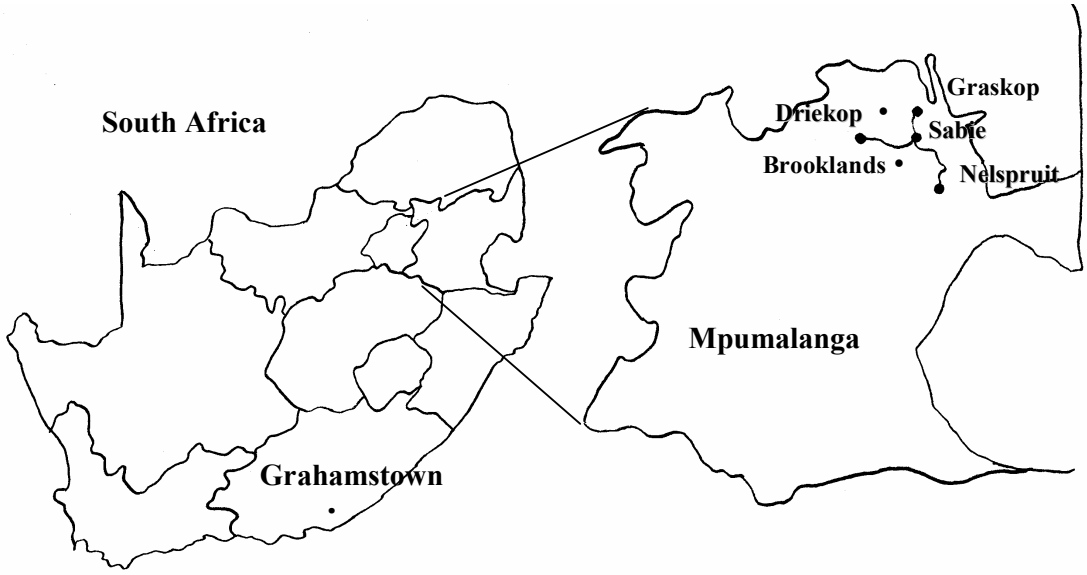


Figure 2.1 Map of South Africa, and an enlarged insert of Mpumalanga Province indicating the Brooklands and Driekop study sites.

The Institute for Commercial Forestry Research (ICFR) currently has 55 *Pinus* field trial sites, two of which were selected according to the fertilisation applications used and the stand age differences. Both experimental trials were already in progress and were monitored by the ICFR. The sites were chosen as both were securely located, were both second rotation *Pinus patula* and experienced similar fertilisation applications, although not simultaneously. The sites are at a similar altitude as it has been shown that litter accumulation

is affected by altitude (Dames *et al*., 1999). The sites were chosen for accessibility. The study sites were approximately 1200km from Grahamstown, which made regular monitoring difficult. The different stand age allowed comparison of any differences between a young and an older plantation stand. The first of the study sites (Brooklands) was located close to Sabie, the second (Driekop) is situated closer to Graskop (Figure 2.1). These trials were selected, as they were both *Pinus patula* compartments exploring similar fertilisation regimes that included N and P. Brooklands is well established while Driekop is a young plantation.

2.3.2 Brooklands trial (Compartment E23 A)

The Brooklands (Fig 2.1.) compartment was planted in 1983, and established as a field trial in 1991 as an 8-year-old stand. The compartment is a second rotation *Pinus patula* Schelcht. et Cham. stand, at an altitude of 1410m located at 25°11.2'S, 30°43.5'E. The trial compartment consists of 32 plots in a 2^5 factorial design (Table 2.1). The nutrients applications were added as combinations of nitrogen, phosphorus, potassium and calcium applied (Table 2.2).

The experimental design consisted of two replicates of each treatment. The trial plots were fertilised twice at 8 and 16 years, in 1991 and 1998, respectively. The treatment plots are 10x10 metres.

Figure 2.2 The fertilised Brooklands trial compartment at 20 years (2002). The trees with white rings are positioned in the centre of each treated plot. Samples were taken 0.5 m from the base of these trees.

Table 2.1 Experimental design of the trial stand for Brooklands compartment (E23). Combinations of potassium (K), calcium (Ca), nitrogen (N), phosphorus (P) were applied to randomised plots in duplicate, including control plot (L) in a 2^5 factorial design (ICFR).

4 K	5 PKCa	4 NK	5 NCa	4 NPK	5 Ca	4 L	5 NK
3 Ca	6 NKCa	3 PK	6 PCa	3 NKCa	6 NPCa	3 KCa	6 NP
2 N	7 P	2 NP	7 L	2 N	7 PKCa	2 NPKCa	7 NCa
1 NPCa	8 NPK	1 KCa	8 NPKCa	1 P	8 K	1 PK	8 PCa
Block I		Block II		Block III		Block IV	
Replicate 1				Replicate 2			

Table 2.2 The nutrient quantities and the respective percentage of nutrient applied to the Brooklands trial.

	Percentage nutrient applied (%)			
Treatment	N	P	K	Ca
Control	0	0	0	0
150kg N ha^{-1} LAN	28	0	0	0
150kg P ha^{-1} MAP	11	22	0	0
150kg K^{+} ha^{-1} KCl	0	0	50	0
140kg Ca^{++} ha^{-1} Gypsum	0	0	0	19

2.3.3 Driekop Trial (Compartment C25)

The trial was planted in February 2000, using a combination of *Pinus patula*, *P. elliotii* and *P. taeda*. The compartment is situated at an altitude of 1600m located at 25°02.9'S, 30°53.4'E. The previous rotation, of *Pinus patula*, was planted in 1971 and clear felling was conducted in October 1999. The trial design combines treatments of fertilisers and fungicides and are replicated three times (Table 2.3). The treatments included a phosphorus treatment (as a double superphosphate) and an optimal nutrition treatment (applied as 2:3:2, N:P:K) that were applied at planting and at 10 months (Table 2.4). Fungicide and insecticide treatments were also applied at planting for pest control trials. These plots were originally sampled but were later disregarded to minimise the number of factors that might adversely affect ECM colonisation. The plots were planted with 8x8 trees where two outer rows of trees acted as buffers between plots between each treatment plot, therefore the centre trees were sampled (Fig. 2.3). Only the *P. patula* plots were sampled for this study (Table 2.3).

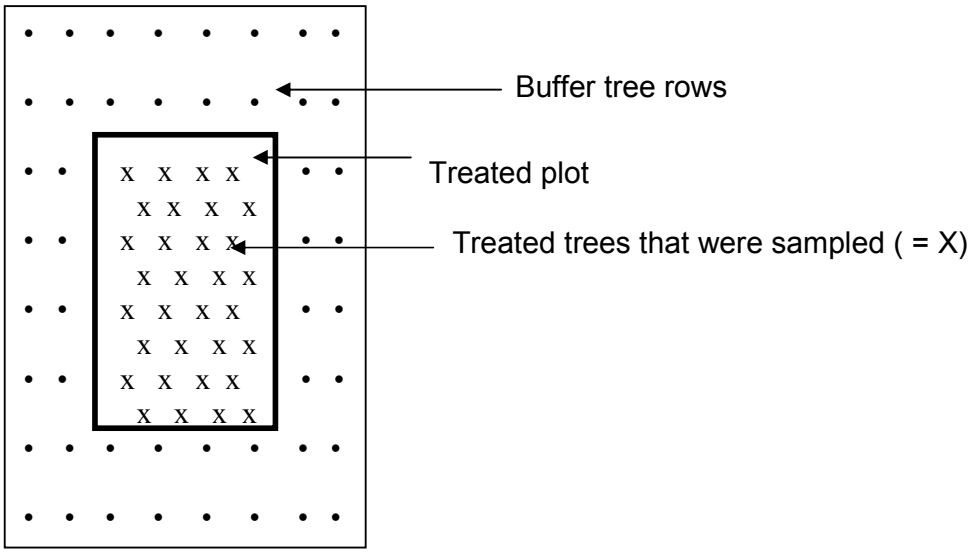


Figure 2.3 Plot design for the trial at the Driekop study site

Figure 2.4 The fertilised Driekop trial compartment of *P. taeda*, *P. elliotii* and *P. patula*. The picture was taken two years after planting (March 2002). Each treatment plot was surrounded by two rows of untreated trees acting as buffers between the treatments.

Table 2.3 Experimental design for Driekop compartment (C25). The plots were treated with superphosphate (P), 2:3:2 (OPT) and control (ZERO). The blank cells denote plots containing species other than *P. patula* and plots treated with fungicides that were not sampled. Yellow, Blue and Pink blocks are replicate 1, 2 and 3 of the trial, respectively. Blocks denoted with an F represent plots that were treated with fungicide.

1 P	10	11	20 OPT F	21	30	31	40	41 ZERO	50	51
2	9	12 ZERO	19	22	29	32	39 P F	42	49 P	52
3	8 P F	13	18 OPT	23	28 OPT	33	38	43	48 ZERO F	53 OPT F
4	7	14	17 ZERO F	24 P	27 ZERO F	34 P F	37	44	47 OPT	54
5 OPT F	6	15	16	25	26	35 ZERO	36	45	46	

Table 2.4 The nutrient quantities applied to the Driekop trial. The mass quantities are given per tree.

	P (double superphosphate)	Optimal (2:3:2)
At planting	20g	200g
At 10months	20g	250g

2.4 Sampling

2.4.1 Brooklands study site

A 5x5 metre area was measured in the centre of each treatment plot and data for fertilisation effects were derived only from these areas. In April and June 2002, two soil core (5cm in diameter) samples were taken from each plot (32 plots in total) and initial sporocarp diversity and abundance were recorded. Sampling was conducted again in December 2002, April 2003 and December 2003. However, only the plots that were treated with N, P, NP, and the control were sampled (Table 2.1) during these times in order to focus on the effect of N and P. The reduced number of plots sampled was coupled with an increase in the number of soils cores taken from each plot (Table 2.5). This resulted in sampling eight plots, at which five soil cores were taken, from the centre of each plot, taken approximately 0.5-1m from the base of a tree. The cores

were placed in plastic bags, labelled and stored at 4°C until processed. All the root material was gently removed from the soil by using a 2mm sieve and rinsing the soil core samples individually. Only vital root tips, both ectomycorrhizal and non-ectomycorrhizal were enumerated. The ECM root tips were microscopically examined fresh where possible or stored in 50% alcohol until examination was possible.

Sporocarp surveys were conducted during each sampling event. An additional sporocarp collection was carried out in March 2002. Although the experimental design was factorial, the focus on just four of the nutrient applications and the data retrieved from these plots did not require factorial statistical analysis.

Table 2.5 ECM root tip analysis and sampling strategies at the Brooklands study site

Analysis	March 2002	April 2002	June 2002	December 2002	April 2003	December 2003	April 2004
Plots sampled	All	All	All	N, P, NP, Control	N, P, NP, Control	N, P, NP, Control	Random
Sporocarp enumerations	Yes	Yes	Yes	Yes	Yes	Yes	No
Number of soil cores taken per plot	none	2	2	5	5	5-10	10 cores in total
ECM Root tip morphotyping	No	Yes	Yes	Yes	Yes	Yes	Yes
ECM root tip enumeration	No	No	Yes	Yes	Yes	Yes	No
Molecular identification	No	Yes	Yes	No	Yes	No	No
Morphological characterisation	No	Yes*	Yes*	Yes*	Yes*	Yes*	Yes*

*Characterisation of selected ECM root tip morphotypes.

2.4.2 Driekop study site

The plots in this field site were treated similarly to the Brooklands study site, with a 5x5m area measured in the centre of each treated plot. During the time between the initial sampling and the second sampling, the 5x5m perimeter markers went missing. Subsequently, the five centre-most trees were sampled. Two soil core samples were taken from the centre of each plot in April and June 2002 (18 plots in total) and sporocarp diversity and abundance recorded. In December 2002 and April 2003, plots that were treated with pesticides were not sampled to eliminate any factors that may mask the nutrient effect of the fertiliser on the ECM fungi. The remaining nine plots were sampled (Table 2.3). Five soil cores were taken approximately 0.5m from the base of the trunk in the centre of each plot. The cores were placed in plastic bags, labelled and stored at 4°C until processed. Sporocarp data was once again recorded. Only vital root tips, ectomycorrhizal and non-ectomycorrhizal were enumerated. The ECM root tips were microscopically examined fresh where possible or stored in 50% alcohol until examination was possible.

Table 2.6 ECM root tip analysis and sampling strategies in the Driekop study site

Analysis	April 2002	June 2002	December 2002	April 2003	December 2003
Plots sampled	All	All	All except fungicide treatments	All except fungicide treatments	All except fungicide treatments
Number of soil cores taken per plot	2	2	5	5	5-10
ECM Root tip morphotyping	Yes	Yes	Yes	Yes	Yes
ECM root tip enumeration	No	Yes	Yes	Yes	Yes
Molecular identification	Yes	Yes	No	Yes	No
Morphological characterisation	Yes*	Yes*	Yes*	Yes*	Yes*

*Characterisation of selected ECM root tip morphotypes.

3. EVALUATION OF ECTOMYCORRHIZAL SPOROCARP DIVERSITY

3.1 Introduction

3.1.1 Sporocarp morphology and related taxonomy

Sporocarps can be broadly grouped according to morphology and habitat. Epigeous sporocarps are found above ground and include a variety of sporocarp morphologies. Members of the Class Agaricales (genera *Amanita* and *Cantharellus*, sub-phylum Basidiomycota as examples) are predominantly gilled and have stipes. Members of the genera *Boletus* and *Suillus* (Basidiomycota) are stiped and pored (Courtecuisse, 1999). The sporocarps of *Thelephora* spp. (sub-phylum Basidiomycota) are dimorphous, forming either a pored bracket or a resupinate layer. Resupinate fungi grow as a thin spore-forming layer along or beneath dead plant matter (Kõljalg *et al.*, 2000). Coralloid fungi, such as *Clavulina* spp., form highly branched sporocarps bearing spores at the tips of their branches (Courtecuisse, 1999). ECM fungi also include the genera *Helvella* (order Pezizales, sub-phylum Ascomycota) and *Morchella* (Dahlstrom *et al.*, 2000) that present a convoluted and globed morphology, mainly of the pileus.

In all the above cases, the hymenium is exposed. Enclosed sporocarps formed by members of the class Gastromycetes, e.g. genera *Rhizopogon* and *Scleroderma* (sub-phylum Basidiomycota), may be epigeous, as in the case of the latter, or found below ground, as found in the genera *Tuber* and *Terfezia* (both occurring in the sub-phylum Ascomycota). These sporocarps are referred to as hypogeous (Valentine *et al.*, 2004).

ECM fungal species can often be distinguished from non-ECM fungi based on the taxonomic inference. Specific genera have been identified, exclusively, or by majority, to be ectomycorrhizal forming. These include the following genera: *Amanita*, *Boletus, Cantharellus*, *Clavulina*, *Inocybe*, *Lactarius*, *Rhizopogon, Russula*, *Scleroderma*, *Suillus, Thelephora* and *Xerocomus* (Basidiomycota) (Cairney and Chambers, 1999), and *Helvella, Tuber* and

Terfezia (Ascomycota) (Schenck, 1982). Not all the species within these genera are mycorrhizal, e.g. *Amanita foetidissima* (Reid and Eicker, 1991). Pande *et al*., (2004), reported a number of mycorrhizal and non-mycorrhizal species in the Western Himalayan temperate forest within the genera *Boletus*, *Inocybe*, *Lactarius* and *Russula*.

3.1.2 Identification

Morphological identification begins with the macro-morphology of the sporocarp, and may eventually require micro-morphological examination for complete confirmation. The main macroscopic features that determine species identification are the characteristics of the pileus, cap margin, lamellae, flesh, rings spore prints and the stipe (van der Westerhuizen and Eicker, 1994). The important features of the pileus are the colour, shape, development of the margin and the presence or absence of scales or fine hairs (Brundrett *et al*., 1996). In the case of hypogeous and exposed sporocarps, the spore bearing tissue normally lies underneath the pileus and may be lamellate or poroid. The colour, position, frequency, and attachment of lamellae and size of pores are all indicative characteristics utilised for species identification. The colour of sporocarp flesh bruising and lactation may also contribute information regarding identification. The flesh within the stipe and pileus may be fibrous or fleshy and could undergo a colour change once exposed to air. Spore prints allow an accurate account of spore colour, which can be used to differentiate between closely related species e.g. *Russula* spp. Stipe ornamentation, colour, length, shape (bulbous or clubbed), presence/absence of partial veil, and presence/absence of a remaining ring are all informative features. The main microscopic/anatomical features considered to be important are spore size, spore ornamentation, amyloid vs. non-amyloid spores, basidial morphology and hyphal configuration in the pileus and stipe (van der Westhuizen and Eicker, 1994).

3.1.3 Disturbance factors and the effect on sporocarp production

Natural environmental gradients, such as moisture, temperature and soil characteristics can affect sporocarp production (Chapter 1). Unnatural events influencing sporocarp formation are currently being considered. Impacts, such

as acid rain, liming, nutrient fertilisers, live-stock waster, industrial effluent and sporocarp harvests (Arnolds, 1991) potentially affect the above and below ground ECM fungal species present.

The response of ECM sporocarp diversity and community composition to various disturbance factors has been addressed by a number of authors. Pampolina *et al.*, (2002) found a decrease in the number of ECM sporocarps in *Eucalyptus* plantations of up to 31% in high P treatments and a related decrease in sporocarp biomass recorded between 25-40%, 24 months into the study. The same authors recorded variable response to P addition both between, and within, *Pisolithus*, *Scleroderma* and *Laccaria* species that were analysed. The response of sporocarp biomass was unaffected, increased or decreased, and no decrease in species diversity was observed. Lilleskov *et al.*, (2001) found a large decrease in species diversity along a steep N gradient resulting from atmospheric NH_3 deposition. The study reported a 10-fold decrease in total species richness, but highlighted that different responses were found between species. *Lactarius*, *Laccaria*, *Paxillus* and *Hygrophorus* species abundance were either not correlated, or were positively associated with N deposition. In contrast to other studies, Agerer *et al*., (1998) reported that after acid irrigation and liming, sporocarp frequencies of *Russula ochroleuca* and *Hygrophorus pustulatus* increased. The number of sporocarps of *R. orcholeuca* increased from 3.3 in the control to 14.5 in response to acid irrigation. *H. pustulatus* increased from 3.7 in the control to 118.7 in the acid irrigation and lime plot. The discussed studies have shown a variation in response of sporocarp production to nutrient and pH factors, and no definite conclusions can be made, as the results appear conflicting. Thus the effect of various fertilising regimes on the ectomycorrhizal ecology in terms of sporocarp diversity and richness may depend on environmental and soil conditions. Even though sporocarp data may not reflect below ground population structure (Dahlberg, 2001, Jonsson *et al*., 1999), sporocarp data is still valuable in determining the effect of factors that pertain to sporocarp production by different ECM fungal species.

3.1.4 Objectives

Sporocarps collections were made to ascertain the above ground species diversity. This is a relatively quick and simple method to determine species presence and can be used for comparison with other collections already documented from South Africa. Sporocarp-based studies are also useful in that the data may be indicative of the species composition within managed plantations. Even though sporocarp data only confirms the presence of fungal species, this data may also indicate potential fertiliser treatment effects and reflect conditions resulting in poor sporocarp formation.

Therefore, the objectives of this study were to assess the general above ground ECM fungal species richness associating with *Pinus* spp. in selected areas of South Africa. ECM fungal sporocarps were collected at a number of sites with the aim of determining the general ECM fungal species diversity on *Pinus* spp. All ECM fungi were then morphologically identified to achieve this objective. A minor objective was to determine the effect of nutrient addition and/or climatic effects on ECM sporocarp abundance and species richness. This was achieved by sampling sporocarps within the Brooklands and Driekop trial plots and were enumerated both in terms of the diversity and frequency of each species within the plots.

3.2 Material collection and methods

3.2.1 Above ground ECM fungal diversity

Sporocarp material was collected at both the Brooklands and Driekop study site (Section 2.3) in March 2002, April 2002, June 2002, April 2003, December 2003 and April 2004. To increase the species database, pine plantations around the Sabie area were sampled at the same time, as well as a pine forest close to Grahamstown. This served to not only determine, on a broader scale, the above ground ECM fungal species within South Africa on *Pinus* species, but also to identify fungal symbionts that could potentially be found below ground within the study sites.

Samples were identified morphologically using field guides (van der Westhuizen and Eicker, 1994, Courtecuisse, 1999). Features of the cap,

hymenium, stipe, partial veil, base, universal veil, flesh and gills were recorded and used for identification (Brundrett *et al.,* 1996). A species list of all the sporocarps collected was compiled and compared with the South African literature in order to update the list. Digital images of fresh specimens were captured using a JVC GC-X3 digital camera. Representatives of each species were dried in an oven set at 30°C, or in plastic bags filled with silica gel and deposited as voucher specimens in the Selmar Schonland Herbarium, Grahamstown (Appendix 1). Once dried, DNA was extracted from selected sporocarps and the sequences were used to verify identification and to link fruiting bodies to ECM root tips for positive identification (Chapter 5).

3.2.2 Effects of climate and fertilisation on sporocarp abundance

All analysis was conducted on the sporocarp enumeration from the Brooklands study site, as no sporocarps were recorded within the sample area of the plots in the Driekop study site. ECM sporocarps were enumerated from the fertilised trial sites only within the inner 5x5m plot (Chapter 2). Enumeration in the Brooklands study site was modified after the third sporocarp-sampling event in June 2002. In the first three sampling events, all the treated plots were used for data collection (Chapter 2) to determine the collective species present within the area. The sampling strategy then focused on nutrient plots that have been found in the literature to affect sporocarp abundance. Therefore, only the N, P, NP and control plots were sampled (see section 2.4), at subsequent sampling events in December 2002 and April 2003. No sporocarps were recorded in the plots in December 2002 and as a result, this sampling event was not included in the analysis of fertiliser effects. The sporocarps of *Scleroderma citrinum* were found to persist for long periods of time in dry conditions. Therefore, after every sampling event, sporocarps were harvested from the sample plot to ensure that re-counting was avoided.

3.2.3 Statistical analysis

Data collected from sporocarp collections were then statistically analysed using Statistica 7 (Statsoft, Inc, 2004). To test for differences in sporocarp production between the wet and dry periods, total sporocarp counts were

modified to a mean number of sporocarps per plot for each sampling event and were compared to each other. Additionally, to determine the variation in sporocarp production between these sampling times of *Scleroderma citrinum*, an ANOVA was performed. The remaining ECM fungal sporocarp species were encountered too rarely to draw any statistically significant conclusions of climate effect between wet and dry periods. *Scleroderma citrinum* abundance was analysed for March, April and June 2002 using all the sampled plots and then analysed together with December 2002 and April 2003. This was conducted such that the March, April and June 2002 data sets only contained sporocarp enumeration for the N, P, NP and control plots to correspond with the sampling procedure executed in December 2002 and April 2003. One-way ANOVAs were conducted with 'sampling event' as the categorical factor in each analysis.

The nutrient effect of applications of N, P and NP on ECM sporocarp production was investigated. All sampling events were used, i.e. March, April, and December 2002, and April 2003. Treatment effects were analysed by comparing the differences of the means using an ANOVA (Statsoft, Inc, 2004). One-way ANOVAs were then conducted for each sporocarp species for all sampling periods.

3.3 Results

3.3.1 Above ground species diversity

Sporocarp collections were identified using field guides (van der Westhuizen and Eicker, 1994, Courtecuisse, 1999),. A total of 26 potential ECM species were recorded from 13 genera. Digital images of 14 specimens, representing 13 species and seven genera are presented in Plate 1, showing key characteristic features.

Amanita muscaria was identified from the red cap and white scale remnants of the universal veil (Plate 1.1a). *Amanita rubescens* (Plate 1.1b) was identified by the pinkish tinges occurring on the cap and stipe. *Amanita excelsa* (Plate 1.1c) had a dark grey-brown cap, a potato-like odour, and

amyloid spores. *Boletus edulis* and *Suillus granulatus* are depicted by Plate 1.1d and Plate 1.1e, respectively. *Suillus salmonicolor* (Plate 1.1f) was identified by the persistent universal veil that adheres to the stipe, the porous hymenium and salmon-ocherous coloured cap. A Tomentelloid sp. (Plate 1.1g) was a resupinate, found on a dead branch and was dark brown-grey. *Scleroderma citrinum* (Plate 1.1h) was identified as having a yellow-brown outer layer and black spore mass when mature. *Lactarius deliciosus* (Plate 1.2i) was identified and was different to *L. hemicyaneus* (Plate 1.1j), which was identified from the green-blue tinges on the cap and the blue colouration that developed after bruising of the flesh. *Lactarius hepaticus* is easily identified from the liver coloured pileus (Plate 1l). *Russula* specimens (Plate 1.2 m,n and o) represent three species collections, comparing to *R. sororia* (Plate 1.2 m), *R. capensis*/*R. drimeia* (Plate 1.2 n) and *R. sardonia* (Plate 1.2 0).

Plate 1.1 and 1.2 ECM fungal species as identified from sporocarp collections associated with *Pinus* in Mpumalanga and the Eastern Cape, South Africa.

Plate 1.1 and 1.2 Sporocarp specimens found in *Pinus* spp. plantations. a) *Amanita muscaria* with remnants of the universal veil as white scales, b) *A. rubescens*, demonstrating the signs of pinkish tinges that occurred along the stipe, c) *A. excelsa*, d) *Boletus cf. edulis*, e) *Suillus granulatus*, f) *Suillus salmonicolor*, note the persistent universal veil and salmon-coloured pileus are distinguishing characteristics, g) *Thelephora/Tomentella* collection in resupinate form h) *Scleroderma citrinum* featuring a black mature spore mass, I) *Lactarius deliciosus*, j) *L.* cf. *hemicyaneus*, a distinctive blue-green colouration on the pileus indicative of the species, k) *L. semisanguifluus*, l) *L. hepaticus,* m) *Russula sororia* spp. featuring a white stipe and pileus with mottled green patches in the centre and pink on the edges, n) *Russula capensis/R. drimeia* featuring a pink stipe and a dark red to purple coloured pileus, o) *Russula sardonia*, p) *Inocybe* sp.

Most of the diversity was found in well-established *Pinus* stands (Table 3.1: B, GH and SG). The only species found in Driekop was *Thelephora terrestris*. Within a single collection of *Russula* specimens in the Highlands (Grahamstown) area, a number of species appeared to be present.

Table 3.1 List of ECM fungal genera and species of sporocarps collected under *Pinus* found in the various sampling areas.

Species list	**Location**	**Species**	**Location**
1. Amanita		***6. Rhizopogon***	
A. excelsa Fr.	B	*R. roseolus* (Corda) TM Fries	GH, B
A. muscaria (L) Hook.	GH, B	***7. Russula***	
A. rubescens (Pers.) Gray	B, SG	*Russula* (7 specimens, consisting of *R.* cf. *capensis*, *R. sardonia*, *R. sororia* and 3 specimens of unknown species)	GH
2. Boletus		***8. Scleroderma***	
*B.*cf. *edulis* Bull.	GH, B	*S. citrinum* Pers.	B
B. aestivalis (Paulet) Fr.	GH	***9. Suillus***	
3. Clavulina		*S. granulatus* (L.) Snell	B
C. cf. *cristata* (L.) J. Schröt.	B, SG	*S. luteus* (L.) Gray	B
C. cf. *cinerea* (Bull.) J. Schröt.	B	*S. salmonicolor* (Peck) Snell ex Slipp & Snell	B

Species list	Location	Species	Location
4. Inocybe		***10.Tricholoma***	
I. cf. *griseolilacina* J.E. Lange	D	*T. albobrunneun* (Pers.) Fr.	B
Inocybe sp. 2	D	***11. Thelephora***	
Inocybe sp. 3	D	*T. terrestris* Ehrh.	D, B
5. Lactarius		***12. Tomentella***	
L. hepaticus Plowr.	B	*Tomentella* sp1	B
L. cf. *semisangifluus* R. Heim & Leclair	B	***13. Xerocomus***	
L. deliciosus (L.) Fr.	B, GH	*X. badius* Pers.	B
L. cf. *hemicyaneus* Romagn	B	*X. chrysenteron* Bull.	B

* The capital letters annotate the location from which the specimen was present/collected. SG denotes plantations in and around Sabie, B is the Brooklands study site, D is the Driekop study site, and GH is Highlands, Grahamstown.

3.3.2 Effects of climate and fertilisation on sporocarp diversity and abundance

The two species most prevalent in the Brooklands study site were *Scleroderma citrinum* and *Amanita excelsa*. However, data from within the 5x5m plots of sporocarp species and abundance was limited to four species; *A. excelsa*, *Suillus granulatus*, *S. citrinum*, and *Clavulina cristata*. *S. citrinum* was the most dominant.

3.3.2.1 Climatic effects

Climatic effects were analysed for the periods March, April and June, December 2002 and April 2003. The number of sporocarps and the number of species enumerated from within the sampling plots at each sampling event are given (Table 3.2). The data demonstrates a marked decrease in sporocarp abundance from a total of 166 in cooler wet months (March 2002) to 12 sporocarps in the cold, dry months (June 2002) (Table 3.2). The Bonferroni test (Table 3.3) indicated that a significantly higher overall abundance was recorded in March 2002 than in either April 2002 or June

2002. A separate ANOVA analysed data from December 2002 and April 2003, which only contained sporocarp enumeration for N, P and NP sample plots.

Table 3.2 Descriptive statistics for the ECM sporocarp count in the Brooklands study site for the sample periods March, April, June and December 2002 and April 2003.

	March 2002	**April 2002**	**June 2002**	**December 2002**	**April 2003**
Mean sporocarps/plot	5.187	1.375	0.375	0	2.7
Number of plots sampled	32	32	32	8	8
StDev	0.627	0.253	0.154	0	0.978
No. of species *	4	3	1	0	3
Total #	166	44	12	0	31

* Total number of species recorded, including *A. excelsa*, *C. cristata* and *S. granulatus* within the 5x5m plots in the whole of the Brooklands study site at each period. # Total number of sporocarps for all species found at each sampling period within all the plots.

The mean number of sporocarps per plot, calculated at each sampling date, (Table 3.2) indicate higher sporocarp abundance in March 2002 and April 2003, which correspond to cooler, wet periods. The total number of sporocarps was the sum of the number of sporocarps found all in the sampled plots. The number of plots sampled was reduced in December 2002 and April 2003, explaining why fewer sporocarps were counted. The mean number of sporocarps per plot is therefore more informative than overall abundance. The species richness is correlated to the sporocarp abundance, but does not significantly differ between sampling dates.

Four ECM fungal species in total were observed in the sampling plots, namely *Scleroderma citrinum*, *Clavulina cristata*, *Amanita excelsa* and *Suillus granulatus* (Figure 3.1). Species abundance was recorded from the N, P, NP and control sites to enable comparisons between all the sampling events. *S. citrinum* was most common. *C. cristata* and *A. excelsa* were only found in April 2003 and *S. granulatus* was only observed in June 2002 (Figure 3.1).

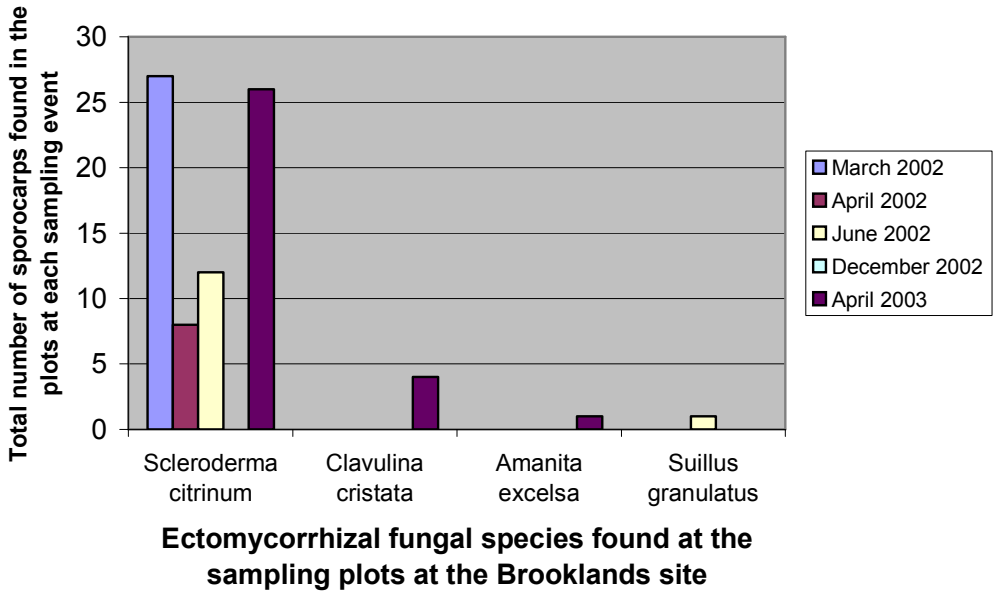


Figure 3.1 The total number of sporocarps of each ECM fungal species recorded within the sample plots at the Brooklands study site from only the N, P, NP and control.

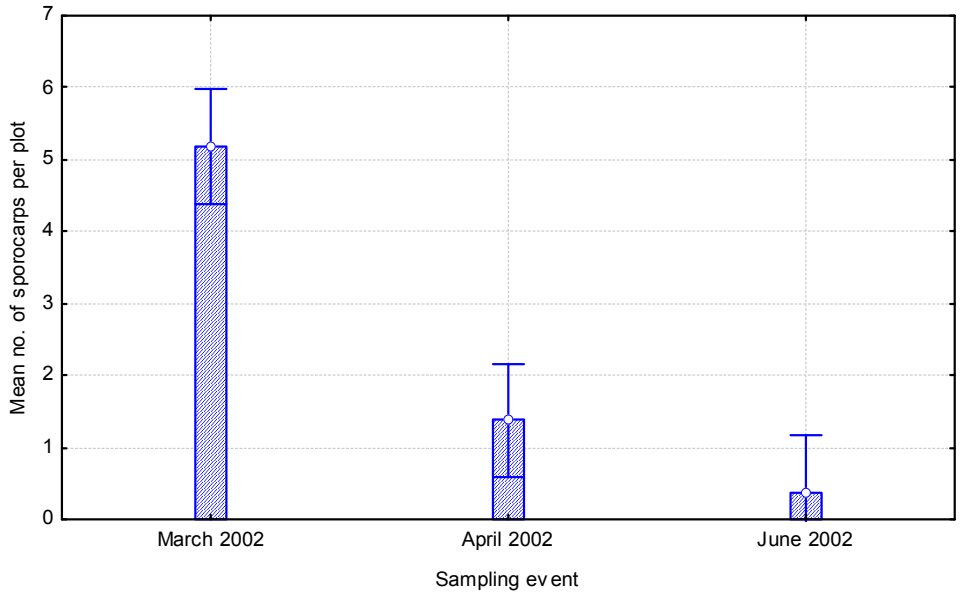


Figure 3.2 Average number of sporocarps of *Scleroderma citrinum* per plot in the Brooklands study site. $F_{(2,93)} = 40.16$, $p < 0.05$. Bars indicate standard error.

Table 3.3 Least square differences were calculated using the Bonferroni test for the sampling events of March 2002, April 2002 and June 2002. Significance is taken at the 95% confidence level. * indicates significant differences.

	March 2002	April 2002
March 2002		
April 2002	0.00*	
June 2002	0.00*	0.24

The ANOVA (Figure 3.2) and Bonferroni test (Table 3.3) confirm significant differences in the abundance of *S. citrinum* between some sampling events. Sporocarp abundance was significantly higher in March 2002. The statistical results confirm the observed differences in the total number of sporocarps of *S. citrinum* collected in each treatment plot (Figure 3.3).

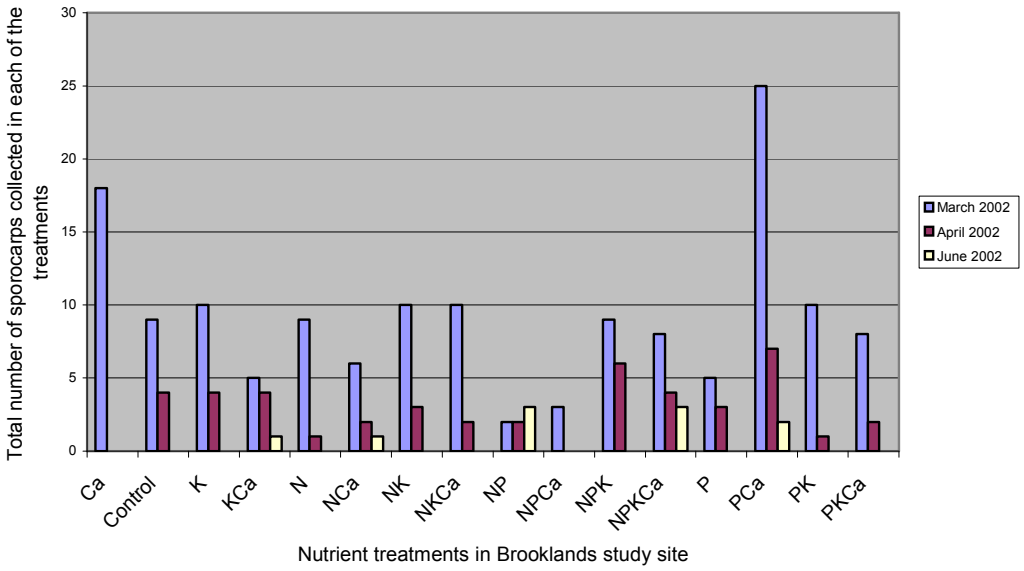


Figure 3.3 Number of *Scleroderma citrinum* sporocarps collected at the Brooklands study site for the periods of March 2002, April 2002 and June 2002. Values are given as the total number of sporocarps found within the 5x5m plots of each treatment.

To test the variation in sporocarp abundance over all the sampling events from March 2002 to April 2003 only the N, P, NP and control plots could be used for comparison. The graph produced by ANOVA (Figure 3.4) and the Bonferroni test (Table 3.3), indicated that sporocarp abundance was significantly higher in March 2002 and April 2003 than June and December 2002, but not from April 2002. These dates correspond to typically cooler, wet months of the year.

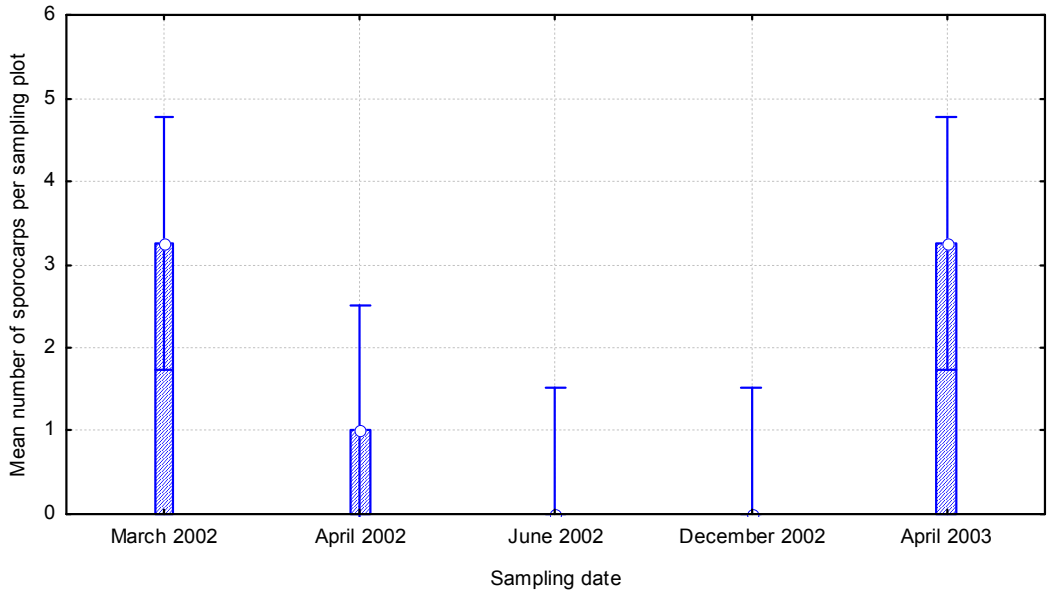


Figure 3.4 One-way ANOVA of the number of sporocarps of *Scleroderma citrinum* per plot at the Brooklands study site. Data from **only** the N, P, NP and control plots were used for comparison. $F_{(4,35)} = 4.84$, $p < 0.05$. Bars indicate standard error.

Table 3.4 Least square differences were calculated using Bonferroni test for the sampling events of March 2002, April 2002, June 2002, December 2002 and April 2003. * indicates significant differences at the 95% confidence level.

	March 2002	April 2002	June 2002	December 2002
March 2002				
April 2002	0.407			
June 2002	0.041*	1.0		
December 2002	0.041*	1.0	1.0	
April 2002	1.0	0.407	0.041*	0.041*

3.3.2.2 Nutrient effects

The effect of nutrient applications of N, P and NP on the ECM sporocarp abundance and species richness was investigated by comparing fertilised plots to control plots, which received no nutrient applications. The initial data distribution (Figure 3.5) suggested that there was a higher overall ECM sporocarp abundance in the control plots. Four species that were collected within the sample plots were subjected to analysis, but the treated plots did not reveal any significant differences in species richness between the nutrient applications (Figure 3.5. Differences in sporocarp abundance were analysed using one-way ANOVA to determine significant differences in the mean abundance of the number of sporocarps per plot.

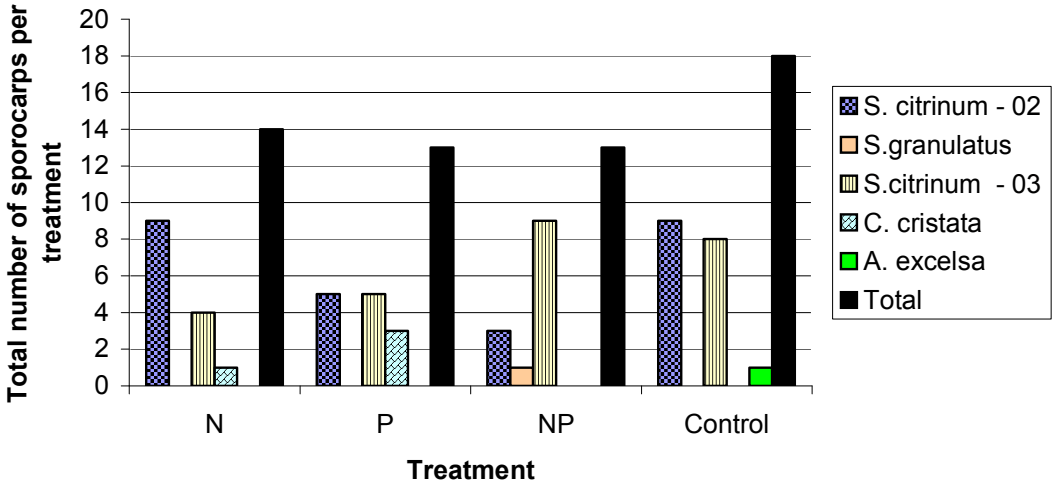


Figure 3.5 Sporocarp abundance for four ECM species recorded in the Brooklands study site in April 2002 and April 2003. The data recorded during the two sampling events for the number of sporocarps per plot are combined for each treatment and presented. *S. citrinum* - 02 represented the sporocarps counted in April 2002 and *S. citrinum* – 03 in April 2003.

Data from the March, April, June and December 2002, as well as from April 2003 were analysed in a one-way ANOVA to detect the treatment effect on sporocarp production. The sporocarp data was pooled over all the sampling events and the mean within each treatment calculated. The analysis was carried out on all the species present during the sampling events.

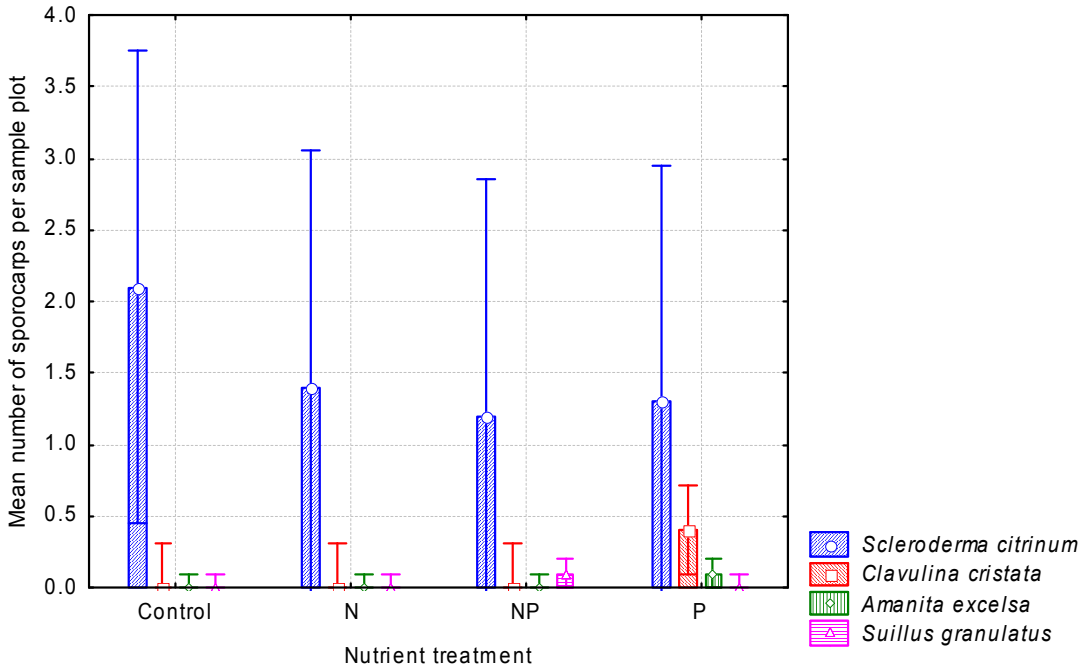


Figure 3.6 Average number of sporocarps per plot of the different ECM fungal species counted in the different treatment plots of N, P, NP and Control at the Brooklands study site for the sampling events March 2002, April 2002, June 2002, December 2002 and April 2003. $F_{(12,87.60)} = 0.96$, $p = 0.49$. Significance observed at 95% confidence level. Bars indicate standard error.

The ANOVA of the means of different nutrient treatment plots and the control plot demonstrated that there was no statistically significant effect of nutrient application on sporocarp abundance of any ECM fungal species (Figure 3.6). The possible effect of climate on the variation of data was noted and therefore an additional ANOVA was run on only data from March 2002, April 2002 and April 2003 to determine whether nutrient treatments affect sporocarp abundance at the time when sporocarp abundance is the highest (Figure 3.7).

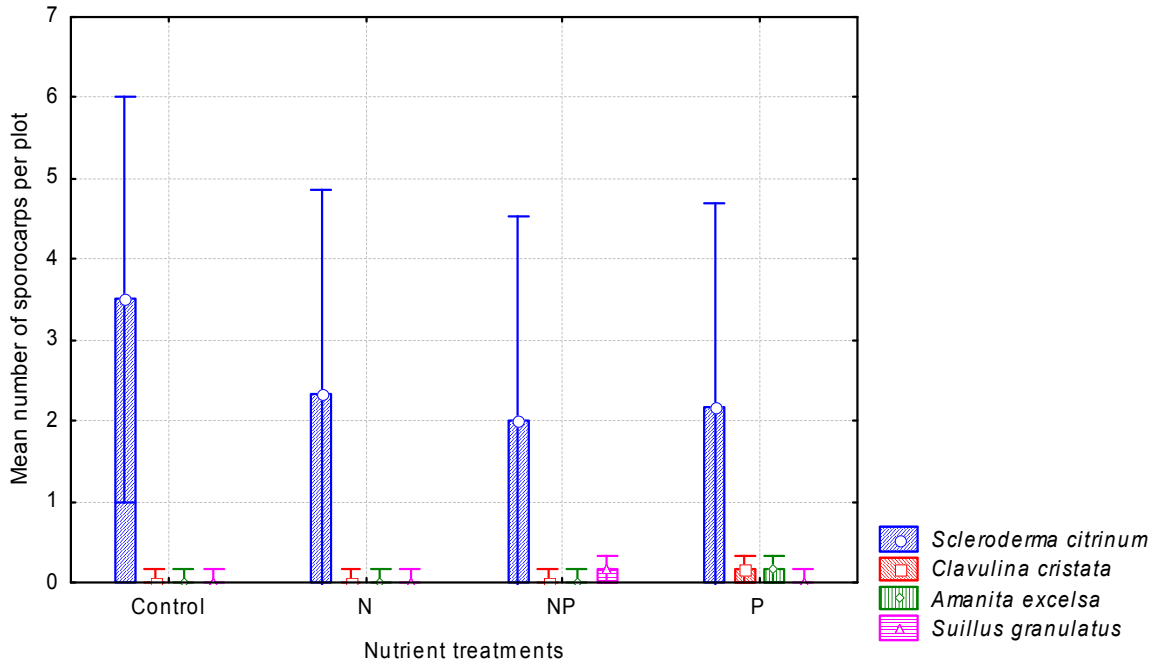


Figure 3.7 One-way ANOVA of the means of total sporocarps produced in the different treatment plots of N, P, NP and control in the Brooklands study site for sampling events March 2002, April 2002 and April 2003. $F_{(18, 87.6)} = 1.12$, $p = 0.35$. Significance taken at the 95% confidence level. Bars indicate standard error.

A one-way ANOVA of data from March 2002, April 2002 and April 2003 demonstrated no significant differences in ECM fungal sporocarp abundance (Figure 3.7) between nutrient treatments.

3.4 Discussion

3.4.1 ECM above ground diversity

A survey of the diversity of ECM sporocarp species was conducted. A representative specimen of each species was silica- or oven-dried and deposited in the Schonland Herbarium, Grahamstown (Appendix 1). Species identification was relatively straight-forward, and was easily achieved, at least down to genus level. Sporocarp species identifications were verified using molecular techniques (Chapter 5).

Amanita muscaria (Plate 1.1a), *A. rubescens* (Plate 1.1b) and *A. excelsa* (Plate 1.1c) were identified from the collections. *A. rubescens* (Plate 1.1b) is distinctly different to *A. excelsa* (Plate 1.1c), in that the sporocarps had a pink tinge on the stipe and pileus that was easily discerned. All three species have already been identified from South Africa, by Doidge (1950) and van der Westhuizen and Eicker (1987), and recorded as occurring in Mpumalanga, the Eastern Cape and the South Western Cape.

Boletus edulis (Plate 1d), found in the Brooklands study site and Highlands area was not common, although there are several companies with harvesting concessions for export purposes in the Mpumalanga and Eastern Cape Provinces (e.g. Bolé, Klamath Investments (Pty) Ltd, Eastern Cape). These species is similar in morphology to *Boletus aestivalis* and is often mis-identified or indicated as sub-species of *B. edulis*. The differences between *B. edulis* and *B. aestivalis* are only discernable at maturity. The flesh of a *B. aestivalis* sporocarp changes from white to pale yellow with age, while the flesh of *B. edulis* remains white (Pegler, 1990). Additionally, the colour of the pileus differs from a greyish brown in the case of *B. aestivalis* and chestnut brown in the case of *B. edulis*.

Two specimens of *Clavulina* (Table 3.1) were collected in the Brooklands study site and in surrounding established *Pinus* plantations and were initially identified as two different species. The specimens in this study were morphologically separated by sporocarp colouring, which in the case of *C. cinerea* is greyer and the tips are lest crested than *C. cristata* (Courtecuisse, 1999). To further confirm the species identity of the specimens, molecular techniques were used (Chapter 5).

Inocybe species (e.g. Plate 1.2 p) were only found in the younger stand, the Driekop study site (Table 3.1), indicating that these species are potentially early stage ECM fungal colonisers. Three species were collected, but only one specimen could be identified solely based on morphology as *I.* cf. *griseolilacina* from the faint lilac tints on the stem and fragile scales on the pileus (Pegler, 1990). The genus *Inocybe* is large and forms a taxonomic

group consisting of over 300 species (Matheny *et al*., 2002). As a large proportion of species have yet to be described (Kropp and Matheny, 2004), morphological identification can be difficult. All three specimens were analysed using molecular techniques to achieve further identification, where possible (Chapter 5).

The identification of *Lactarius hepaticus* (Plate 1.2 l) was relatively straightforward. The specimens of *Lactarius semisanguifluus* (Plate 1.2 k), *L. deliciosus* (Plate 1.2 l) and *L. hemicyaneus* (Plate 1.2 j) (Table 3.1) were, however, more difficult to discern from one another and were differentiated by the change in colour of the flesh when cut or bruised (Courtecuisse, 1999). The flesh of *L. hemicyaneus* was blue just under the external layer. All of the above species have been described as orange with green patches developing on the pileus. The flesh of *L. semisanguifluus* is described as bruising red then fading to green and *L. deliciosus* flesh is carrot-orange and remains unchanged over time (Courtecuisse, 1999). All had an orange pileus, with ocherous-green tinges. In the literature, it appears that three species names are often loosely applied as sub-species to refer to the same species, for example, *L. quieticolor* var. *hemicyaneus*, *L. deliciosus* var. *hemicyaneus* and *L. deliciosus* var. *quieticolor* (Bisby *et al*., 2005), and are apparently synonymous. The literature is confusing as to the delineation of these species and whether they are in fact sub-species in a species complex. Classification within the genus is still under debate, with 122 species allocated by Singer (1986, in Hutchinson, 1999), and 200 species by Hesler and Smith (1979, in Hutchinson, 1999). Further attempts to determine specimen identity and whether the specimens are separate species were made using molecular techniques (Chapter 5).

Rhizopogon roseolus (Table 3.1) was collected in the Brooklands study site and was identified from the enclosed, red-brown tuberous sporocarp (Garnweidner, 2001). This species has previously been recorded in South Africa (van der Westhuizen and Eicker, 1994)

The *Russula* collection made in the Highlands stand (Table 3.1) appears to consist of three species (Plate 1.2 m, n and o). Specimens that were characterised as featuring a white stipe, ochorous pileus and pale lemon yellow spores (Plate 1.2 m), were comparable to *R. sororia*. A specimen that featured a broader pink stipe, a dark purple pileus and darker apricot-coloured spores (Plate 1.2 n), fit the description of *R. capensis* (van der Westhuizen and Eicker, 1994). *Russula capensis* was described by Pearson, based on collections in Pine plantations around Cape Town (Dunstan *et al*., 1998). It is possible that the description made by Pearson in South Africa already coincides with a species described in Europe, *R. drimeia*, but further investigation into spore morphology and molecular sequencing would be necessary to determine the validity of this species. The remaining collection included a specimens characterised by a white stipe and dark purple pileus (Plate 1.2 o), which were similar to *R. sardonia*. The specimens that compared favourably to *R. sororia* (Plate 1.2 m) were morphologically characterised as having cream coloured lamellae, a patchy green-yellow coloured pileus and a white stipe. The specimens identified as *R. sardonia* were characterised by yellow lamellae, a pink coloured stipe and a red coloured pileus. These two species have been documented as occurring in South Africa (Lundquist, 1987a, van der Westhuizen and Eicker, 1994), but the validity of the identification of these species is yet to be verified.

Sporocarps identified as *Scleroderma citrinum* (Plate 1.1 h) were characterised as having a yellow-brown, enclosed sporocarp formation and an inner spore mass that turned black at maturity. This species was particularly common in the Brooklands study site. The sporocarps were tough and persistent.

Several species of *Suillus* were identified. *S. salmonicolor* (Plate 1.1 f), was identified by features of the partial veil that remain attached to the stipe and the ochereous-salmon colour of the pileus (Smith and Thiers, 1971). Reid and Eicker (2000), identified this species as a ‘new’ Bolete in South Africa, and have also been found to occur in *Pinus* plantations in Natures Valley, Tsitsikamma (personal communication, Dames 2005). *Suillus granulatus*

(Plate 1.1 e) and *Suillus luteus* have been recorded in South Africa (van der Westhuizen and Eicker, 1994).

A Tomentelloid specimen was found in resupinate form (Plate 1.1 g). The sporocarp was light brown in colour forming a thin layer on leaf litter and dead wood. Resupinate Tomentelloid fungi are geographically widely distributed. Kõljalg *et al*., (2000), demonstrated the ECM association of four *Tomentella* species in Swedish boreal forests for the first time. Both the resupinate and bracket form of *Thelephora terrestris* were collected in both study sites. *Thelephora terrestris* is common in nurseries and plantations in Mpumalanga as discussed by van Greuning and van der Westhuizen (1990). The presence of this fungus in the 3-5 year stand, Driekop (Table 3.1), is therefore not surprising.

A single species of *Tricholoma* and two species of *Xerocomus* were identified in the sporocarp collections of this study. *T. albobrunneum*, has been recorded in South Africa, but *X. badius* and *X.* cf. *chrysenteron* have not, and can therefore be added to the current understanding of ECM fungal diversity.

Compared to the species list derived during previous studies (van der Westhuizen and Eicker, 1987, Lundquist, 1986, Reid and Eicker, 2000, Doidge, 1950), the diversity observed in this study does not record the presence of any new species found in South Africa. The results is this study, however, confirm the association of all the fungal species found with *Pinus* in South Africa and therefore the association of the following species are new in a South African context: *Boletus aestivalis, Inocybe* cf. *griseolilacina, Lactarius hemicyaneus, Xerocomus badius* and *Xerocomus chrysenteron*. Levin *et al*., (1985) in their non-peer reviewed field guide have recorded the presence of *X. badius* and *X. chrysenteron* in South Africa. It is, however, unclear from the authors whether these specimens were in fact expertly identified and where the collection material could be accessed. Additionally, Levin *et al*., (1985) make reference to mycorrhizal fungi, but do not mention this genus (*Xerocomus*) as part of the mycorrhizal trophic group, nor do they associate this fungus with *Pinus* spp.

The survey of diversity is limited in this study as sampling was sporadic and ideally, should have occurred over an extended time period, but has none-the-less contributed to the list of species associating with *Pinus* spp. Sporocarp formation is also sporadic, requiring a specific set of environmental conditions particularly involving moisture (O'Dell *et al.*, 1999). An additional limitation to sampling the total diversity is that some sporocarps are very short-lived or sporulate beneath the soil surface. The diversity of species recorded is however of interest. The majority of the species found are derived from Europe, with the exception of *Suillus salmonicolor (also known as S. subluteus)*, which is described from Northern America, associated with jack pine species east of the Rocky Mountains (Smith and Thiers, 1971).

A comparison of the above ground ECM fungal species diversity can be made between other exotic, managed *Pinus* plantations internationally. In South America 52-55 ECM fungal species have been identified from *Pinus* spp. New Zealand and Eastern Australia compare closely with 30-32 and 32-34 ECM fungal species, respectively (Dunstan *et al*., 1998). Western Australia has only recorded ten fungal species (Dunstan *et al*., 1998). Fungal ECM sporocarp diversity in *Pinus patula* plantations of varying age in India (Natarajan *et al*., 1992) reports only ten species collected, from sampling events every forty days over a 2-year period, but similarities of species composition, such as *Amanita muscaria*, *Scleroderma citrinum*, *Tricholoma* sp., *Russula* sp., *Rhizopogon* sp. and *Suillus* sp are evident. The species richness recorded in this study compares with that found in other exotic managed *Pinus* plantations. ECM fungal species diversity in exotic managed plantations is relatively low compared to areas where ECM species recorded in exotic plantations originate, e.g. Sweden (Erland and Taylor, 2002). In this study only 26 morphological species were identified, which agrees with the comparatively low level of species diversity encountered in exotic managed plantations (Dunstan *et al*., 1998). There are a number of explanations for this phenomenon. A native species pool of ECM fungi is thought to be absent. This is especially highlighted in South Africa, where no indigenous ECM fungi have been identified. As a result the ECM host rely on fungal species that

have been imported. Secondly, the management practices applied to intensive forest plantations may not be conducive, in particular ECM fungi sensitive to forestry practices such as fertilisation and pesticide application. This would result in the continued presence of only a few ECM fungi that are capable of adapting to such disturbance factors.

3.4.2 Effects of fertilisers and climatic changes

3.4.2.1 Climatic effects

A climatic effect on sporocarp production has been demonstrated (Table 3.2), as a decrease from a total of 166 sporocarps in March 2002 to 12 sporocarps in June 2002, within the 5x5m plots (Figure 3.1, 3.2, 3.3 and 3.4). Statistical significance of this pattern was demonstrated (Table 3.3 & 3.4) and correlates to climatic changes in the study site area, from early autumn (March) to mid-winter (June), characterised by a decrease in moisture and temperature (Section 2.2.1). The implication of this result for further consideration of sporocarp-collecting research is that sampling events should concentrate in the months of March to April. These findings are not unexpected, as sporocarp production has been correlated with environmental factors such as temperature and moisture. The results confirm the finding of Agerer *et al.*, (1998), where it was demonstrated that irrigation (and therefore soil moisture) increased sporocarp abundance from 3.3 to 12.3 sporocarps of *Russula ochroleucra* and 3.7 to 24.2 sporocarps of *Hygrophorus pustulatus* relative to non-irrigated plots.

3.4.2.2 Effects of nutrient applications

It should be noted that observations carried out in this study are limited by sporadic sampling. Additionally, larger diversity of fungal species collected outside the plots over a larger area (Table 3.1) indicated that the treated plot sizes were possibly too small for adequate statistical data collection. The plot size used to determine nutrient effects was, however, unavoidably restrictive due to the pre-existing trial design, and future research should consider using entire plantations of the same age and geographical position with varying fertiliser treatments for more conclusive results. Effects of fertiliser treatments on ECM fungal species diversity were not apparent (Fig 3.5, 3.6 & 3.7).

Although this study does not bring to light any fertiliser effect on either species diversity or sporocarp abundance, several authors provide evidence to the contrary. Peter *et al*., (2001) found a large reduction of ECM species diversity, from nine in untreated plots to two species in response to N application, two years after application in a Norway spruce stand. Lilleskov *et al*., (2001), also reported a reduction from 144 ECM fungal species at low N inputs to 14 species at high N inputs, associated with white spruce in Alaska. Additionally, Pampolina *et al*., (2002) reported a significant reduction in sporocarp production of up to 14% in P fertilised *Eucalyptus* plantations, but without change in above ground ECM fungal diversity.

Although this study does not concur with the above research, the possible lack of nutrient effect on sporocarp production may be due to the time since the last treatment was applied to the trial stand, in 1998. This implies that changes in sporocarp production by particular ECM fungal species as a result of fertilisation may not be sustained over longer periods of time, in South Africa. The abundance of the four fungal species in the N, P, NP and Control plots appeared to be unaffected, however, the sample data size was too small to be statistically analysed.

Most ECM fungi are sporocarp-forming basidiomycetes, allowing visual observations of site condition. The low sporocarp frequency within the sample plots made conclusion regarding nutrient affects difficult. Sporocarp formation is triggered by environmental conditions, mainly rainfall, temperature/season and soil chemistry. Should the environment not provide favourable conditions, fruiting will not occur. As sampling events occurred over short, intermittent time periods, prolific fruiting times could have been missed. Sporocarp longevity varies and this may contribute to the lack of diversity and abundance observed in the trial stands. Collections should, therefore, be carried out over a number of successive seasons and years to achieve a more conclusive species richness measure, which is likely to be higher than recorded in this study. Although sporocarp presence provides a strong indication of the presence of the fungal species below ground, sporocarp data may not fully reflect the diversity of the ECM root tip community.

4. ECTOMYCORRHIZAL ROOT TIP CHARACTERISATION

4.1 Introduction and objective

Ectomycorrhizal (ECM) characterisation is primarily the establishment of morphological and anatomical features of the fungus in association with the plant host, occurring mainly along fine feeder roots and root tips. The analysis involves anatomical characterisation of features occurring in the mantle layers, the Hartig net, extra-radical rhizomorphs and emanating hyphae (Mohan *et al.*, 1993a,b, Taylor and Alexander, 1989 & 1990, Massicotte, *et al*., 1999, Kraigher *et al*., 1995, Agerer 1987-2002).

De Roman *et al.*, (2005) reports that over 1244 Ectomycorrhizal root tip descriptions have been published from 1961 to the present, the majority of which were published after 1986, as a result of the Colour Atlas of Ectomycorrhizae (Agerer, 1987-2002) that photographically portrays both morphological and anatomical features characteristic to ECM fungal species or genera, which will henceforth be referred to as the Colour Atlas. Using the features as defined by Agerer (1987-2002), as well as those contributed by Ingleby *et al.*, (1990), has provided standardised criteria for the examination of ECM root tips. The features of the root morphology, mantle texture, mantle pattern and structure, as well as the examination of specialised cells, graphically presented in the Colour Atlas, have been used to identify ECM root tips, at least to genus level (Agerer, 1987-2002) and may be used to key out field-collected ECM roots and compare its morphology to identified species plates.

Species diversity is an integral part of conservation biology and ecology management, with particular reference to species abundance and distribution (Begon *et al.*, 1990). Key indicator species, once identified and characterised, can help to monitor environmental health and responses to disturbance factors. ECM fungal species diversity in commercially managed plantations is not well documented in South Africa (Dames, 1996) inhibiting further research

and management within this field. In many cases, information about mycorrhizal structures was limited, hence hindering the identification of associated ECM fungi using key characteristics. Diversity studies have therefore relied on sporocarp data to represent both above ground and below ground diversity (Lundquist, 1986, van der Westhuizen and Eicker, 1987). Although sporocarp data has been collected and analysed in this project (Chapter 3), it cannot be used as a reliable source of rhizosphere community diversity (Dahlberg, 2001). Sporocarp collections fall short of representing the species of ECM that do not form fruiting bodies. Abiotic factors (such as rainfall) have a large influence that may inhibit sporocarp production. Some species do not or very rarely form fruiting bodies. Sporocarp data may represent the dominant fungal species that fruit, and not necessarily the dominant underground community structure (Jonsson *et al.*, 1999). Therefore the absence of a sporocarp species is not indicative of the absence of that species in the rhizosphere. For these reasons, sporocarp enumeration does not necessarily, or accurately, represent below ground species.

Currently, six fungal species have been identified on *Pinus patula* as a host, using ECM root tip characterisation. *Amanita muscaria*, *Laccaria laccata*, *Suillus brevipes* and *Thelephora terrestris* was identified by Mohan *et al*. (1993a,b) after inoculating nursery seedlings with sporocarp material and harvesting the roots after five months. Dames *et al*., (1999), characterised two species from field samples growing in association with *P. patula*, which were identified as *Scleroderma citrinum* and *Boletus pinicola*.

The objective of this study was to identify the prominent morphotypes of ECM fungal species present in the *Pinus patula* plantations by characterising ECM root tips using morphological characteristics as described in the guidelines of the Colour Atlas (Agerer, 1987-2002) and to derive a morphological identification key. ECM root tip morphologies documented in this study can therefore be utilised in future ECM root studies in South Africa, creating a database of recorded South African species from which to consult and make taxonomic comparisons.

4.2 Methods

4.2.1 Extraction of root material

Four collections of root material were made in June 2002, December 2002, April 2003, and December 2003 and from the Brooklands and Driekop study sites (Chapter 2). Additionally, a random collection of roots was collected in April 2004 for confirmation of morphological features as depicted from prior observations of morphotypes. Roots were washed clean of any soil and organic matter using a 2mm sieve and were inspected under a dissecting microscope (Leica S4E). Where possible the material was observed fresh or preserved in 50% alcohol for future examination (Brundrett *et al*., 1996). ECM root tips exhibiting similar morphological characteristics such as mantle colour, mantle surface features, branching type and extra-radical hyphae, were grouped into types and are, from here on, referred to as morphotypes (Agerer, 1987-2002). The codes given to each morphotype were derived from these observations.

4.2.2 Sectioning and staining

Cross and longitudinal sections were made using a freeze microtome (Mectron model 150, Frigistor LTD). ECM root tips were mounted in 2% glycerol and sections were then placed in a drop of water in 1.5ml eppendorf tubes. The lactoglycerol trypan blue staining procedure of Koske and Gemma (1989), and Smith and Dickson (1997), for whole root staining was followed and modified by extending the hydrogen peroxide soak for darker sections to allow for better bleaching. Each staining procedure was done in the 1.5ml eppendorf, and any solutions to be added or removed, were done so using an autopipette. During the course of staining, if root sections became suspended, the samples were centrifuged at 5000rpm for 30 seconds. Sections were slide mounted in lactoglycerol and anatomical characteristics were examined (Table 4.1) using a compound microscope (Olympus BX50). Mantle sections were peeled off whole roots and mounted in lactoglycerol or water, and examined for surface hyphal arrangement and key distinguishing features (Fig 4.1).

4.2.3 Morphological features of ECM root tips

Roots were first annotated with features derived from root branching and mantle morphology. Cross- and longitudinal sections were then examined to characterise various layers for hyphal arrangement and presence of specialised cells (Agerer, 1987-2002).

Important features described in the Colour Atlas (Table 4.1) include root tip branching, shape of branches, mantle colour and surface texture, presence of rhizomorphs and emanating hyphae (Agerer, 1987-2002). Anatomical characteristics that are routinely examined include detailed records of mantle morphology, characteristics of emanating hyphae and rhizomorphs and structure of the Hartig net in terms of hyphal arrangement, hyphal size, presence of specialised cells and mantle layering (Mohan *et al.*, 1993 a, b, Taylor and Alexander, 1989 & 1990, Massicotte *et al.*, 1999, Kraigher *et al.*, 1995).

Table 4.1 Characteristics recorded for each ECM morphotype.

ECM root tip morphology (Dissecting microscopy)	ECM anatomical morphology (Compound microscopy)
• Branching type (ramification) Simple, pinnate, irregular, dichotomous, coralloid or tubercle-like	• **Inner surface** (hyphal arrangement) (Figure 4.1) • Specialised cells (dimensions) • Cystidia, lactifers, tannin cells • Hyphal dimensions (Width and length of cells)
• Length and diameter of branches	• **Middle layers** (hyphal arrangement) (Figure 4.1) • Hyphal dimensions • Specialised cells (dimensions and frequency) • Wall thickness
• Axis: last branch axis (last order branch)	• **Outer surface** (hyphal arrangement) (Figure 4.1) • Hyphal dimensions
• Shape of ramified ends: Straight, bent, tortuous or beaded	• **Rhizomorph type and specialisation** • Emanating hyphae (wall thickness and shape) • Specialised hyphae: Cystidia, lactifers, tannin cells
• Mantle surface texture: Smooth, reticulate, grainy, spiny, cottony or woolly	• **Other**: surface oil droplet, crystals, soil particles
• Mantle colour	
• Mantle colour (aged)	
• Soil particles (where): Between branches, on mantle, surrounded by rhizomorphs	
• Rhizomorphs: colour, attachment and shape • Attachment: restricted, angled or fanned • Shape: filamentous, smooth or hairy	
• Emanating hyphal features	

(Agerer, 1987 -2002)

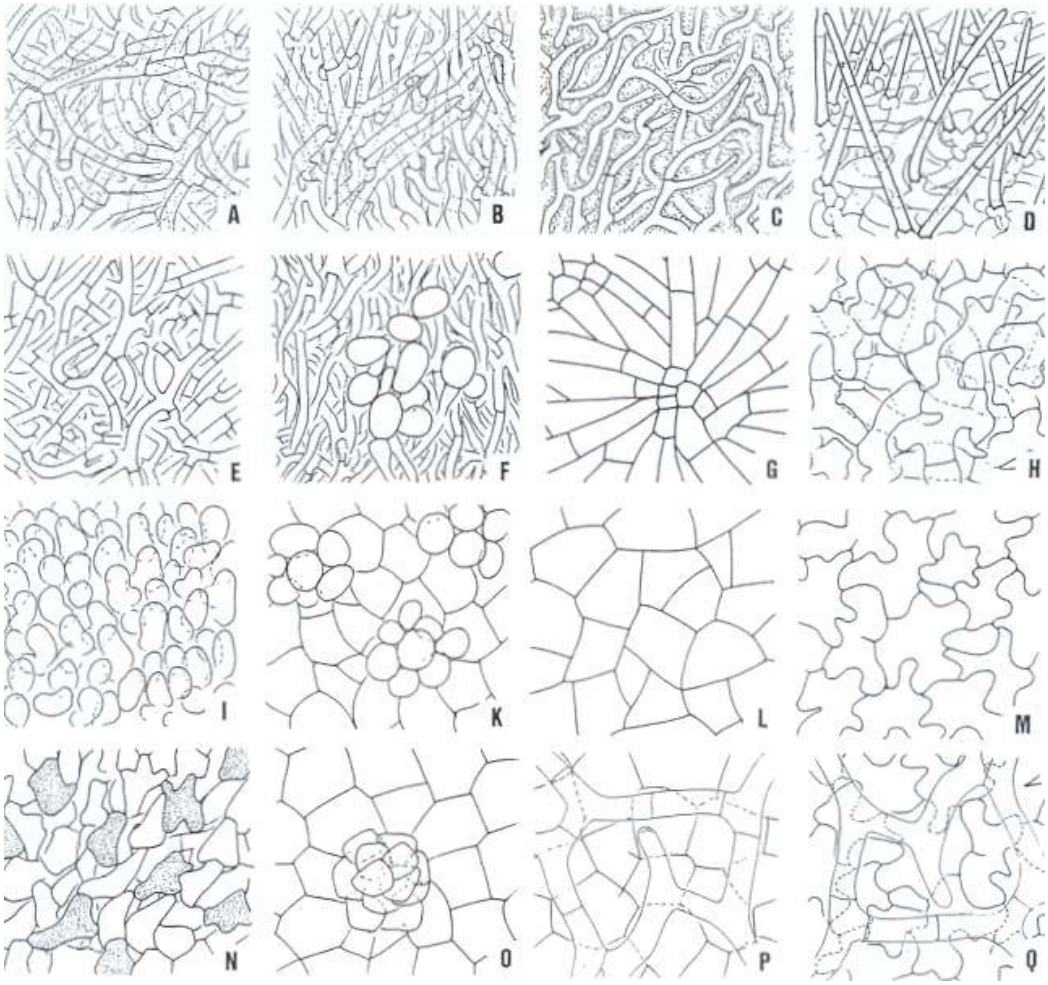


Figure 4.1 Mantle types as seen from mantle scrapings. (a) plectenchymatous, (b) irregular plectenchymatous, (c) plectenchymatous in gelatinous matrix, (d) net-like plectenchymatous with prominent cystidia, (e) sqarrosely branched and net-like plectenchymatous, (f) round cells on a plectenchymatous layer, (g) arrangement is star-like, tightly plectenchymatous, (h) transitional plectenchymatous to pseudoparenchymatous, irregular, (i) plectenchymatous, hymeniform, (k) angular pseudoparenchymatous, with mounds of round cells, (l) angular pseudoparenchymatous, (m) epidermoid pseudoparenchymatous, (n) pseudoparenchymatous, shape variable and staining with sulfo-vanillin, (o) angular pseudoparenchymatous, with mounds of flattened cells, p) angular pseudoparenchymatous, with hyphal net, (q) epidermoid pseudoparenchymatous, with hyphal net (Agerer, 1987-2002).

4.2.4 Photography

ECM root tip morphology was photographed using dissecting and compound microscopes (Wild Photomakroskop M400 and Olympus BX50 respectively). Root tip morphology was photographed using 100 ASA daylight slide film. Differential interference contrast (DIC) and light microscopy was used to record microscopic anatomy. Photographs were taken with black and white 100 ASA film.

4.3 Results

4.3.1 Morphotypes and morphological descriptions:

Twenty-two distinct morphotypes were isolated from the Brooklands and Driekop study sites. Seven of the most dominant morphotypes were selected for further investigation and characterisation. Characterisation and identification of the following morphotypes was undertaken: 'White', 'BSY', 'Black', 'Orange', 'Yelsil', 'Grey' and 'Tuberculate', and are presented with descriptions on Plates 2-8 respectively. From the Brooklands plots the following dominant morphotypes 'White', 'BSY', 'Orange' and 'Grey' were selected. Three dominant morphotypes from Driekop, 'Black', 'Yelsil' and 'Tuberculate', were characterised. Of the above types, 'White', 'Orange', 'Yelsil', 'Black' and 'Tuberculate', were common morphotypes between the two sites, but varied in frequency between the sites (Chapter 6, Section 6.2). Additionally, selected dominant morphotype were briefly characterised.

4.3.1.1 White morphotype: morphological and anatomical features.

Plate 2 Photographic presentation of ECM morphotype 'White': morphological and anatomical characteristics.

Plate 2 Morphotype 'White' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a) ECM root tips with white mantle sheath and numerous rhizomorphs (**rh**), dichotomous branching (**db**); (b) loosely arranged, clamped hyphae (**arrow**) on surface of mantle; (c) branching of rhizomorph; (d) loose plectenchymatous hyphal arrangement of outer mantle layer; (e) tight plectenchymatous hyphal arrangement of inner mantle, net-like and squarrosely branched; (f-h) Hartig net as seen in cross section, in a palmetti arrangement (**p**). Scale bar represents 10µm.

Root tip morphology

The branching of the root tip was dichotomous, long and straight (Plate 2 a); and the dimensions of the last order axis tips ranged from 0.50 to 1.20mm in length and 0.25 to 0.30mm in diameter. The mantle surface consisted of loosely arranged 'stringy' hyphae that trap air, making the mantle hydrophobic, and giving the root tips a 'silvery' sheen. The mantle was white, bruising dull yellow with no soil attachments on the mantle. Frequent rhizomorphs emanating from the mantle were white to light yellow/opaque, and had smooth margins with few hyphal branches. Emanating hyphae were found along the length of the colonised root.

Anatomical morphology

The mantle was composed of two plectenchymatous layers. The outer layer was loosely arranged (Plate 2 d) with hyphal dimensions at 2.90 to 3.90µm in width and 14.55 to 33.95µm in length. The inner layer was more compact (Plate 2 e), 2.9 to 3.9µm in width and 9.70 to 21µm in length. Rhizomorphs were observed to be thicker in diameter at branching points, forming nodia, and were differentiated by randomly thickened hyphae (Plate 2 c). The diameter of the rhizomorph ranged from 33.95 to 38.8µm. Emanating hyphae were clamped (Plate 2 b) and were 2.5 to 4.9µm in width and 43.6 to 58.2µm in length from septa to septa. The Hartig net was in a palmetti arrangement, which developed from branching hyphae aligned alongside each other to form a single layer between the cells (Plate 2 f-h). Hartig net hyphae penetrated 1 to 2 layers of the plant cortical cells.

4.3.1.2 BSY morphotype: morphological and anatomical features.

Plate 3 Photographic presentation of ECM morphotype 'BSY': morphological and anatomical characteristics.

Plate 3 Morphotype 'BSY' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a) ECM root dirty yellow with a silver sheen, adhesive soil particles at the base of dichotomous branching, (b) cystidia (**cy**) and cell wall thickening (**arrows**) on rhizomorph, (c) emanating hypha, septate at the base (**arrow**), (d) inner mantle layer of angular pseudoparenchymatous hyphal pattern, (e) outer mantle layer of loosely arranged plectenchymatous hyphae, cystidia with thickened cell walls (**arrows**), (f) inner 'tube' of rhizomorph and an outer cystidia with thickened cell walls (**cy**), (g) cross-section showing soil particles, mantle (**m**) and Hartig net (**p**). Scale bar represents 10µm.

Root tip morphology

Branching of the root tips were coralloid to irregularly pinnate (Plate 3 a); with the last axis ranging from 2 to 2.5mm in length and 0.5mm in diameter. The mantle was a dirty yellow in colour with a slight sheen, indicating hydrophobic properties. The ramification was straight and the mantle surface texture smooth to reticulate. Soil particles covered the entire root, concentrating in the base of the root branches. Rhizomorphs were dark yellow to tan in colour (not shown in Fig 4.3), but infrequent.

Anatomical morphology

Emanating hyphae were infrequent, and were septate at the base of attachment (Plate 3 c). No clamps were apparent from the emanating hyphae. The outer mantle was arranged in a loose delicate plectenchymatous hyphal net (Plate 3 e), with hyphal dimensions of 1.8 to 2.9µm in width and 10 to 11.64µm in length. The inner mantle consisted of strongly angular pseudoparenchymatous cells (Plate 3 d), 6 to 10µm in width and 9 to 11µm in length. The rhizomorphs were highly differentiated (Plate 3 f). There appeared to be an inner thin walled tube surrounded by shorter thick-walled hyphae (Plate 3 f). Thick walled cystidia were observed on the outer mantle and the outer most cells of the rhizomorph (Plate 3 b,f). The Hartig net was in a palmetti arrangement and penetrated 1 to 2 layers of the cortical cells (Plate 3 g).

4.3.1.3 Black morphotype: morphological and anatomical features.

Plate 4 Photographic presentation of ECM morphotype 'Black': morphological and anatomical characteristics.

Plate 4 Morphotype 'Black' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a) Dark mantle, dichotomous branching (**db**), mantle surface woolly. (b) root tip showing dark pigmentation, (c) emanating hyphae from the mantle surface, characterising bulb shape cystidia (**arrows**), (d) Palmetti arranged Hartig net (**p)**, tannin cells present as dark cells just below mantle, (e) view of longitudinal section showing mantle and Hartig net, (f) inner mantle layer showing characteristics of parenchymatous/pseudoparenchymtous irregular hyphae, (g) emanating hyphae from the mantle surface. Scale bar represents 10µm.

Root tip morphology

Branching of the roots was unramified to short dichotomous and straight (Plate 4 a). The last order axis was 0.5 to 0.8mm in length and 0.3 to 0.4mm in diameter. The mantle was highly pigmented (Plate 4 b) and black in colour. The texture of the mantle surface was reticulate to woolly in texture. The woolly texture was formed by emanating hyphae that then extended from the mantle to form spine-like structures and was observed along the surface of the mantle. Soil particles were held between emanating hyphae. No rhizomorphs were observed, but emanating hyphae were found along the length of the ECM root tip, concentrating around younger, acropetal areas. Emanating hyphae formed fan-like morphologies.

Anatomical morphology

Emanating hyphae were frequent, dark and clamped (Plate 4g). Cystidia were observed attached to the mantle by a globular base (Plate 4 c). The spine-like hyphae emanated from the globular base of the cystidia, which were separated at the base by septa. The spine-like hyphae measured 75µm in length from septa-septa and 3µm in diameter. The mantle consisted of one layer of irregularly shaped transitional hyphae from plectenchymatous to pseudoparenchymatous arrangement (Plate 4 f). The hyphal dimensions in this layer were 4 to 8µm in width and 7 to 10µm in length. Tannin cells were present as a single layer just below the mantle (Plate 4 d). The Hartig net formed a typical palmetti arrangement (Plate 4 d,e) and penetrated 3 to 4 layers of the plant cortical cells.

4.3.1.4 Orange morphotype: morphological and anatomical features.

Plate 5 Photographic presentation of ECM morphotype 'Orange': morphological and anatomical characteristics.

Plate 5 Morphotype 'Orange' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a-b) Smooth orange mantle, dichotomous branching (**db**); (c) outer mantle layer showing loose hyphal network and cystidia with what appeared to be a pigment release (**arrow**); (d-e) pseudoparenchymatous network with angular cells in middle mantle layer; (e) irregular pseudoparenchymatous hyphae of inner mantle layer (f) loose hyphae on outer mantle surface, angular pseudoparenchymatous cells below; (g) cross section of mantle; (h-i) cross section of root showing mantle and Hartig net (**hn**). Scale bar represents 10µm.

Root tip morphology

The branching associated with the 'Orange' morphotype was short dichotomous to coralloid, straight (Plate 5 a,b). The length and diameter of the last order axis was 0.5 to 0.7mm and 0.2 to 0.5mm respectively. The mantle surface was smooth with a few hyaline emanating hyphae. The mantle was bright orange in colour, dulling to a darker orange with age. No rhizomorphs were observed.

Anatomical morphology

Emanating hyphae lacked clamps. A cystidium (Plate 5 c) with what appeared to be a pigment at the tip was observed The outer mantle layer consisted of a delicate net ranging from 5 to 7µm in width and 15 to 20µm in length calculated from septa to septa (Plate 5 c). The net covered a middle mantle layer of pseudoparenchymatous cells with an angular configuration and hyphal dimensions of 8 to 15µm in width and 14 to 18µm in length (Plate 5 d,e,g). The inner layer was pseudoparenchymatous to plectenchymatous, in another net-like arrangement (Plate 5 f) with hyphal dimensions of 3 to 5µm width and 10 to 25µm length. No rhizomorphs were observed. The Hartig net, in palmetti arrangement, penetrated 3 to 4 layers of the plant cortical cells (Plate 5 h,i).

4.3.5 Yelsil morphotype: morphological and anatomical features.

Plate 6 Photographic presentation of ECM morphotype 'Yelsil': morphological and anatomical characteristics.

Plate 6 Morphotype 'Yelsil' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a) bright yellow dichotomously branching root (**db**), (b) cystidia (**cy**) on the outer mantle were infrequent, (c) middle mantle layer showing a tight plectenchymatous arrangement, (d) cross-section of root tip showing a dense mass of emanating hyphae, clamped (**arrow**) hyphae and a loose plectenchymatous outer mantle layer, (e-g) inner mantle layer presenting a pseudoparenchymatous arrangement. Scale bar represents 10µm.

Root tip morphology

The root of the morphotype was a luminescent-lemon yellow that branched dichotomously (Plate 6 a). The last order axis was 0.7 to 1.2mm in length and 0.4 to 0.5mm in width. The mantle was smooth with infrequent short, emanating hyphae.

Anatomical morphology

Three layers were observed in the mantle of the 'Yelsil' morphotype. The outer layer is arranged in a loose plectenchymatous network and emanating hyphae (Plate 6 d). The hyphal dimensions of the outer mantle layer were 2 to 3µm in width and 23 to 40µm in length measure between septa. Infrequent cystidia with globular base were observed on the outer mantle (Plate 6 b). The middle layer was tightly plectenchymatous and the hyphae were observed between 3 to 4µm in width and 10 to 15µm in length (Plate 6 c). The inner layer was pseudoparenchymatous (Plate 6 e,f,g), and 4 to 7µm in width and 8 to 10µm in length. Rhizomorphs were not observed in the samples examined. The emanating hyphae were clamped (Plate 6 d). The Hartig net penetrated 2 layers of the plant cortical cells.

4.3.1.6 Grey morphotype: morphological and anatomical features.

Plate 7 Photographic presentation of ECM morphotype 'Grey': morphological and anatomical characteristics.

Plate 7 Morphotype 'Grey' showing morphological and anatomical features as observed under the dissecting and compound microscope. (a) grey mantle, root tip branching is dichotomous, soil attached along length of ECM root tip (**arrow**), (b) emanating hyphae are clamped, (c) cross-section of the root tip showing the mantle (**m**), and the Hartig net in palmetti arrangement (**p**), (d) the outer mantle has loose net of emanating hyphae that become more tightly arranged, (e) tightly arranged transitional pseudoparenchymatous/plectenchymatous hyphal arrangement in the inner mantle layer, (f) the Hartig net in a palmetti arrangement. Scale bar represents 10µm.

Root tip morphology

The 'grey' morphotype was dichotomously branched (Plate 7 a) with straight unramified ends. The length and width of the last order branches were 0.6 to 0.7mm and 0.25 to 0.3mm, respectively. The mantle surface was smooth to slightly woolly. Soil particles were attached to the length of the mantle.

Anatomical morphology

Two layers were observed in the mantle (Plate 7c). The outer mantle layer had a plectenchymatous arrangement with squarrose branching (Plate 7 d), with hyphal length and width ranging from 15 to 20µm and 3 to 4µm respectively. The inner mantle depicted a transitional arrangement in between pseudoparenchymatous and plectenchymatous (Plate 7 e) and had hyphal dimensions of: 7 to 9µm in length and 5 to 7µm in width. No rhizomorphs were observed. Emanating hyphae were clamped and long (Plate 7 b), ranging between 60 to 75µm in length from septa-septa and 3 to 4µm in diameter. The Hartig net was in a palmetti arrangement penetrating 2 layers of the plant cortical cells (Plate 7 f).

4.3.1.7 ‘Tuberculate’ morphotype: morphological and anatomical features.

Plate 8 Photographic presentation of ECM morphotype ‘Tuberculate’: morphological and anatomical characteristics.

Plate 8 Morphotype 'Tuberculate' showing morphological and anatomical features as observed under the dissecting and compound microscope. Scale bar represents 10µm except for (c). (a) white/grey with tubercle-like ramification, (b) cross section showing tightly coralloid branching, (c) cross section showing oil droplets on surface of the mantle (**arrows**), scale bar = 100µm, (d) cross section of mantle hyphae showing a plectenchymatous hymeniform arrangement, (e) cross section of mantle, oil droplets on surface (**arrows**), (f) hyphae in mantle in surface view, (g) loose hyphal arrangement between root tips, (h) Hartig net with a palmetti arrangement (**p**).

Root tip morphology

The 'Tuberculate' morphotype was greyish-white in colour (Plate 8 a) and was densely coralloid (Plate 8 b), becoming a dark grey with age. The tubercle-like aggregations were roughly oval in shape and measured between 2 to 3.5mm in width and 3 to 5mm in length. The mantle was smooth to slightly reticulate.

Anatomical morphology

The mantle hyphae protruded perpendicularly to the root surface (Plate 8 d) in a plectenchymatous hymeniform arrangement, 5.8 to 10.6µm in width and 18.4 to 24.5µm in length. The hyphal ends contained frequent amounts of oil droplets (Plate 8 c). From a surface view of the mantle, the hyphae appeared round (Plate 8 e,f). The hyphae of the mantle between the branches were tightly arranged and plectenchymatous (Plate 8 g). The rhizomorphs were differentiated with thickened hyphae. No clamp connections of the hyphae were encountered. The Hartig net was in a typical palmetti network (Plate 8 h), penetrating 4 to 5 layers of the plant cortical cells.

4.3.1.8 Descriptions of additional morphotypes

Table 4.2 Brief descriptions of some remaining dominant morphotypes found in the Brooklands and Driekop study site.

Morphotype	Macro-morphological features	Micro-morphological features	Study site
Partial white	Monopodial to dichotomous branching, partially covered with white-ish mantle, short emanating hyphae along the length of the root tip, reticulate mantle. Final axis root tips 4-5mm in width, 6-11mm in length.	Pseudoparenchymatous mantle layer, mixture of clamped and unclamped emanating hyphae.	B, D
DSY	Grey-yellow coloured, dichotomously branching, adhering soil particles, mantle texture smooth and loose, final axis root tips 4-5mm in width, 15-16mm in length.	Minute particles adhering to hyphae, clamped emanating hyphae, loose outer plectenchymatous mantle layer and tighter plectenchymatous inner mantle layer.	D
Beige	Larger beige-coloured, dichotomously branching, smooth mantle, final axis root tips 4mm in width, 8-15mm in length	Emanating hyphae clamped, outer mantle of linear plectenchymatous, inner mantle pseudoparenchymatous	B
Bt (wt)	Root tips small, white not covering entire root, very tip of root tan brown, long dichotomous branching, opaque rhizomorphs observed, final axis root tips 2mm in width, 9-13mm in length.	Clamp connections apparent, outer mantle loose linear plectenchymatous, inner mantle irregular pseudoparenchymatous	B, D
Dnorm	Root tip not easily discernable, no swelling observed, monopodial to dichotomous branching, final axis root tips 3-4mm in width, 10-16mm in length.	No emanating hyphae observed, outer mantle loose plectenchymatous, a tighter plectenchymatous middle layer and pseudoparenchymatous inner layer.	D
Dwhite	Root tips white, dichotomously branched, adhering soil particles, final axis root tips 3-5mm in width, 10-11mm in length.	Frequent emanating hyphae, clamped. Single mantle layer in pseudoparenchymatous pattern.	D

4.3.1.9 Morphological key

The seven morphotypes described were combined to the morphotypes cited by Dames *et al*., (1999), to provide an ECM root tip identification key for South African *Pinus* plantations. This provides a platform from which further characterisation and identification of ECM roots on *Pinus* spp. in South Africa can develop.

Key to South African ectomycorrhizas associated with *Pinus patula* collected from field material.

Ectomycorrhizas with a distinction mantle

1 Mycorrhizas with clamp connections

2 Rhizomorphs present, white-yellow, differentiated, cell walls not thickened, no cystidia present

Scleroderma citrinum

2* Rhizomorphs lacking

3 Mantle smooth, yellow, hyphae clamped, outer mantle plectenchymatous, middle layer tightly plectenchymatous and inner layer pseudoparenchymatous

Thelephora terrestris

3* Emanating hyphae with globular base, mantle is irregular, transitional plectenchymatous to pseudoparenchymatous, mantle is reticulate, black

***Tomentella* sp.**

3**Mantle smooth to woolly, grey, outer mantle layer loose plectenchymatous, inner layer is transitional between pseudoparenchymatous to plectenchymatous

Albatrellus ovinus

1* Mycorrhizas without clamp connections

4 Rhizomorphs present

5 Mantle plectenchymatous hymeniform, branching densely coralloid

Rhizopogon roseolus

5* Outer mantle layer plectenchymatous, inner layer strongly angular pseudoparenchymatous

Amanita excelsa

4* Rhizomorphs lacking

6 Angular pseudoparenchymatous mantle, mantle is smooth orange

***Amanita* sp.**

Ectomycorrhizas with no distinct mantle

1 Root tips dichotomously branched, no mantle or rhizomorphs detected, emanating hyphae sparse, no cystidia, but a well developed Hartig net present (Dames *et al*., 1999)

Cf. *Boletus pinicola*

4.4 Discussion

In this study, seven morphotypes have been characterised from *Pinus patula* plantations in South Africa, adding to the two morphotypes described by Dames *et al*., (1999). Also, a brief account of six morphotypes and the morphological features were given (Table 4.2).

The morphotype 'white' was identified as *Scleroderma citrinum* according to the Colour Atlas key (Agerer, 1987-2002), in that clamps were present (Plate 2 b), but lacked contact-clamps and contact septa, the mantle being plectenchymatous in surface view, with no cystidia and an absence of sclerotia (Plate 2). There were, however, differences in the branching. The morphotype examined in this study branched dichotomously (Plate 2 a); whereas the branching presented in the Atlas is monopodial-pyramidal. This may be attributed to the genus of the host, *Pinus*, the roots of which are often dichotomous with no fungal mycorrhizal association (Kaska *et al*., 1999). The 'White' morphotype is also similar to morphotype WITS 01, also described as *S. citrinum*, by Dames *et al*., (1999), from *Pinus patula*, but differs in the colour of the rhizomorph. Dames *et al*., (1999) found the rhizomorphs to be yellow, but notes that the variation in colour could be attributed to age. The

white ECM root tip identified by Mohan *et al*., (1993b) from *Pinus patula* as *Amanita muscaria*, was compared to the morphotype in this study, but differed in that the specimen in this study contained clamp connections and lacked tannin cells, that would have appeared as dark plant cells in the outer cortex (Plate 2 f-h). Sporocarp collections in the Brooklands site contained a prolific frequency of *S. citrinum* (Chapter 3). The presence of this species below ground was therefore, not surprising.

The morphology of the mantle of 'BSY' is remarkably similar to 'Orange' in that the mantle has an angular pseudoparenchymatous hyphal arrangement in the middle to inner layer. For this reason, the morphotype was compared to the *Amanita* species, presented in plate form, in the Colour Atlas. However, when compared to an *Amanita* species described by Mohan *et al.*, (1993b), *A. muscaria*, and few similarities were recorded. The distinguishing characteristic in which they differed was the strongly angular pseudoparenchymatous mantle layer observed in the mantle of 'BSY' that was not recorded in the mantle of *A. muscaria*.

The 'Black' morphotype was identified as a Tomentelloid ECM fungus. Keying out to genus or species was not possible, however, comparisons with *Tomentella* species were made. Occasional specimens of *Cenococcum geophilum* were observed. This species is characterised by stellate pseudoparenchymatous mantles (Agerer and Gronbach, 1988). Other, rarely found, black root tip types were comparable to *Hymenoscyphus ericae* and were characterised by plectenchymatous hyphal arrangement in the mantle (Brand *et al*., 1992). The similar morphologies of these black root tips may result in morphotyping inaccuracies. The morphotype described in this study, however, did not resemble either species. The morphotype 'Black' did compare to *Tomentella albomarginata* in that emanating hyphae extended from a globular hyphae in the mantle. Although not all the mantles layers of the description of *T. albomarginata* given by Agerer (1996) correlate with the current morphotype, a similar pattern of irregular, transitional type hyphae were comparable.

Morphotype ‘orange’ was compared to *Amanita* genera in the Colour Atlas. Although there is only one *Amanita* species graphically presented as a plate in the Colour Atlas (Agerer, 1987-2002), *A. citrina*, the key to mycobionts of *Pinus* represents two *Amanita* species. A comparison of mantle structure between *A. citrinum* and morphotype ‘orange’ can be made. Both species share loose, ring-like plectenchymatous surface hyphae and a definite angular pseudoparenchymatous middle layer as well as clamped emanating hyphae.

The morphotypes ‘Yelsil’ was keyed out in the Colour Atlas (Agerer, 1987-2002), to *Thelephora terrestris* and was compared to the photographic plate provided. The description, in association with *Picea abies*, differs very little from the ECM root tip in this study in terms of mantle morphology. Both mantles have three layers comprising of an outer loose arrangement of clamped hyphae, and then forming a middle layer of transitional pseudoparenchymatous and plectenchymatous thick hyphae. The inner layer is more tightly arranged with thinner hyphae. The ECM root colours, a silvery yellow, are the same, but ramification is different in that the specimen in this study branched dichotomously as opposed to monopodial-pinnate. The ECM root characterised in this study was comparable to the *Thelephora terrestris* described on *Pinus patula* by Mohan *et al*., (1993a). Similarities include the colouration of the mantle as light yellow, an outer mantle layer consisting of a loose net of emanating, clamped hyphae, a middle layer of tightly plectenchymatous septate hyphae and an inner layer of pseudoparenchymatous hyphae.

The morphotype ‘grey’ was not keyed out on the *Pinus* key in the Colour Atlas (Agerer, 1987-2002) although it was compared *Albatrellus ovinus* plate. Similarities and dissimilarities were noted. Both specimens were grey in colour, and were described as having a loose layer of emanating hyphae. In both cases, the outer mantle was plectenchymatous, the arrangement becoming tighter towards the inner mantle. The differences between the descriptions were cystidia that were not reported from this morphotype. The branching reported in association with *Picea abies*, was represented as

monopodial, where the present description associated with *Pinus patula* was dichotomously branched.

The morphotype 'Tuberculate' was keyed out as *Rhizopogon roseolus*, in that the ECM root tip branching was tightly coralloid, the emanating hyphae contained oil droplets and were not embedded in a gelatinous matrix, the mycorrhizal system was smooth and clamps were absent (Agerer, 1987-2002). Sporocarps of this species were found in the Brooklands study site (Chapter 3), but not in Driekop, where these suilloid-like morphotypes were also frequently found. The morphotype may also be a tubercle-forming *Suillus* species as frequent accounts of this species was made in and around the Brooklands field trial (Chapter 3). Morphological features that distinguish between the genera *Suillus* and *Rhizopogon* in the Colour Atlas (Agerer, 1987-2002) are obscure and the genera share many characteristics such as densely coralloid systems, unclamped hyphae and a matrix embedding emanating hyphae. The only characteristic that seems to separate the genera is a red-brown pigment in the matrix found with some *Suillus* spp. Therefore the identification in this study is inconclusive.

The characterisation of seven morphotypes has led to the identification of *Scleroderma citrinum*, two *Amanita* species ('BSY' and 'Orange'), a Tomentelloid species, *Thelephora terrestris*, *Albatrellus ovinus*, and a suilloid species. The staining technique used for the ECM root tip cross-sections in this study was suitable, especially since all the tannin in the plant root cells was removed and hyphal cells were emphasised. Chemical reactions were not taken into account, as this has become a redundant technique that has been replaced with molecular techniques.

5. MOLECULAR ANALYSIS OF ECM ROOT TIPS AND SPOROCARPS: DNA SEQUENCING

5.1 Introduction

In recent years, the molecular techniques of DNA amplification have been used to solve or aid a number of systematic and ecological challenges associated with the identification of closely related organisms (Bruns and Gardes, 1993), especially when morphological data is non-informative or not sufficiently refined. Although the morphological identification of ECM fungal sporocarps is very advanced, the identification of the corresponding ECM root tips is challenging. Using morphological characteristics to identify an ECM mycobiont down to species level is difficult, even if provided with a comparison in the Colour Atlas of Ectomycorrhizae (Agerer, 1987-2002). The comparisons in the Colour Atlas (Agerer, 1987-2002) are limited, in that *Pinus* is not well represented and may require examination of keys to other host genera.

Some groups within the Ascomycota that produce ECM associations are difficult to identify at the species level (Mello *et al*., 1999). Bertini, *et al.*, (1998) found that identification of *Tuber magnatum* and *Tuber borchii* using root tip morphology was ambiguous and non-distinguishing as during the mycorrhizal stage these species are in practice morphologically indistinguishable. Because of these limitations, a number of molecular techniques have been employed, such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (Kårén *et al.*, 1997, Martin *et al.*, 1998, Timonen *et al.*, 1997), DNA probing (Bruns and Gardes, 1993), and eventually sequencing. DNA sequence data is generally used for initial identification and may serve as a cornerstone for other molecular identification techniques.

The Internal Transcribed Spacer (ITS) is a very well known and widely used DNA region in the field of fungal systematics. It is situated between the small subunit (SSU) and large subunit (LSU) of ribosomal DNA genes. The region contains two non-coding spacer regions (ITS1 and ITS2) separated by the

5.8S rRNA gene and flanked on either side by the 18S and 28S genes (Figure 5.1) (Horton and Bruns, 2001). The region that is commonly used ranges from the 18S (SSU) across the ITS1, 5.8S, ITS2 to the 28S (LSU). This region is approximately 600-800 bp and as a result of its non-coding areas, is highly divergent, making the region a useful tool when analysing closely related species (White *et al*., 1990). The SSU and LSU regions are more highly conserved and are useful above the species level, normally used at family level (Spatafora and Blackwell, 1993, Swann and Taylor, 1993, Vilgalys and Sun, 1994). Sequences of the ITS region can be compared with the sequences deposited in databases such as Genbank (Altschul *et al*., 1997), and can generally be used for phylogenetic analysis and determination of species identity.

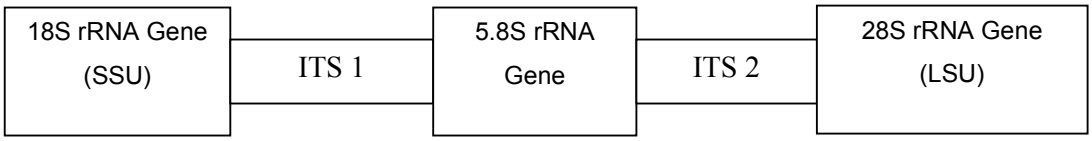


Figure 5.1 Regions targeted in molecular fungal identification studies. ITS 1 and 2 are separated by the highly conserved 5.8S gene and flanked by the 18S (SSU) and the 28S (LSU) gene. Modified from Gardes and Bruns (1993).

Genbank (Altschul *et al*., 1997) is a sequence database that allows for the comparison of generated sequences to those already deposited in the database. This database already contains many ECM mycobionts/sporocarps, as well as other fungi. Another more recently established website, which solely contains sequences of ectomycorrhizal fungi, UNITE (Kõljalg *et al*., 2005), has also been used. Derived sequences may then be aligned with similar sequences from these databases by subjecting a sequence to a Basic Logical Alignment Search Tool (BLAST). The top ten most similar sequences are then presented together with score bits and e values. The score bit value is calculated using the number of gaps and substitutions required to align the query sequence and the database sequence. Higher scores indicate more significant similarities between the sequences. The e value represents the likelihood of a sequence with a similar score occurring in the database by chance. A lower e value is therefore desirable. As errors may occur when

forward and reverse sequences are aligned and subsequently submitted to databases, along with the strong possibility that material on the databases may be wrongly identified, BLAST results from both websites should be regarded as a guide rather than a determinate tool. Another reason to be cautious of sequence database BLASTs is that they do not have deposits of all fungal species. In spite of the possible errors, BLAST results provide an important starting point when attempting to identify and describe ECM root tips and fungal diversity.

BLAST results need to be analysed according to the output data. Not only are the score bit and e values important to consider, but the region of alignment must be reviewed too. As discussed earlier, the sequenced ITS region contains areas of highly conserved genes and non-coding, highly divergent ITS spacers. Close (near identical) matches and alignments of the gene coding areas are expected between very diverse groups of fungi, therefore, caution is advised when query and matched sequences are only aligned along these areas of the sequences. It is important that the ITS regions align at some level along the more variable (less conserved) regions (ITS 1 and 2) for a more confident species match. Both SSU and LSU genes are a mosaic of highly conserved "core" segments and hypervariable expansion segments that are commonly used for phylogenetic inference (Hillis and Dixon, 1991).

The main objective of this study was to determine species identification by studying sequence data extracted from ribosomal DNA from both sporocarp and root tip material, specifically the ITS region. This was conducted in order to confirm the morphological identifications of both sporocarps and ECM root tips.

The aims were to confirm morphological identification of sporocarp material; to confirm the identity of ECM fungal species occurring on the root tip material which would aid in the morphological characterisation and to use this data to expand the fungal species list in the Sabie study area. Additional objectives included comparisons between specimens that resulted in similar sequence alignments on Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al*.,

2005); to establish the accuracy of morphotyping techniques and lastly, to link sporocarp and morphotype sequences, thereby relating the above and below ground diversity.

5.2 Methods

DNA extraction from root tip and sporocarp material was carried out at the Department of Forest Pathology and Mycology, Uppsala, Swedish University of Agricultural Science Sweden, in August 2002 and again in June 2003. The root tip and sporocarp material was collected from the study sites in Sabie (Section 2.3). In order to expand the local sequence database, sporocarp material was collected outside the primary study sites in *Pinus* plantations/forests in a variety of areas including Sabie and Grahamstown. DNA was extracted using a modified CTAB method (modified from Zeze *et al.*, 1998 (Appendix 2). Specimen samples were homogenised in a 2% CTAB solution and incubated at 65°C for no less than 30 minutes. Proteinase K was added only to the root material before the incubation, to degrade any proteins produced by the plant root, which could inhibit the Polymerase Chain Reaction (PCR). As a result of the relatively low phenol - high DNA concentration of the sporocarp material, the chloroform procedure was not necessary. The sample DNA was then precipitated with isopropanol. The DNA was re-suspended in sterilised milli-Q water. The primers used in the PCR were ITS1F, specific for fungi for the forward transcription and either ITS4B/ITS4 for the reverse (Gardes and Bruns, 1993). Primer ITS4B is Basidiomycota specific and was used for the sporocarp amplification. ITS4 was used for the roots and is not limited to the phylum Basidiomycota. It was necessary to include the phylum Ascomycota and phylum Zygomycota, as members of these groups are also known to form ECM (Carlile *et al.*, 2001, Brundrett *et al.*, 1996, Smith and Read, 1997). The PCR amplification reactions contained both positive and negative controls, of which the DNA template contained a positively amplified ITS region and autoclaved milli-Q water respectively. Products of the PCR reactions were fractionated on a 2% agarose gel. Double DNA bands that were observed on the gel were considered as contamination by non-ECM fungi or co-amplification of co-habiting endophytes and other ECM species and were discarded. Successful

single band amplifications were sequenced using the ABI 310 DNA sequencer (Perkin Elmer). Sequences were aligned using Sequence Navigator (ABI) and DNA star or Vector NTI suite package (InforMax, Inc). Sequences were then submitted to Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al*., 2005) and compared to similar reference sequences, making potential identification possible. Both score bit and e-values of matched sequences were consulted to determine good matches. E-values closer to zero combined with high score bit values of at least 800 were considered as a strong alignment and acceptable measures as a means for species inference. E-values other than 0.0 were considered a poor match, suggesting that the retrieved sequence from the database is from a close relative of the sequence being identified. Identity percentages were used to compare sequences within genotypic groups of the genera *Amanita* and *Thelephora*. Alignment identity percentages below 98% was considered as different species in this study. BLAST alignments of both sporocarp and root tip material, which appeared to be in the same genus, were aligned using ClustalW (Chenna *et al*., 2003) (Appendix 3).

As a result of differing sequence lengths, BLAST alignment results can be mis-leading by mis-aligning with closely related species. To resolve the taxonomic position of the sequences generated in this study, similarity trees were derived. This was accomplished by downloading several sequences from Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al*., 2005), which were aligned in ClustalW (Chenna *et al*., 2003) and visualised in Treeview (Win32, 1.6.6, 2001). The accession numbers of these sequences are presented in Appendix 7. The ITS region, although useful for species distinction, should not be used for higher order resolution as the variation between different genera, particularly in the ITS 1 and ITS 2 regions, would not allow for homologous base-pair alignment. Therefore, species within the same genus were aligned (Appendix 3) and analysed with appropriate outgroup species from sister genera.

5.3 Results

DNA was extracted from 40 sporocarp samples, consisting of a potential 25 species. Only 20 species (27 sporocarps) (Table 5.1) were successfully amplified and sequenced (Appendix 5). Of the 92 root tips that were sampled, 27 were successfully amplified and produced clean DNA sequences (Appendix 5) that could be aligned (Table 5.2). Failed sequencing was observed as 'noisy' and ambiguous base pair positions that could not be edited using chromatographs, nor could the forward and reversed sequences be aligned. These sequences were not carried forward into the molecular analysis.

5.3.1 Sporocarp DNA sequence results

Table 5.1 DNA sequence BLAST results of the ITS region extracted from collected sporocarp material. The values following the species name of the BLAST results refer to the score bit and the e-values, respectively. The Brooklands study site, Driekop study site and Sabies Hoek are located around Sabie. Highlands and the Botanical Gardens are located in the Grahamstown area.

Sporocarp Morphological identification	**Location collected**	**Genbank BLAST**	**UNITE BLAST**
Amanita excelsa (1)	Brooklands	*A. spissa* 339 6e-90	*A. rubescens* 270 1e-73
Amanita excelsa (2)	Brooklands	*A. spissa* 1179 0.0	*A. rubescens* 1249 0.0 *A. spissa* 910 0.0
A. muscaria	Brooklands	*A. gemmata* 1427 0.0 *A. muscaria* 1285 0.0	*A. regalis* 1013 0.0
A. rubescens	Brooklands	*A. flavipes* 1033 0.0, *A. rubescens* 955 0.0	*A. spissa* 1342 0.0 *A. rubescens* 1181 0.0
Clavulina cf. cinerea	Brooklands	*C. cinerea* 1132 0.0	*C. cristata* 989 0.0
Clavulina cristata	Brooklands	*C. cinerea* 1144 0.0	*C. cristata* 1019 0.0
Inocybe cf. griseolilacina	Driekop	*I. flocculosa* 313 4e-82	*I. lacera* 309 2e-85
Inocybe specimen 1	Brooklands	*I. lacera 327* 3e-8	*I. fuscidula* 319 2e-88
Lactarius hemicyaneus	Brooklands	*L. quieticolor* 1421 0.0	*L. deterrimus* 981 0.0
L. hepaticus	Brooklands	*L hepaticus* 1374 0.0	*L. subdulcis* 1400 0.0
L. semisanguifluus	Brooklands	*L. quieticolor* 1374 0.0	*L. deterrimus* 938 0.0
Scleroderma citrinum	Brooklands	*S. bovista* 462 e-127	*S. verrucosum* 301 4e-83

Sporocarp Morphological identification	**Location collected**	**Genbank BLAST**	**UNITE BLAST**
Suillus granulatus	Brooklands	*S. granulatus* 1338 0.0	*S. granulatus* 1261 0.0
S. luteus	Brooklands	*S. luteus* 1211 0.0	*S. luteus* 1316 0.0
S. salmonicolor	Sabies hoek	*S. cothurnatus* 932 0.0	*S. sibiricus* 486 e-138
Tricholoma albobrunneum	Highlands	*T. fracticum* 1289 0.0	*T. fracticum* 1296 0.0
Xerocomus badius	Brooklands	*X. badius* 1041 0.0	*X. badius* 1380 0.0
X. cf. chrysenteron	Botanical Gardens	*X. pruinatus* 1552 0.0 *X. chrysenteron* (not a significant alignment)	*Boletus pruinatus781 0.0/* *Xerocomus690 0.0*
Russula specimen 1 *Specimen 3* *Specimen 4* *Specimen 6* *Specimen 7*	Highlands	*R. laricina* 1114 0.0 *R. laricina* 1086 0.0 *R. laricina* 1152 0.0 *R. laricina* 1136 0.0 *R. laricina* 1120 0.0	*R. caerulea* 519 e-148 *R. caerulea* 464 e-132 *R. caerulea* 549 e-157 *R. caerulea* 533 e-153 *R. caviceps* 426 e-120
R. drimeia	Highlands	*R. drimeia* 1404 0.0	*R. sardonia* 1104 0.0

The BLAST alignment results of the two websites (Table 5.1) concur with the sporocarp identification at genus level. However, many species names do not necessarily correspond with those that have been morphologically identified and frequently the specific names retrieved from the databases differ.

5.3.2 ECM root tip sequence results

The derived sequences of the ECM root tips were last submitted to and BLAST performed on both UNITE (Kõljalg *et al.*, 2005) and Genbank (Altschul *et al*., 1997) on the 13 August 2006, to update the sequence match results. Twenty-five specimens were successfully amplified, of which 21 matched with known ECM forming fungal species.

Sequences matching *Thelephora terrestris* were obtained from four different morphotypes: 'Partial white B', 'black (D35a)', and 'Yelsil' (diff)' and 'Yelsil A' (Table 5.2). 'Fwhite' was aligned with *Thelephora caryophyllea* (Yellow highlight). *Xerocomus badius* sequences matched two specimens of 'BSY' (2 & 6), 'beige' and 'dull orange' (Blue highlight). All other 'BSY' morphotypes, 'Grey-pink', 'Yellow-orange' and 'White A' aligned with *Amanita spissa* (Pink highlight). The genus *Scleroderma* aligned poorly to both morphotypes 'white B' and 'Yelsil'. *Albatrellus ovinus* matched sequences from morphotype 'Grey'.

'DSY' aligned well with *Clavulina cristata*. The morphotype 'beige/white' resulted in a good alignment with *Heterobasidion annosum*. 'Partial white A' and 'White C' aligned with *Hymenoscyphus ericae*. The mycorrhizal status of *Phialocephala fortinii*, which was aligned to 'Partial white C' and 'Black', is not yet known.

Table 5.2 DNA sequence BLAST results of the ITS region extracted from ECM root tips. The values following the species name are the score bit and the e-values, respectively.

Site	Morphotype	Genbank BLAST	UNITE BLAST
Driekop	Grey tuber (D17a)	*Scleroconidioma sphagnicola* 821 0.0	*Hymenoscyphus ericae* 250 1e-67
Brooklands	White C	*Hymenoscyphus sp* 900 0.0	*Hymenoscyphus ericae* 646 0.0
Brooklands	White A	*Amanita spissa* 825 0.0	*A. rubescens* 718 0.0 *A. spissa* 644 0.0
Brooklands	White B	*Scleroderma bovista* 585 e-164	*S. verrucosum* 301 3e-83
Brooklands	Fwhite	*Thelephora/ Tomentella* 997 0.0	*Thelephora caryophyllea* 698 0.0
Brooklands	Partial white A	*Hymenoscyphus sp* 833 0.0	*Hymenoscyphus ericae* 601 e-173
Driekop	Partial white C	*Phialocephala fortinii* 900 0.0	*Hymenoscyphus ericea* 392 e-111
Brooklands	Partial white B	*Thelephora terrestris*1183 0.0	*T. terrestris* 1084 0.0
Driekop	Black A	*Thelephora terrestris* 936 0.0	*T. terrestris* 842 0.0
Brooklands	Black B	*Phialocephala fortinii* 930 0.0	*Hymenoscyphus ericae* 365 e-102
Brooklands	Yelsil C	*Thelephora terrestris*1100 0.0	*Thelephora terrestris* 1045 0.0
Driekop	Yelsil A	*Thelephora terrestris* 1082 0.0	*Thelephora terrestris* 1013 0.0
Driekop	Yelsil B	*Scleroderma bovista* 466 e-128	*Scleroderma verrucosum* 301 3e-83
Brooklands	Dull orange	Xerocomus badius *1051 0.0*	*X. badius 1110 0.0*
Brooklands	Beige	*Xerocomus badius* 1031 0.0	*Xerocomus badius* 1037 0.0
Brooklands	BSY 2	*Xerocomus badius* 779 0.0	*Xerocomus badius* 831 0.0
Brooklands	BSY 1	*Amanita spissa* 1322 0.0	*A. spissa* 1322 0.0
Brooklands	BSY 3	*Amanita spissa* 1328 0.0	*A. spissa* 1328 0.0
Brooklands	BSY 4	*Amanita spissa* 1166 0.0	*A. rubescens* 952 0.0 *A. spissa* 852 0.0
Brooklands	BSY 5	*Amanita spissa* 1312 0.0	*A. spissa* 1312 0.0
Brooklands	BSY	*Amanita spissa* 12370	*A. spissa* 1237 0.0

Site	Morphotype	Genbank BLAST	UNITE BLAST
		0.0	
Brooklands	Orange	*Amanita spissa* 1261 0.0	*A. spissa* 1261 0.0
Brooklands	Grey/ Pink	*Amanita spissa* 1261 0.0	*A. spissa* 1261 0.0
Brooklands	Grey	*Albatrellus ovinus* 1150 0.0	*Russula firmula* 287 5e-79
Driekop	DSY	*Clavulina cristata* 965 0.0	*Clavulina cristata* 977 0.0
Brooklands	Beige/white	*Heterobasidion annosum* 801 0.0	*Entoloma serrulatum* 262 2e-71

5.3.3 Comparisons of sporocarp and morphotype sequence data

To link sporocarp and ECM morphotype material, pair-wise comparisons of the sequences were carried out (Table 5.3) on the Genbank website using the "Align two sequences" tool (Altschul *et al*., 1997).

Table 5.3 Pairwise comparisons of sporocarp and ECM root sequences. Column 1 provides a species list derived from BLAST results of both sporocarp and ECM root material. Column 2 represents corresponding sporocarp material from the Brooklands study site, and field identification. Column 3 represents morphotypes corresponding to the species derived from BLAST searches. Column 4 represents the results of the sequence comparison between sporocarp and ECM root sequences where both are available.

Specimen BLAST results	Identified by Sporocarp as species	Identified by root tip morphotype	Alignment values *
Amanita spissa/*A. rubescens*	*Amanita rubescens*	BSY1, Grey/Pink	S = 1261, E = 0.0 I = 671/676 (99%) S = 1185, E = 0.0 I = 641/646 (99%)
Amanita muscaria	*Amanita muscaria*		
Amanita rubescens	*Amanita excelsa*	BSY4 White	S = 1163, E = 0.0 I = 635/646 (98%) S = 798, E = 0.0 I = 466/480 (98%)
Clavulina cinerea/ cristata	*Clavulina cristata*	DSY	S = 1148 bits (597), E = 0.0 I = 618/621 (99%)
Inocybe sp1 (inconclusive match)	*Inocybe cf. griseolilacina*		
Inocybe sp2			
Lactarius quieticolor,	*Lactarius hemicyaneus,*		

Specimen BLAST results	Identified by Sporocarp as species	Identified by root tip morphotype	Alignment values *
Lactarius deterrimus	*L. semisanguifluus*		
Lactarius hepaticus	*Lactarius hepaticus*		
Scleroderma sp bovista/verrucosum (inconclusive match)	*Scleroderma citrinum*	Wool white, Yelsil	S = 1058, E = 0.0, I=626/649 (96%) S = 717, E = 0.0, I = 467/489 (95%)
Suillus granulatus	*Suillus granulatus*		
Suillus luteus	*Suillus luteus*		
Suillus cothurnatus	*Suillus salmonicolor*		
Thelephora terrestris	•	Fwhite, Black, Yelsil	
Tomentella (Thelephora caryophyllea)	#	Fwhite	
Xerocomus badius	*Xerocomus badius*	BSY2, Beige, Dull Or	S = 835, E = 0.0, I = 553/585 (94%) S = 996, E = 0.0, I = 552/559 (98%) S = 1071, E = 0.0, I = 589/595 (98%)
Albatrellus ovinus	#	Grey	

• Unable to amplify sporocarp, # sporocarp not found. * S = score bit value, E = e values and I = identity.

The morphotype 'white' in Table 5.3 is a relatively poor match when compared to the alignment values of 'BSY1' and 'Grey/pink' with the sporocarp *A. rubescens*. 'DSY' aligns well with the *Clavulina cristata* sequence. *Scleroderma citrinum* aligns well with 'Woolly white', and to a lesser degree 'Yelsil'. *Xerocomus badius* aligned well with 'beige' and 'Dull orange' with high score bit values and good e-values. 'BSY' has a lower score bit value, but still has a good e-value.

5.3.4 Similarity trees of the derived genera from sporocarp and ECM root tip sequences

Similarity trees were derived from sequence alignments executed in ClustalW (Chenna *et al*., 2003) and visualised in TreeView to determine the relative position of the specimens in this study within genera. All the sequences

derived in this study were grouped according to BLAST results and analysed together with sequences downloaded from both Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al.*, 2005). The UNITE database (Kõljalg *et al.*, 2005) is more reliable in terms of species identification as all sequence deposits are correctly identified before submission is permitted. Therefore, where possible, as many sequences from UNITE (Kõljalg *et al.*, 2005) were preferred for use in the alignment analysis. In the absence of relevant species on the UNITE database, sequences from Genbank were downloaded. The trees for 5 cladograms are given with the purpose of resolving the taxonomic position of ECM sporocarp and root tip material, respectively. No bootstrap or confidence values were derived for the tree branches, as stringent taxonomic classifications were not the objective, but functioned to create a stronger case that would confirm BLAST analysis. Trees for the following clades were generated: *Amanita*, *Clavulina*, *Albatrellus-Russula-Lactarius*, *Suillus-Xerocomus* and *Thelephora-Tomentella* and the outgroup species used were: *Limacella glioderma* (Schüßler *et al*., 2001), *Ramaria stricta* (Thacker and Henker, 2004), *Albatrellus* spp. (Miller and Buyck, 2002), *Rhizopogon roseolus* (Grubisha *et al*., 2002) and *Pseudotomentella* spp. (Kõljalg *et al.*, 2005), respectively.

All downloaded sequences are annotated with the last four digits of the accession number that may be found in full in Appendix 6. All other sequences have been derived from this study and are either presented as sporocarp species names or coded as ECM root tip morphotypes (Table 5.1 & 5.2).

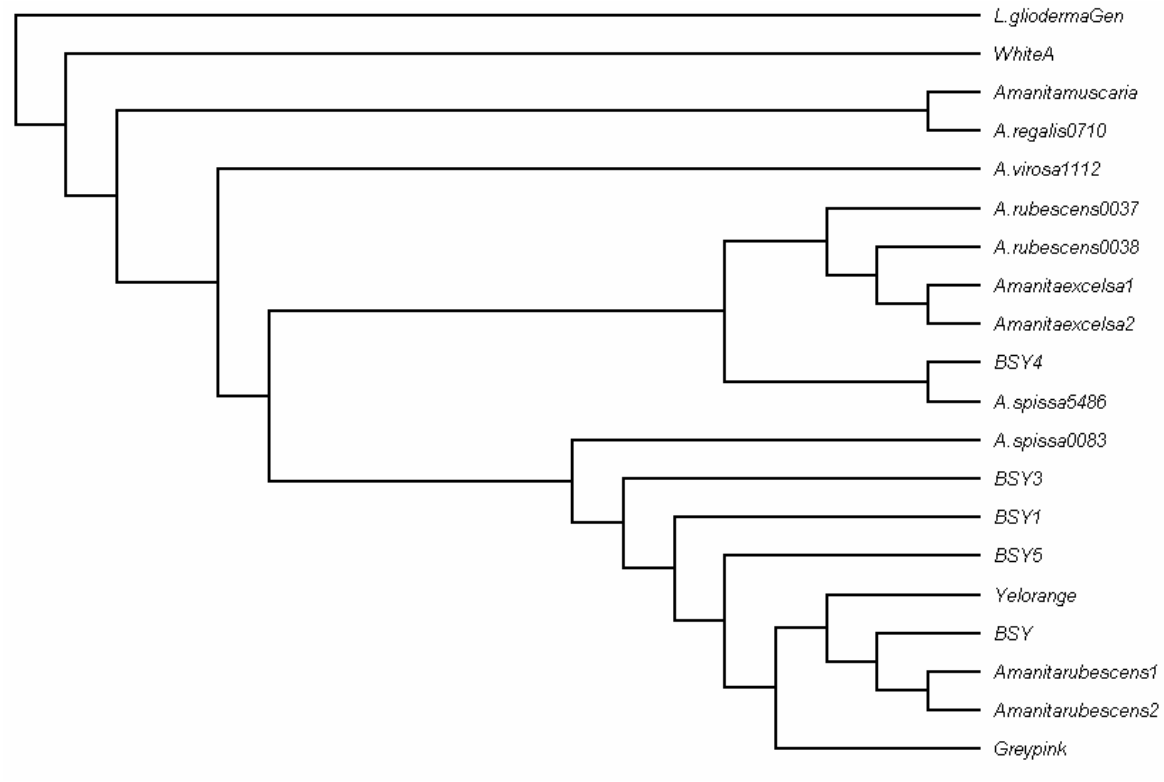


Figure 5.2 The *Amanita* cladogram.

The species and morphotype codes correspond to those found in Table 5.1 and 5.2. Species names followed by a 4-digit number were downloaded from databases.

The derived tree (Figure 5.2) groups 'BSY1', 'BSY3', 'BSY5', 'Yellow-orange', 'BSY' and both *A. rubescens* sporocarp sequences in a group associated with the downloaded sequence of *A. spissa* (UNITE). The morphotype 'BSY4' was associated with *A. spissa* downloaded from Genbank, but groups with *A. rubescens* from UNITE and *A. excelsa* (synonym = *A. spissa*) sporocarps.

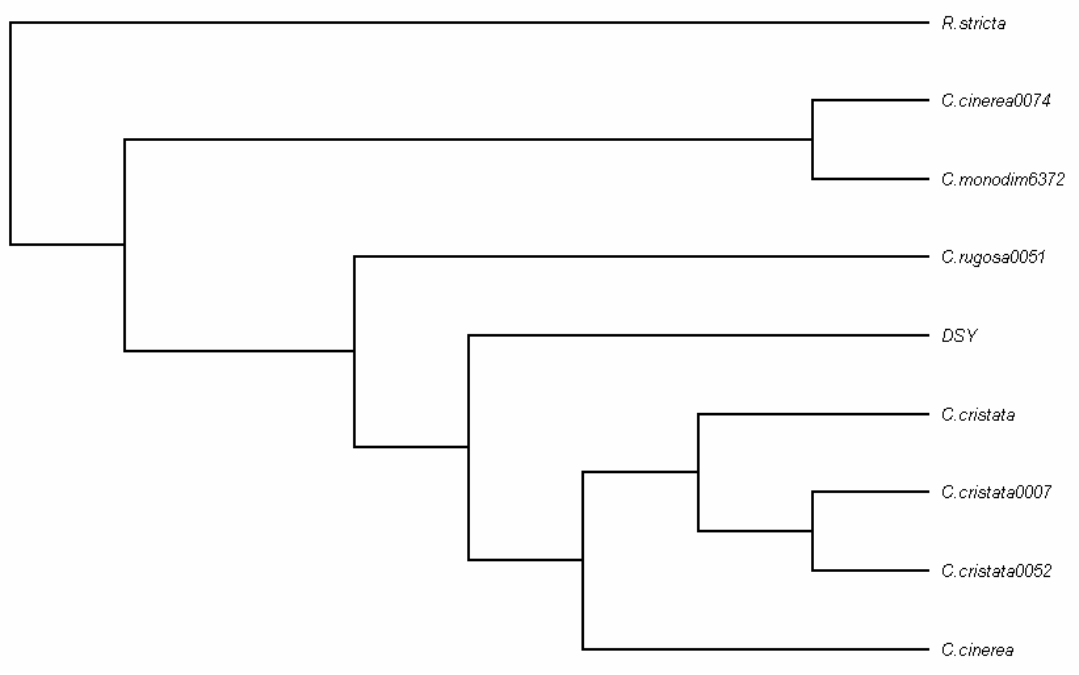


Figure 5.3 The *Clavulina* cladogram.

All the *Clavulina* sequences derived from this study, namely two sporocarp specimens of *Clavulina* (Table 5.1) and morphotype 'DSY' (Table 5.2), formed a group with *C. cristata* (Figure 5.3).

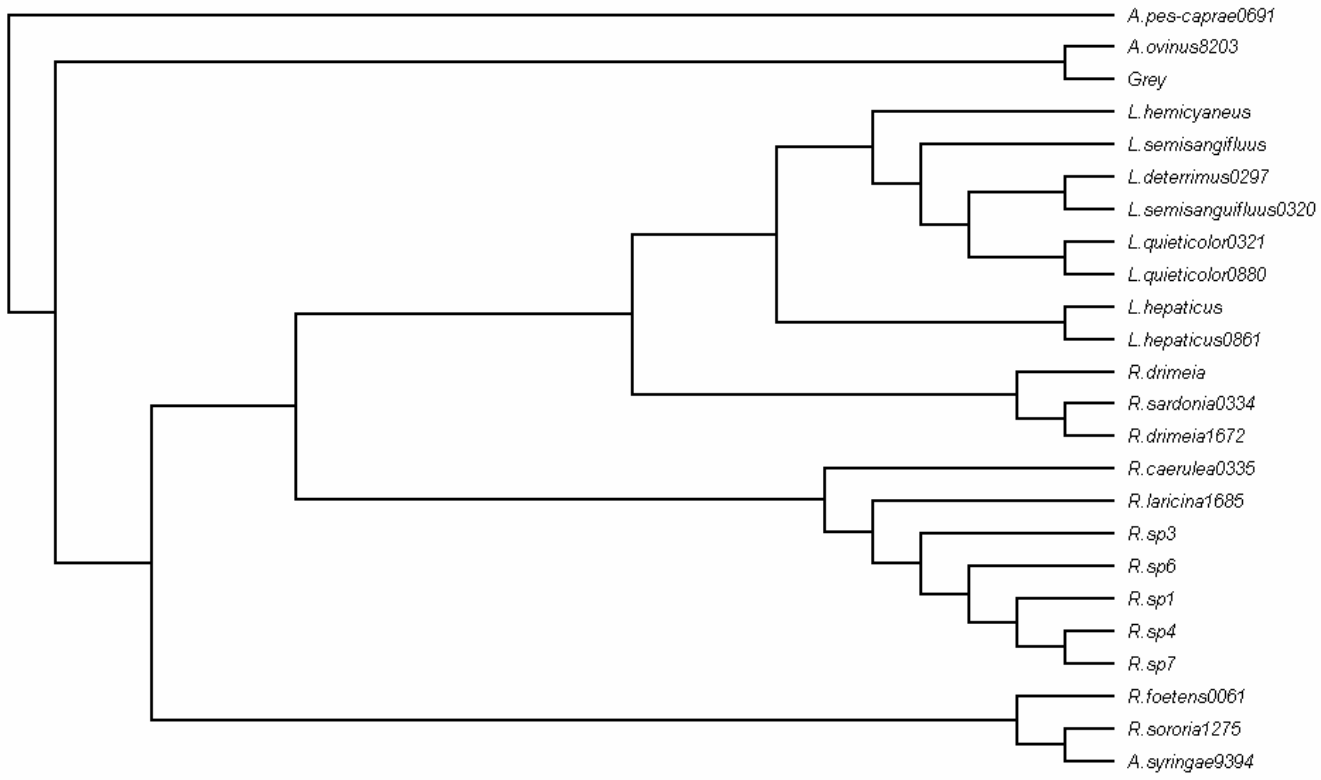


Figure 5.4 The *Russula*, *Lactarius* and *Albatrellus* cladogram. (A = *Albatrellus*)

The similarity tree of the three genera, *Russula*, *Lactarius* and *Albatrellus* (Figure 5.4), confirmed the position of the morphotype 'Grey' within the genus *Albatrellus*. Additionally, all the *Lactarius* sporocarp species sequenced in this study grouped with *L. semisanguifluus*, *L. quieticolor* and *L. deterrimus* (UNITE). *L. hepaticus* species grouped together. The species of *R. drimeia* in this research grouped with *R. sardonia* and *R. drimeia*. The remaining *Russula* specimens from this study grouped together with *R. laricina* and *R. caerulea*. The appearance of *Albatrellus syringae* with *R. foetens* and *R. sororia* is unusual and probably a result of mis-identified deposits in the Genbank database.

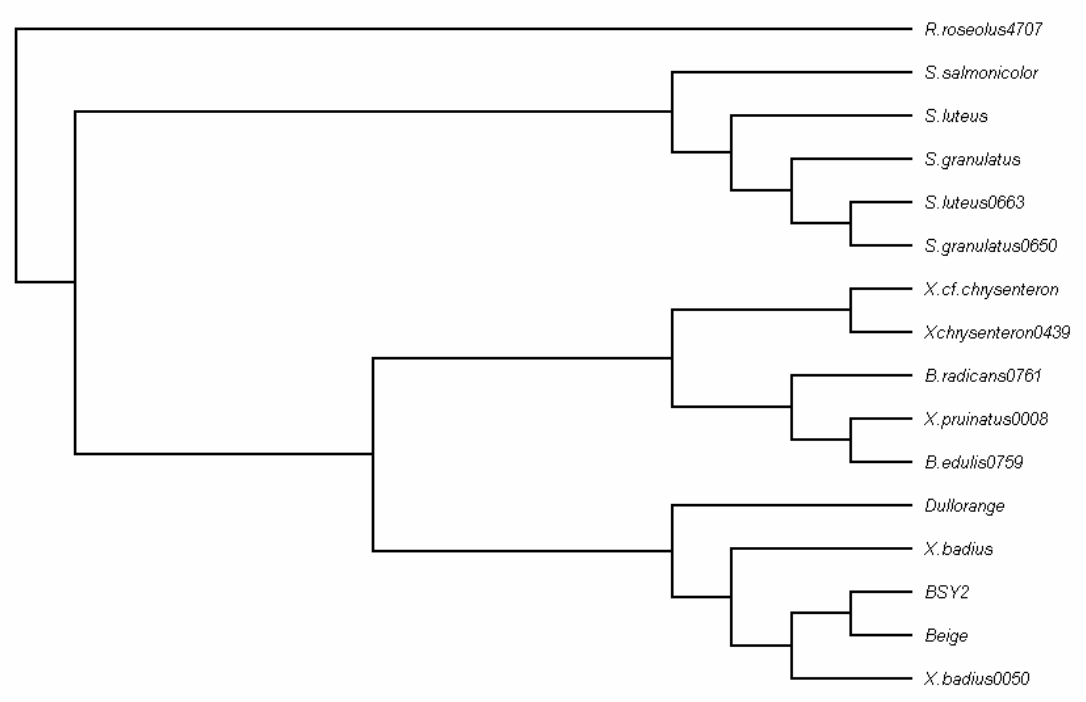


Figure 5.5 The *Suillus-Xerocomus* cladogram. The genera are abbreviated. R. = *Rhizopogon*, S. = *Suillus*, X. = *Xerocomus* and B. = *Boletus*.

The taxonomic position of *Suillus* and *Xerocomus* species were investigated by aligning the sequences together with species of *Boletus* in an attempt to better resolve the similarity tree (Figure 5.5). It is apparent that the sporocarp specimen identified as *X.* cf. *chrysenteron* groups with the downloaded sequence of *X. chrysenteron* and not with *X. pruinatus* as the BLAST alignments seem to indicate (Table 5.1). The morphotypes 'Dull orange', 'BSY2' and 'Beige', together with the sporocarp specimen of *X. badius* all form a monophyletic group with *X. badius* (UNITE). The *Suillus* species formed a separate monophyletic group, the resolution of which however was not high enough to derive definite conclusions as to the taxonomic position.

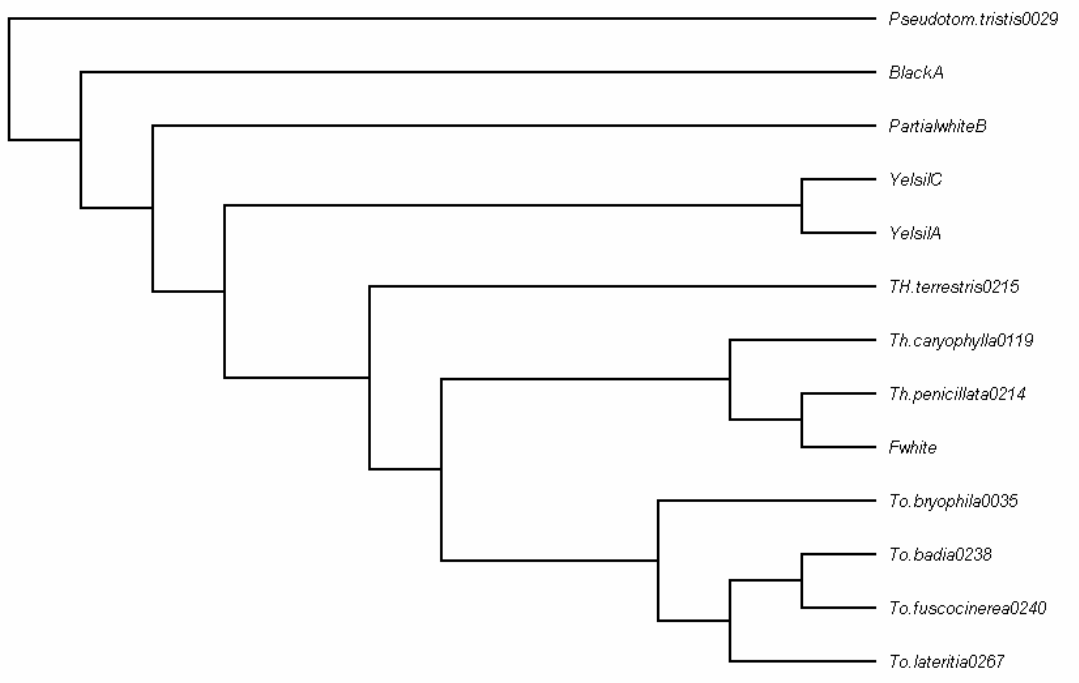


Figure 5.6 The *Thelephora-Tomentella* cladogram. The genera are abbreviated. *Pseudotom*. = *Pseudotomentella*, *Th*. = *Thelephora* and *To*. = *Tomentella*.

The morphotype sequences that aligned with *Thelephora* species were aligned with *Tomentella* and *Thelephora* species downloaded from UNITE (Kõljalg *et al.*, 2005). The morphotype 'Fwhite' was grouped together with *Thelephora penicillata*, indicating that the alignment with *T. caryophylla* (Table 5.2) was potentially inaccurate. All the other morphotypes; 'YelsilA', 'YelsilC', 'Partial whiteB' and 'BlackA', group with *Thelephora terrestris* at basal nodes. A large amount of paraphyly is evident in this cladogram (Figure 5.6).

5.4 Discussion

The sporocarp species that failed to amplify included specimens of *Boletus* and *Thelephora* (both in resupinate and bracket form, Chapter 3), despite numerous attempts. Kõljalg *et al*., (2000) extracted DNA from *Thelephora* spp. successfully, by using the CTAB method and purifying the DNA isolated. There may be residual compounds in the DNA extract that inhibit PCR. Alternative extraction or PCR conditions would need to be optimised for

successful molecular analysis, but were not carried out. Although these methods were not utilised in this study, a modified protocol would be necessary for further research concerning these groups of fungi.

Extraction from root tip material proved to be more difficult than from sporocarp material. The smaller amount of DNA extracted from the root tip relative to sporocarp material called for additional steps in the extraction process, such as the Proteinase K and a chloroform/phenol step. This proved essential for successful extraction and amplification (Appendix 4). Amplification of ECM root tip DNA was often altogether unsuccessful, i.e. no amplification of DNA, or if successful, represented two fungal species detected by the separation of two distinct bands when electrophorised on an agarose gel. A number of reasons can be attributed to the above results. Root material was stored in 50% alcohol over an extended period before the DNA was extracted and this may have lead to DNA degradation. Additionally, difficulties associated with the DNA amplification of particular fungal species (e.g. *Boletus* and *Thelephora* species, as experienced from the sporocarp extractions) may be encountered in ECM root tip material and result in failed DNA amplification. Even though in some cases extractions and amplifications were successful, the presence of a diversity of fungi in the rhizosphere led to co-amplification of more than a single fungal species. It is common scientific knowledge that ECM root tips can host more than one species of ECM fungus and that dark septate endophytes co-habit the plant root tips. These associations are not necessarily morphologically distinct. Rosling *et al*., (2003) found that up to 38% of the root tips analysed were dually colonised and were represented by double bands after gel separation. Many PCR products in this study contained more than one fungal species, observed as double bands on gels. These samples were not, unfortunately carried forward for any further analysis. Furthermore, single bands that were observed after gel separation, could potentially have contained co-amplified ITS regions of the same size. Such DNA bands could explain sequences containing highly erratic, ambiguous base-pair positions, which could not be edited from ‘noisy’ sequence chromatographs. This was the case with approximately 10% of the PCR products that were sequenced and were discarded as no further analysis

could be carried out. Cloning would have contributed enormously to the separation of several species within the PCR products visualised as containing two bands, as only an individual DNA stretch could be inserted into the vector during ligation. Once inserted into a target organism, such as *Escherichia coli*, the vector containing a unique DNA stretch would be reproduced and with additional selection pressure would result in transformed colonies. The harvesting and enzyme digestion of the DNA from several colonies would have separated the DNA of distinct species types (Barnum, 1998).

Table 5.1 and 5.2 have indicated, for some of the BLAST results, two species names. This is a result of the close alignment values and scores of more than a single species listed in either database and it was therefore appropriate to include them. The comparisons of BLAST results from both databases serves to confirm species identification and concur with morphological identification at least to genus level (Table 5.1 and Table 5.2). The species level match, however, is not always congruent between the two. For example, with the two *Clavulina* spp. in Table 5.1, Genbank (Altschul *et al*., 1997) retrieved a close match to *C. cinerea*, whereas UNITE (Kõljalg *et al.*, 2005) retrieved a close match to *C. cristata*. A Genbank (Altschul *et al*., 1997) pairwise comparison analysis was conducted on reference sequences of *C. cinerea* and *C. cristata* from the database (Appendix 4.1). The sequences from *C. cinerea* and *C. cristata* revealed a low identify of 95%, 474 Score bit and an e value of e-130 to each other, indicating that the two species are relatively distinct. This rules out the possibility of the incongruence between the databases being attributed to comparable ITS sequences of the two species in question.

The sequence BLAST results from sporocarps identified morphologically as *Amanita excelsa* (synonym = *A. spissa*) from the two databases resulted in well-aligned matches to several different species. Genbank (Altschul *et al*., 1997) retrieved a match to *A. spissa* and UNITE (Kõljalg *et al.*, 2005) retrieved a match to *A. rubescens* (Table 5.1). The same discord was noted for the BLAST results of *A. rubescens*: Genbank (Altschul *et al*., 1997) BLAST retrieved a close match to *A. flavipes*/*A. rubescens* (Table 5.1) and UNITE

(Kõljalg *et al.*, 2005) retrieved a close match to *A. spissa*. Reasons for this mismatch between the two databases may be a result of mis-identified sequence material being deposited in the databases. The deposited reference sequences of *A. spissa* from Genbank (Altschul *et al*., 1997) were compared to *A. rubescens* from UNITE (Kõljalg *et al.*, 2005) and resulted in a sequence dissimilarity (94% identity), large enough for the two species to not be confused with each other. It is important to note that all three of these species are closely related and have been placed in the Sect *Validae* (Fr.) Quél. (Weiβ *et al*., 1998). Within the genus, the ITS region displays high rates of divergence that do not allow proper species distinction. For this reason, the ITS regions may not be sufficiently informative for species level identification. The derived similarity tree (Figure 5.1) indicated that there may be some confusion of species taxonomy when using the ITS region. The LSU region, which is more conserved, is currently used in the literature for the phylogenetic analysis of this genus (Weiβ *et al.*, 1998).

Clavulina cristata and *C. cf. cinerea* matches found at both study sites have high score bit values and e-values compared to sequences from both websites (Table 5.1). However, Genbank (Altschul *et al*., 1997) matches both sporocarps closely to *C. cinerea* and UNITE (Kõljalg *et al.*, 2005) matches both sporocarps closely to *C. cristata*. Furthermore, the alignment of the sequences from this study were compared on the National Centre for Biotechnology Information BLAST website (Altschul *et al*., 1997, Internet 3) aligned with a high score bit value, and no e-values and 99% identity, indicating that the two specimens are likely the same species. This suggests that what was originally thought to be two species based on morphology appears to be a single species. The similarity tree (Figure 5.2) indicates that the two specimens should be classified as *C. cristata* as they group with this species.

The *Inocybe* spp. BLAST results matched poorly with Genbank and UNITE reference sequences, but appropriate genus identification was achieved. The genus has approximately 250-350 species (Matheny *et al*., 2002), and many species are currently still being described and sequenced. It is possible that

the species analysed in this study have not yet been sequenced. Currently, sequences from only 75 species have been submitted to the Genbank database (Altschul *et al*., 1997).

Based on the sequence BLAST result for sporocarps of *Lactarius hemicyaneus* and *L. semisanguifluus*, these specimens appear to be the same species (Table 5.1). Genbank (Altschul *et al*., 1997) acquired a very close match to *L. quieticolor*, whereas UNITE (Kõljalg *et al*., 2005) retrieved a close match for *L. deterrimus*. *L. quieticolor* retrieved higher score bit values, which would indicate a stronger alignment with this species. The morphology of both *Lactarius* species was very similar and also had similarities to *L. deliciosus* (Section 3.3.1). The use of the species names; '*quieticolor*', '*hemicyaneus*' and '*deliciosus*', appear to be loosely applied as sub-species (Internet 2). For example, synonyms used for the valid species *L. quieticolor* are *L. hemicyaneus*, *L. deliciosus* var. *hemicyaneus*, *L. deliciosus* var. *quieticolor*, *L. quieticolor* var. *hemicyaneus* and *L. deliciosus* var. *quieticolor*. The similarity tree generated (Figure 5.3) suggests that the two specimens of *L. hemicyaneus* and *L. semisanguifluus* identified in this study are related to the group formed by species sequences from *L. quieticolor*, *L. deterrimus* and *L. semisanguifluus*. Taxonomic revision of this group is necessary to achieve definitive identification.

The *Scleroderma citrinum* sporocarp sequence resulted in poor matches to *S. bovista* and *S. verrucosum*, on the Genbank (Altschul *et al*., 1997) and UNITE database (Kõljalg *et al*., 2005) respectively (Table 5.1). This is most probably due to the fact that neither of the websites have accessions of sequences from this particular species. The morphotype 'WhiteB', when aligned with the sporocarp sequence provided high scores (Table 5.3), indicating a strong probability that they are the same species. Deposits of these sequences will be made in both Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al*., 2005) databases.

Sequences from *Suillus salmonicolor* retrieved poor matches on both sites (Table5.1). Upon further inspection, it was noted that neither of the sites

contain *S. salmonicolor* sequences. The specimens were identified in the field (personal communication Taylor, 2002) and the morphological identification appears to be more accurate (Chapter 3). Information on *S. cothurnatus* (Genbank BLAST, Altschul *et al*., 1997) seems limited, so no morphological comparison could be made. The similarity tree (Figure 5.4) indicated that *S. luteus* and *S. granulatus* sequences generated in this study were homologous to those of the respective species downloaded from the UNITE database, although the species did not correspond exactly.

Tricholoma albobrunneum, as identified from morphological features, should be renamed *T. fracticum* as both databases retrieved high alignment scores to this species (Table 5.1). The sequences of both the above species are available on Genbank (Altschul *et al*., 1997) and were compared to reveal a large enough difference between the sequences for species separation (94% identity). The specimen in this study was also compared to *T. albobrunneum* on Genbank (Altschul *et al*., 1997) and retrieved a 95% identity (Accession no. AF377243), ruling out the possibility of missing sequences on the database.

The sporocarp identified as *Xerocomus* cf. *chrysenteron* aligned with *X. pruinatus* with high scoring BLAST results on both databases (Table 5.1). In spite of these results, the similarity tree (Figure 5.4) indicates that the specimen was correctly morphologically identified, as the specimen groups with the UNITE sequence of *X. chrystenteron* and not *X. pruinatus* (Kõljalg *et al.*, 2005).

The *Russula* specimens that were collected were morphologically indistinct (Chapter 3) and were therefore listed as specimens, rather than allocating species names. Based on descriptions in a South African field guide (van der Westhuizen and Eicker, 1994), the species were identified as *Russula capensis* (Chapter 3), but the species description did not correspond to all the collected material. The description also needs to be validated as *R. capensis* was described from South African *Pinus* stands, but has probably already been described in Europe (Chapter 3), as *R. laricina*, as indicated by the

sequence analysis, from where the majority of South Africa's ECM fungal diversity was introduced. The BLAST matches retrieved from Genbank (Altschul *et al*., 1997), (Table 5.1), would appear to be more reliable as the score bit and e-values are high. Genbank BLAST identified *R. laricina* as the closest match. Pair-wise alignment analysis of the specimens (Table 5.4) showed high identity of 97-99% and score values of 1281-1444. Specimen 3 appears to have a lower identity similarity to all the other specimens of 97% and lower score bit values that may indicate a poorer quality of sequence. *R. drimeia* is a clear mismatch (Table 5.4) to the other specimens as the alignments were very poor.

Morphotyping is often inaccurate and can lead to a mis-representation of below ground species diversity. Morphotyping is largely based on colour, branching and mantle surface features (Chapter 4). ECM root tip colour can change due to aging, storage or bruising. From Table 5.2, it is clear that there is some variation within morphotypes (e.g. 'BSY'), and yet some conformity between morphotypes (e.g. 'Grey/Pink', 'BSY' and 'Yellow-orange' all resolve as *Amanita spissa*). A possible 11 species may be identified from the BLAST results of the root tip sequences.

The BLAST matches of several ECM root tips (Table 5.2) resulted in interesting alignment retrievals. The 'Tuberculate' sequence was aligned, on Genbank (Altschul *et al*., 1997) with *Scleroconidioma sphagnicola*, which is a fungal pathogen that has been found in mosses, *Sphagnum fuscum* (Tsuneda *et al*., 2001). The alignment with *S. sphagnicola* was strong and it is therefore concluded, that the DNA amplified from the morphotype 'Tuberculate', was not an ECM fungus but an amplification of a cohabiting fungus, as a result of the general fungal primers used in the ECM root tip PCR reaction. The alignment on UNITE (Kõljalg *et al.*, 2005), a purely mycorrhizal fungi database, retrieved a poor alignment with *Hymenoscyphus ericae* as a result. Rosling *et al*., (2003) experienced fungal co-amplification. The authors then re-amplified the DNA using Basidiomycota-specific primers (ITS1F and ITS4B). As a result, Rosling *et al*., (2003) was able to separate the Basidiomycetous ECM fungi species from co-colonising Ascomycete fungi. In

this study, such technique may have been useful where double amplification was experienced but this was however was not the case with the amplification of *S. sphagnicola*.

BLAST matches of a number of morphotypes led to further investigation of *Phialocephala fortinii* and *Hymenoscyphus ericae*. The of these mycobionts has only recently been elucidated. *H. ericae* represents a number of closely related taxa that form an aggregate and it was previously thought that these fungi were primarily ericoid mycorrhizal (Grünig, 2003). Strains from this aggregate recently have been shown to produce ECM associations with *Betula pubescens*, *Picea abies* and *Pinus sylvestris*, and ericoid mycorrhizas with *Vaccinium vitis-idaea* (Vrålstad *et al*., 2002). Villarreal-Ruiz *et al*., (2004) showed that the same strain of *H. ericae* can form both ECM and ericoid mycorrhizas with *P. sylvestris* and *Vaccinium myrtillus*, respectively. The BLAST alignment results retrieved from both the website of the morphotype 'White C' and 'Partial white A' (Table 5.2), match the morphotype sequences to *Hymenoscyphus* sp. indicating the presence of this species in association with *P. patula* in South Africa.

The 'Partial white C' morphotype found in Driekop and the 'Black' morphotype found in the Brooklands study site (Table 1) both have a strong BLAST match to *Phialocephala fortinii* from sequences in Genbank (Altschul *et al*., 1997). The UNITE database (Kõljalg *et al*., 2005) retrieved a relatively poor match to *H. ericae*. On closer investigation it was found that *Phialocephala* is related to *Hymenoscyphus* (Grünig, 2003). The high scores of the match retrieved from Genbank (Altschul *et al*., 1997) (Table 1) of these morphotypes identify them as specimens of *P. fortinii*. *P. fortinii* is a dark septate endophyte (DSE), found globally, colonising numerous plant species (Yu *et al.*, 2001). The functional biology of DSE's is uncertain, and whether they are pathogenic or symbiotic still needs to be ascertained (Addy *et al*., 2005). The mechanism by which the hyphae colonise the root was examined by Yu *et al.*, (2001), and found to be similar to that of arbuscular mycorrhizal fungi. The results indicated that the *P. patula* host plants in South Africa are associating not only with ECM, but other types of endophytic fungi.

Five morphotypes, ‘Black’ (Driekop), ‘Fwhite’ (Brooklands), ‘Yelsil’, ‘Yelsil A’ and ‘Partial white B’ were matched to the genus *Thelephora* species (Table 5.2). ‘Fwhite’ matched a different species, *T. caryophyllea*, according to the UNITE database (Kõljalg *et al.*, 2005), which corresponds to the ambiguous BLAST result from Genbank (Altschul *et al*., 1997). The BLAST results of the remaining morphotypes strongly matched *T. terrestris*, a resupinate/bracket ectomycorrhizal fungus (Chapter 3). Comparisons between the alignments of all the morphotypes (Appendix 6) indicate that the morphotype ‘Fwhite’ is potentially a different species, as score bit values and e-values were weak and percentage identity was as low as 88%, when compared to each of the other morphotypes. The similarity tree (Figure 5.5) indicates that it is in fact a different *Thelephora* species, but grouped closer to *T. penicillata* than to *T. caryophyllea*.

Xerocomus badius was represented by three morphotypes (Table 5.2) according to the concurring results of both Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al.*, 2005). ‘Dull orange’, ‘Beige’ and ‘BSY2’ all aligned with high score bit values and low e values with *X. badius*. Although this species was represented by three separate morphotypes, this is not surprising as changes in macromorphological characteristic such as mantle colour occur as a result of aging and bruising, leading to potential inaccuracies during quick and crude morphotyping methods. All three morphotypes formed a monophyletic group with *X. badius* (Figure 5.4), strengthening the allocation of this species name to the morphotypes. The presence of this species in the rhizosphere was expected as sporocarp collections of the species were made (Chapter 3).

Four morphotypes have been identified as *Amanita spissa* on Genbank (Altschul *et al*., 1997), with different levels of alignment: ‘Grey/pink’, ‘White’, ‘BSY 1, 3, 4 & 5’, ‘BSY’ and ‘yellow-orange’. BLAST results of ‘BSY4’ and ‘White’ from the UNITE database (Kõljalg *et al.*, 2005), which were aligned with *A. rubescens* (Table 5.2), lead to a pair-wise comparison between the morphotypes. From these results (Appendix 4.2), it can be concluded that

morphotype ‘BSY4’ and ‘White’ are more similar to each other, with a 98% identity but low score bit value, than to any of the other morphotypes in the ‘*Amanita*’ group, the alignment identities reflecting only a 92-94% level. This indicates that they are potentially a separate species, *A. rubescens*. The remaining morphotypes, ‘Grey/pink’, ‘BSY’, ‘yellow-orange’ and ‘BSY 1, 3 & 5’, form the ‘*A. spissa*’ group and aligned strongly with each other (Table 5.6). The *A. spissa* group’s position on the similarity tree (Figure 5.2) reflected, once again, confusing results. The morphotype ‘WhiteA’ was basal to the whole *Amanita* clade. The morphotype ‘BSY4’ grouped with a Genbank (Altschul *et al*., 1997) sequence of *A. spissa*, the sporocarp species of *A. excelsa* (synonym = *A. spissa*), but UNITE (Kõljalg *et al.*, 2005) grouped ‘BSY’ with sequences of *A. rubescens*. Similarly, the remaining morphotypes forming the ‘*A. spissa*’ group, were grouped with the sporocarp sequences of *A. rubescens* derived from this study and a UNITE (Kõljalg *et al.*, 2005) sequence of *A. spissa*. Accessioning of incorrectly identified specimens of *A. spissa* and *A. rubescens* to either of the databases appears to have occurred, creating confusion concerning the taxonomic position of the sequences in this research. As mentioned before, the ITS rDNA region is not ideal for the taxonomic resolution within this genus, and the LSU is a more conserved, informative region for species resolution. Further molecular analysis of this genus should concentrate on more conserved regions of DNA to avoid phylogenetic ambiguity and ensure morphological classification of material.

‘WhiteB’ and ‘Yelsil B’ aligned poorly with *Scleroderma bovista* and *Scleroderma verrucosum* on Genbank (Altschul *et al*., 1997) and UNITE (Kõljalg *et al.*, 2005), respectively. The same poor alignment was observed for the *S. citrinum* sporocarp (Table 5.1). A comparison of the morphotype sequence to the sporocarp sequence (Table 5.3) indicated that while the ‘woolly white’ morphotype appears to be closely aligned to *S. citrinum*, the ‘Yelsil’ morphotype appears to be more distantly related as indicated by poor alignment values (Table 5.3) and suggests it is another *Scleroderma* species.

The ‘Grey’ morphotype was strongly aligned with *Albatrellus ovinus* on Genbank (Altschul *et al*., 1997) and poorly with *Russula firmula* on UNITE

(Kõljalg *et al.*, 2005) (Table 5.2). The correct identity of 'Grey' is considered to be *A. ovinus* due to the high score bit and e-values retrieved from Genbank (Altschul *et al*., 1997). The sequence similarity tree (Figure 5.4) confirmed this result by grouping the morphotype with *A. ovinus*. Although *A. ovinus* has never been reported in South Africa, the finding is not surprising as to date, very little research has covered the ECM community diversity and structure. Although sporocarp collections have been made (Lundquist, 1986, 1987a,b, van der Westhuizen and Eicker, 1987), the absence of sporocarps of this species in prior collections does not rule out the possibility of this finding. 'DSY' was conclusively identified as *Clavulina cristata*, as both databases retrieved good alignments with the species (Table 5.2).

The morphotype 'Beige/white' retrieved a good alignment from Genbank (Altschul *et al*., 1997), with *Heterobasidion annosum*, a wide-ranging basidiomycetous plant pathogen that has been studied in an infected site of *Pinus sylvestris* and *Betula pendula* (Lygis *et al.,* 2004) and considered to be highly infective over extended periods of time. The fungi are known to colonise fresh plant tissue normally exposed as stumps during felling and infects surrounding plants through the rhizosphere. The consequences of infection are reduced wood quality, growth losses and mortality of *Pinus sylvestris* (Pukkala *et al*., 2005). No record of this fungal infection has previously been made from South Africa. The match retrieved from the UNITE database (Kõljalg *et al.*, 2005) aligned poorly with *Entoloma serrulatum* (Table 5.2). Given that this is a purely ECM database, this alignment was not allocated significance, especially since the Genbank match was high. Amplification of non-ECM fungi is from field samples is virtually impossible to eliminate as clearly shown by the results. The technique employed by Rosling *et al*., (2003), using Basidiomycete-specific primers, would have failed in this case as *H. annosum* is a Basidiomycete.

The sequencing of ECM root tip morphotypes enabled them to be linked to sporocarp species. DNA amplification of the *Thelephora terrestris* sporocarps found in the Brooklands and Driekop study sites (Table 3.1) was not successful, and therefore not presented in this study, but sequences

comparable to *T. terrestris* were amplified from root samples found in both the Brooklands and Driekop study site (Table 5.2). The alignment of sporocarp and ECM morphotype sequences of *A. spissa* (Table 5.3) demonstrated a strong similarity of sporocarp sequences to sequences from morphotypes 'BSY1' and 'Grey/Pink', with high Score bit values and 99% identity. 'Beige' and 'Dull Orange' aligned well with sporocarp sequence of *Xerocomus badius*, both of which were collected from the Brooklands study site. All the ECM morphotypes, apart from the non-mycorrhizal fungi, can be linked to sporocarp collections, indicating a link to the above ground diversity in the Brooklands study site.

By combining molecular and morphological (Chapter 3 and 4) techniques that reinforce one another, a powerful tool used in taxonomy and systematics is created (Kraigher *et al.,* 1995, Eberhardt, 2002, Sakakibara *et al*., 2002). Although the confirmatory quality of utilising both methods produces a strong argument for species delineation, the methods often result in conflicting identification. From this research, it is clear that there is some genotypic variation within morphotypes (e.g. 'BSY') and yet some conformity between different morphotypes (e.g. Grey/Pink, BSY and Orange all resolve as *Amanita spissa*) (Table 5.2). Similar results have observed by Mah *et al*., (2001). In that study, 22 genotypes were derived from eight morphotypes. A genotypic analysis of morphotypes found by Erland *et al*., (1999) recorded eight genetic species within 6 morphotypic groups. Phenotypic variations may be a result of the morphological changes of the ECM root tip that occur during the aging process. Mantle colour may change or may be altered under different environmental conditions. Contradicting these results, however, Sakakibara *et al*., (2002) demonstrated an average of 93% conformity of genotypes within the eight morphotypes examined. These authors utilised techniques of detailed anatomical analysis, which may be impractical for large-scale population surveys. From the genotypic results in this research it is concluded that the morphotypes identified in this study, based on macro-morphology, are not sufficiently accurate for species identification of root tip associated ECM fungi.

Although the ECM fungi were in a few cases linked to sporocarp species found, sporocarp collections were only partially representative of the below ground diversity. All but two species in the sporocarp collection were sequenced, compared to the ECM root tip data where approximately 66% of the root tip extractions and amplifications were unsuccessful. In the event that root tip sequencing was optimised, the species diversity could be predicted to be higher than identified in this study. It is therefore inaccurate to make correlations between above and below ground species diversity and relative abundance. Below ground sampling is necessary to provide a more complete account of ECM fungal diversity and species abundance.

The objective of sporocarp identification using molecular techniques in this study was achieved. Species identification using molecular techniques was often more accurate, as was the case with morphologically identified species of *Lactarius hemicyaneus*, *L. semisanguifluus* and *Tricholoma albobrunneum*. In some cases, molecular taxonomy was not accurate, and the genus *Amanita* appears to be ambiguous. The ITS region does not resolve the phylogeny of the genus *Amanita* and other rDNA regions should be used for better identification accuracy. Six ECM species were identified from the root material. The objective of linking the ECM root tip morphotype species to the sporocarp collections was achieved (Table 5.3). Five of the six ECM root tip morphotypes were linked with sporocarps collected in the field. Although there appears to be higher species diversity above ground, this is almost certainly misleading, as sampling events of below ground material were restricted to 5x5m plots, which limited sampling, while sporocarp collection occurred over the entire field trial and further a field (Grahamstown).

6. EFFECTS OF FERTILISATION ON ECM FUNGAL SPECIES COMMUNITY IN THE RHIZOSPHERE

6.1 Introduction

The extent to which ECM can perform its associated functions depends primarily on the conditions of the soil. The literature supports the view that the main factor influencing mycorrhizal colonisation and fungal growth is soil pH (Hung and Trappe, 1983, Erland *et al*., 1990). The balance is often changed by acidification of forest soils (acid rain), nitrogen deposition from industrial effluent and emission, nitrogen fertilisation in managed plantations, aluminium toxicity and litter accumulation (Arnolds, 1991, Dames, 1996). Brun *et al.*, (1995) showed that mycorrhizal formation of *Paxillus involutus* on *Betula pendula* was inhibited by high ratios of N to P rather than the concentrations of either nutrient.

The enhanced plant growth experienced due to fertiliser additions is well documented and valued in the forestry industry where rapid growth and higher yields are desirable (ICFR, Annual Research Report, 2004). Applications of N and P are now regarded as common practice (Chamberlain *et al*., 2005), although little is known, and even less concern is shown, of the effects of fertilisers on the rhizosphere in South Africa with regard to micro-organisms, and in particular, ECM fungi. The possible negative effects on the microorganisms occurring in the rhizosphere may limit the long-term sustainability, and therefore the viability of land used for plantation agriculture. Considering the intimate and delicate interaction between plant and fungus in an ectomycorrhizal association, concerns regarding the effects of fertilisation, not only on reduced overall sporulation (Section 3.3.3.2), but also of colonisation potential and mycelial growth into the rhizosphere, have been expressed.

Some of the more commonly used inorganic fertilisers include 2:3:2, LAN and Orthophosphate, and are applied to compensate to soils with limiting levels of nitrogen and phosphorus, needed for ideal plant growth (du Toit and Freimond, 1994). Between 20-30kg of fertiliser per hectare is applied in managed plantation, annually in South Africa. As a result, increased soil

acidity is often experienced in areas that already have low pH levels (Dames *et al.,* 2002). Increased soil acidity has direct consequences on soil nutrient availability and may therefore dramatically influence the ECM fungal species community. No comprehensive research has been conducted in South Africa to further investigate the current state of the ECM fungal community in response to high soil acidity. Ring (2004) confirmed the soil altering properties of fertilisers by demonstrating that up to six years after high applications of ammonium nitrate, 1800kg N ha^{-1}, the soil could be characterised as containing higher concentrations of aluminium, manganese, nitrate and a lowered acid-buffering capacity. Although the levels of fertiliser application were remarkably higher in this study (Ring, 2004) than would be used in managed plantations in South Africa, these results suggest that an effect may exist with lower levels of fertilisation.

The effects of fertilisation, liming, and acid rain on the biosphere and the community diversity of the organisms are being hotly debated. Most authors have recorded no change in fungal species diversity and/or only a change in relative abundance of fungal species. Jonsson *et al.,* (1999), found a species shift in composition as a result of liming in a spruce forest. Although there was no significant difference of species diversity between the control and high dolomite treatment, a similarity analysis showed that the species composition was affected. Fransson *et al.,* (2000), found that although there was a community shift in species composition in response to optimal fertilisation in a Norway spruce forest, there was no statistical difference in the species richness, or the number of roots colonised. Neither study detected a decrease in root colonisation or a decrease in species diversity. Arnolds (1991), has recorded large decreases in fungal diversity due to increased acidity, but the theory has been challenged by Agerer *et al.,* (1998), who found an increase in sporocarp production of *Russula orcholeuca* from 3.3 in the control plots to 14.5 in response to acid irrigation. Fransson *et al*., (2000), in a study of Norway spruce, concluded that some ECM species decreased in proportion and others increased, in response to acid irrigation treatments and that liming at the time of irrigation seemed to reduce these effects. Often the studies reveal no decline in species diversity, but rather a change in composition.

Very little is known about the ultimate implications a species shift has for the plant. Trappe (1987), strongly suggests that a high diversity of ECM fungi is preferable, as there is intra- and interspecific variation in the ability and efficiency of nutrient retrieval for host plant nutrition. It would then make sense to have as high diversity as possible to cover all the nutrient requirements for the host plant.

The objective of this study was to determine the effects of nutrient additions (in the form of fertilisers) of N, P and NP on the fungal species community in both a mature and young *Pinus patula* stand and corresponding pH differences between these sites.

6.2 Methods

6.2.1 Sampling

In June (2002) all the plots at the Brooklands study site, and all the *P. patula* plots at Driekop were sampled as described in Section 2.3. This included all the nutrient combinations of Ca, K, N and P in the Brooklands study site and the nutrient (2:3:2 and P) and fungicide treated plots in the Driekop study site. During this sampling event, two soil cores were taken from each plot meaning that 64 core samples from the Brooklands and 36 from Driekop study site were taken, respectively. This served to provide and initial overview of the number of ECM morphotypes and since this field was new to the author, also provided the necessary experience of morphotyping. The subsequent sampling events focused on only on the N, P and NP treatments in the Brooklands study site and non-fungicide fertilised treatments in Driekop study site (Section 2.3). By conducting this reduced number of plots sampled, more core samples could be extracted. Five core samples were taken from each plot equating to 40 cores from the Brooklands and 45 from the Driekop study site, respectively, which allowed more repititions with in each treatment.

The core samples (5cm in diameter) were extracted to a depth of approximately 15-20cm deep, collecting mainly from the litter and organic layer. The cores were taken approximately 1m from the base of the tree. The soil cores were carefully washed of soil particles and the remaining plant

material inspected for ECM root tips. All root material was separated into non-ectomycorrhizal and ECM root tips, which were separated into morphotypes. All the non-ectomycorrhizal root tips were counted, as well as those of each of the morphotypes.

The data was collated from each sampling event and run through a preliminary statistical analysis, which revealed large variance in the data. In an attempt to reduce the variation, a single replicate of each treatment in the Brooklands and Driekop study site was sampled twice; i.e. ten core soil samples were taken instead of five, in December (2003).

6.2.2 Soil pH analysis

Soil samples were taken from each plot in December 2002. The soil core samples were sifted through a 2mm sieve and 20g of soil extracted. 15g of soil was added to 37.5ml of distilled water and placed on a shaker for 15min and left to settle for 30min. The solution was mixed again, and the pH was taken with a pH meter (Hanna 8134 membrane, Hanna instruments) (Okalebo *et al.,* 1993).

6.2.3 Below ground species diversity and abundance

From each core sample, the numbers of mycorrhizal and non-mycorrhizal root tips were recorded. The total abundance of ECM, as a percentage of the total number of root tips per core was ascertained and the root tips were divided into morphotypes (Section 4.3). The Simpson's index was calculated from the following formula: $D = \sum n(n-1)/N(N-1)$, where n = the number of root tips in one morphotype and N = the number of root tips of all the morphotypes within each treatment (Begon *et al*., 1990). The value of D ranges from 0-1 where 0 would represent an infinite diversity and 1 would indicate no diversity, or more simply, values near zero indicate a heterogenous ecosystem and values closer to one would be more homogenous. This index calculates the probability that two individual ECM root samples are the same species (species richness) and takes into account the relative abundance of each species or ECM root morphotype within the population.

6.2.4 Effects of sample event and fertilisers

The trends of the six most dominant morphotypes in the Brooklands and Driekop study site were utilised to determine significant sample event and nutrient effects. Shifts in relative abundance were also investigated. The relative abundance of each morphotype was calculated by dividing the total number of root tips from a single morphotype by the total number of root tips enumerated from the treated plots. The resultant data were then arcsine transformed using the ARCSIN function in Excel for proportionate data, and analysed using a factorial ANOVA. One-way ANOVA was utilised to determine statistical differences in relative abundance of each morphotype.

The data from Brooklands and Driekop were analysed separately. The sampling events used for this analysis were June 2002, December 2002, April 2003 and December 2003.

6.3 Results

6.3.1 Soil pH analysis

The pH in the Brooklands study ranged from pH3.58 - 3.7 between the different treatments, with an average of pH3.6 ± 0.22. The Driekop study site varied slightly, but not significantly, from pH 4.15 ± 0.19 in the Optimum fertilised plots, pH3.9 ± 0.13 in the phosphate enriched plots and pH 4.1 ± 0.22 in the control plots. The addition of phosphate appeared to decrease soil pH, but not significantly.

6.3.2 Below ground species richness and abundance

6.3.1.1 ECM abundance

The overall ECM root tip abundance was measured as a percent of the total number of root tips. ECM abundance was relatively low in the Brooklands study site in June 2002 (Table 6.1). December 2002 and April 2003 yielded not only a larger number of total root tips, but also a larger percentage of ECM. December 2003 was characterised by a decrease in the number of ECM and a total number of root tips, but yielded a noteworthy higher percentage (76%) of ECM. A slightly different pattern was observed in the Driekop study site (Table 6.2), where ECM abundance did not appear to

increase in percentage ECM in December 2002. In April 2003 the percentage ECM increased to 44%, and increased even further to 83% in December 2003 in marked contrast to the previous season (December 2002) of only 20%.

Table 6.1 Data from the Brooklands study site presenting the total number of root tips enumerated at each sampling from all the plots.

	ECM	Non-ECM	Tot Root tips	%ECM
Jun 2002	532	2236	2768	19.21
Dec 2002	8684	17720	26404	32.88
Apr 2003	6853	6966	13819	49.59
Dec 2003	4759	1440	6199	76.76

Table 6.2 Data from the Driekop study site presenting the total number of root tips enumerated at each sampling from all the plots.

	ECM	Non-ECM	Tot Root tips	%ECM
Jun 2002	268	904	1172	22.86
Dec 2002	2496	9796	12292	20.30
Apr 2003	2861	3562	6423	44.54
Dec 2003	5801	1124	6925	83.77

Climatic effects have been demonstrated, not only in terms of the increased number of total roots in December 2002, 2003 and April 2003 (Table 6.1, 6.2) that would normally occur during the root flush in plant growth season (Section 2.2.1), but also a simultaneous increase of ECM fungal abundance. Higher percentages of ECM root tips in both the Brooklands and Driekop study site, in spite of lower total number of roots were noted.

To assess the season changes within each individual treatment, percentage ECM was calculated as number of ECM over the total number of roots counted in a particular treatment (Figure 6.1). A low percentage of ECM was observed in June 2002, where N and NP appeared to have the lowest percentage (15 and 18%, respectively), and P had the highest percentage of just over 40%. In December 2002, however, the highest ECM percentages were recorded in the N and NP treatments. In April 2003 not much difference

was observed, December 2003 demonstrates a marked increase in ECM percentage in N treated plots.

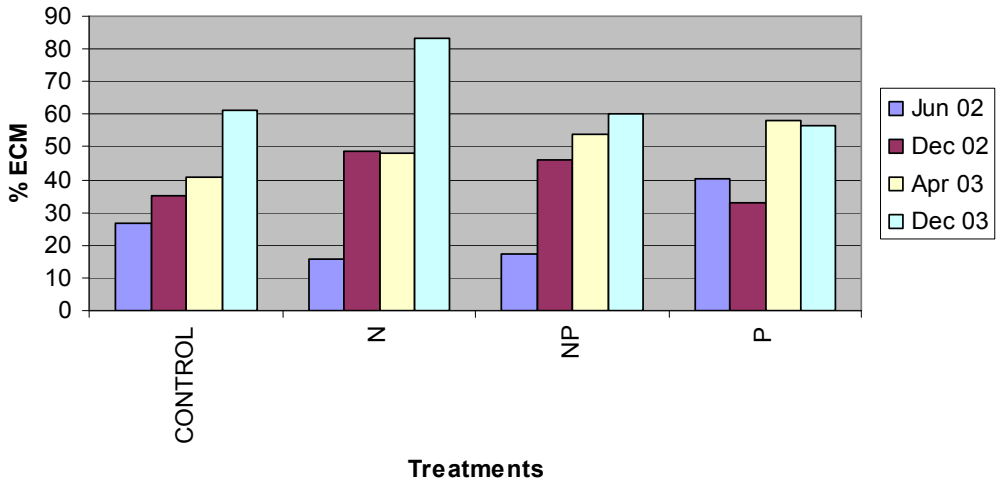


Figure 6.1 Percentage of total ECM colonisation in the Brooklands study site for the periods June 2002, December 2002, April 2003 and December 2003 as calculated from the soil samples collected in the control, N, NP and P plots.

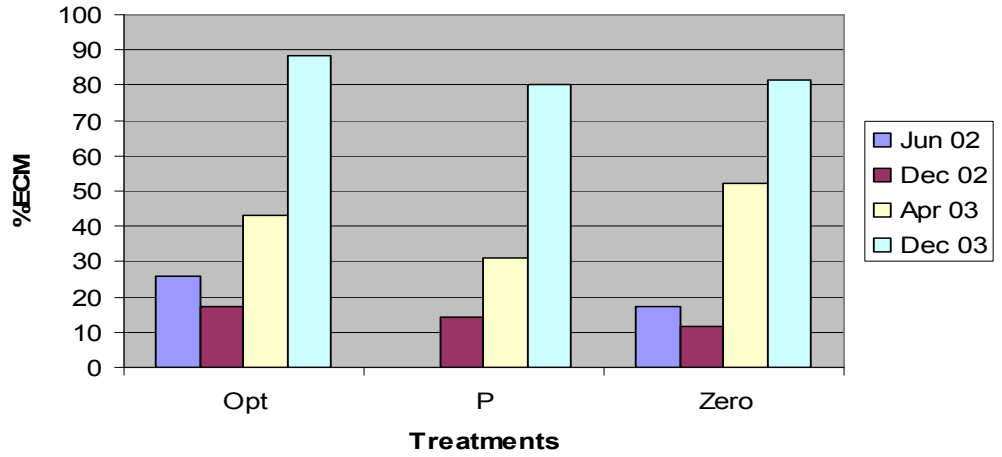


Figure 6.2 Percentage of total ECM colonisation in Driekop study site for the periods June 2002, December 2002, April 2003 and December 2003 as calculated from the soil samples collected in the control, Optimal (NP) and P plots.

Similarly, a marked increase of percentage ECM was recorded in December 2003 (Fig 6.2), increasing by 30-50% from April 2003. In June 2002 and April 2003, the P plots have the lowest levels of colonisation, with no ECM encountered at all in June 2002 (Fig 6.2). In June 2002, the Optimum treatment records the highest ECM percentage (25%) and in April 2003 the Zero (control) treatment recorded the highest percentage ECM (just over 50%). In December 2002, low percentages were recorded compared to December 2003, with not much difference in the percentage ECM between the different treatments. Differences in percentage ECM between the treatments in December 2003 were not large, but the Optimum treatment recorded a higher percentage of (88%).

6.3.1.2 Species diversity

The Simpson's index was measured using the equation: $D = \sum n(n-1)/N(N-1)$, where n = the number of root tips in one morphotype and N = the number of root tips of all the morphotypes within each treatment (Table 6.3 and 6.4).

Table 6.3 Diversity index for the species richness in the Brooklands study site.

	N	**P**	**NP**	**Control**
Jun 2002	0.397	0.729	0.279	0.225
Dec 2002	0.271	0.143	0.158	0.234
Apr 2003	0.349	0.313	0.263	0.276
Dec 2003	0.397	0.450	0.410	0.229

Table 6.4 Diversity index for the species richness in the Driekop study site.

	Opt (2:3:2)	**P**	**Control**
Jun 2002	0.196	No ECM roots	0.846
Dec 2002	0.147	0.233	0.302
Apr 2003	0.234	0.168	0.242
Dec 2003	0.184	0.177	0.185

The species diversity in the Brooklands study site (Table 6.3) and Driekop study site (Table 6.4) demonstrate variable dynamics. No pattern with regards to sampling date or nutrient application can be discerned, but general comment about the overall diversity and the homogeneity of the ECM fungal species in the Brooklands site can be regarded as moderately diverse on the scale of 0-1. The lowest species richness was calculated in the P plots in June 2002. The Driekop site was generally more heterogenous in comparison, the lowest species richness was calculated from the Control plots in June 2002.

The number of morphotypes found in the Brooklands study site were analysed and demonstrate that in June 2002 (Figure 6.3) species richness was low and did not differ between the treatments. December 2002 data indicates a marked increase in the number of morphotypes for all treatments, with almost no difference in the number of morphotypes. In April 2003, the P and NP treatments at the Brooklands study site (Figure 6.3) indicate a lower diversity than the N and Control plots. In December 2003, the number of morphotypes was relatively low, compared with the previous year, December 2002. The species/morphotypes in Fig 6.3 and 6.4 are not subsets of the species recorded in subsequent sampling in December 2002, April 2002, or December 2003. This follows for all the comparisons of diversity. A large increase in diversity in December 2002 and April 2003 at both sites was experienced.

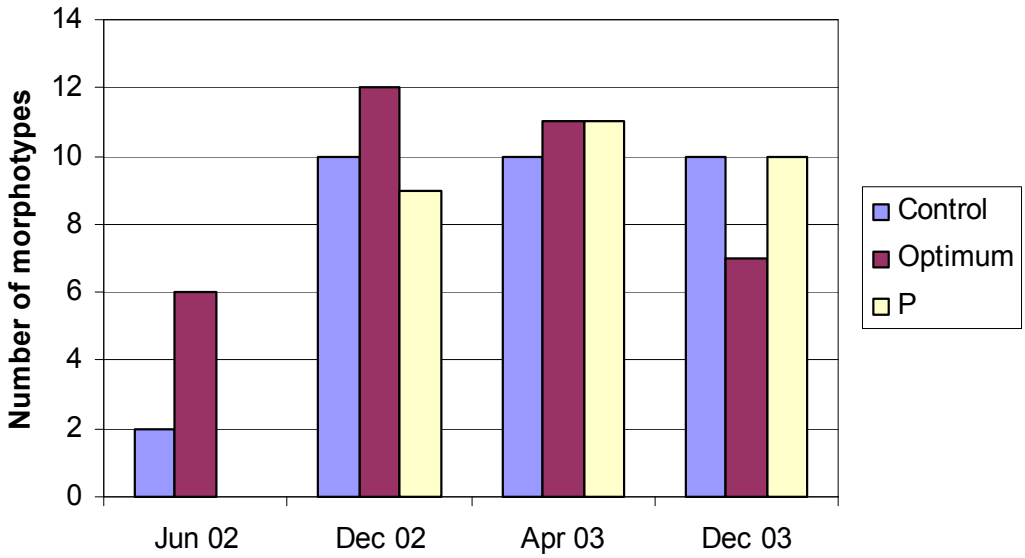


Figure 6.3 Average number of morphotypes in the Brooklands study site between the Control (untreated) plots, the nitrogen (N), phosphorus (P) and combination of nitrogen and phosphorus (NP) for sampling events in June 2002, December 2002, April 2003 and December 2003 are detailed.

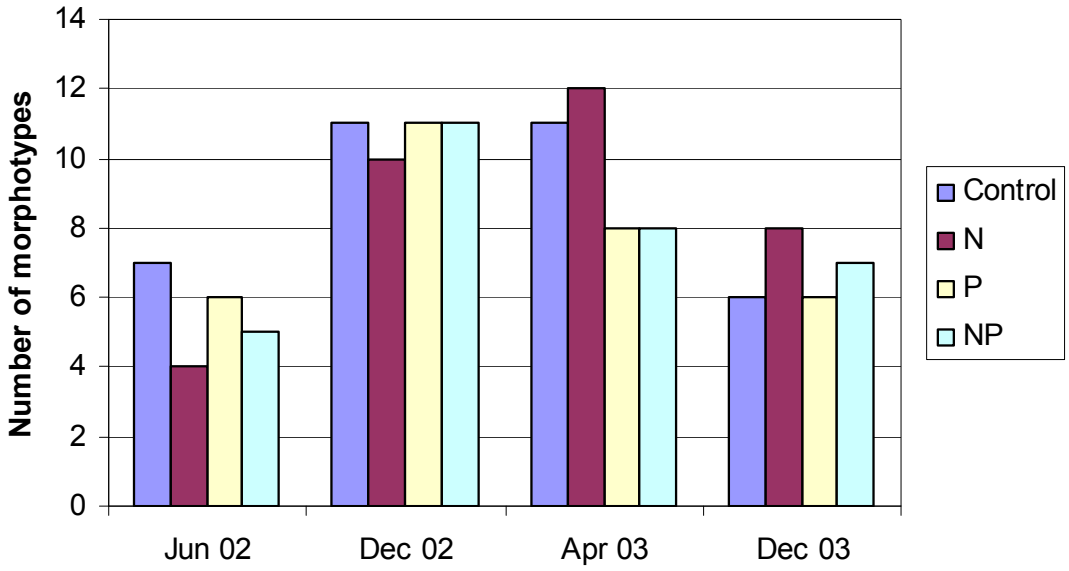


Figure 6.4 Average number of morphotypes found in the Driekop study site between the Control (untreated), Optimum (2:3:2 fertilisation) and P (phosphorus application) for sampling events in June 2002, December 2002, April 2003 and December 2003 are detailed.

The number of morphotypes observed in the Driekop study site in June 2002 demonstrated a comparatively low number of morphotypes (Figure 6.4). The number of morphotypes recorded for December 2002, April 2003 and December 2003 did not appear to differ significantly between treatment plots. In December 2002, the Optimum treatment recorded the highest number of morphotypes (12). April 2003 data did not reveal any differences in the number of morphotypes between treatments, and December 2003 showed a potential negative effect of the Optimum treatment on the number of morphotypes.

The spatial distribution patterns of community species composition can be displayed by rank-abundance graphs of the species present (Figure 6.5 and Fig 6.6). The morphotypes representing more than 5% of the total ECM found in the Brooklands study site (Figure 6.5) were: 'BWh', 'Bt(w)', 'BSY', 'Black' and 'Orange' (Table 6.5). Similarly, the morphotypes found in the Driekop study site (Figure 6.6) were: 'DSY', 'Dnorm', 'Yelsil', 'Tubercle, 'Black' and 'DWh'. The results show few abundant species and a large number of rare species at both sites (Table 6.6).

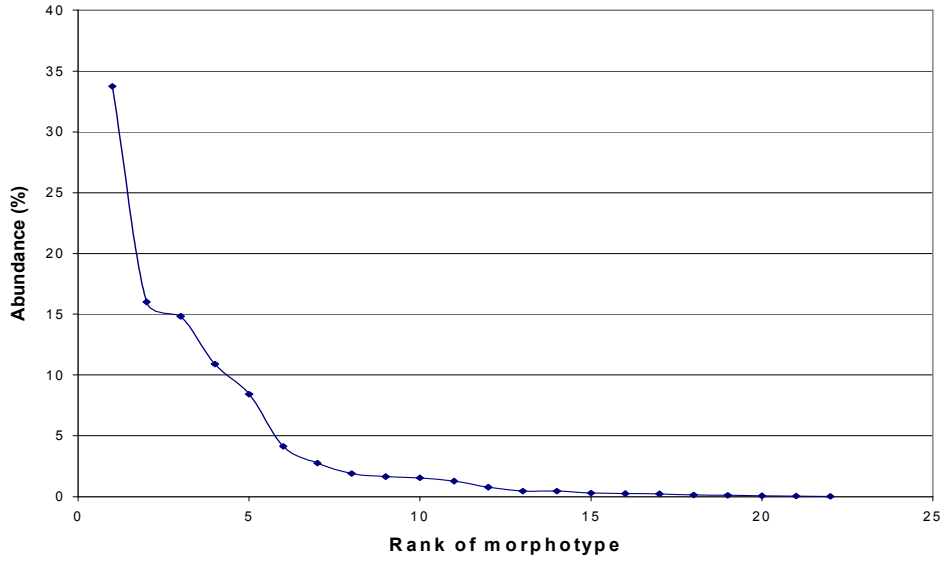


Figure 6.5 Rank-Abundance for the ECM morphotypes in the Brooklands study site. Data combined from June 2002, December 2002 and April 2003 sampling were combined to calculate the average percentage abundance.

Table 6.5 List of morphotypes in the Brooklands study site in rank order of percent abundance for combined treatments and sampling events.

Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	
Morph	White	Bt (w)	Yelsil	Black	Orange	Beige long	Partial white	
*% Rel abun	33.70	16.02	14.82	10.90	8.42	4.13	2.769	
Number	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
Morph	Beige short	Grey	Brown	WrinkSY	Bnorm	Brown tips	Yellow	
% Rel abun	1.88	1.65	1.54	1.27	0.76	0.46	0.44	
Number	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Morph	Woolly white	Brown/ grey	Silver	Skin	Fwhite	Bubb	Grey (tubs)	Pink
% Rel abun	0.28	0.24	0.23	0.13	0.11	0.06	0.05	0.02

* Rel abun = Relative abundance

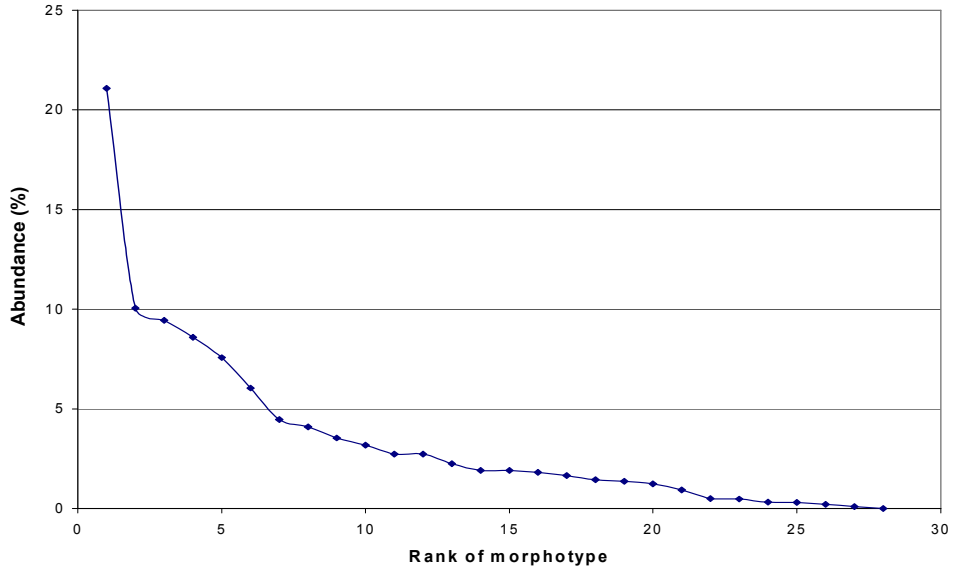


Figure 6.6 Rank-Abundance graph for the ECM morphotypes found in the Driekop study site. Data from June 2002, December 2002 and April 2003 were combined to calculate the average percentage abundance.

Table 6.6 List of morphotypes in the Driekop study site in rank order of percent abundance for combined treatments and sampling events.

Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Morph	DSY	Dnorm	Yelsil	Tuberculate	Black	White	Grey	Bt (w)	Part white	Brown
% relative abundance	21.08	10.04	9.44	8.58	7.57	6.04	4.46	4.10	3.53	3.18
Number	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Morph	Beige	Dyell	SY/ Grey	Black patch	Dark	Dark grey	Mx	Cf Grey tuberculate	Sel	Tri
% relative abundance	2.73	2.73	2.24	1.92	1.92	1.81	1.65	1.44	1.36	1.24
Number	**21**	**22**	**23**	**24**	**25**	**26**	**27**			
Morph	Orange	Cotton wool	Pale brown	Bubb	Skin	White et	Dodd			
% relative abundance	0.94	0.49	0.48	0.32	0.30	0.21	0.10			

* Rel abun = Relative abundance

6.3.3 Effects of sample events and fertilisers

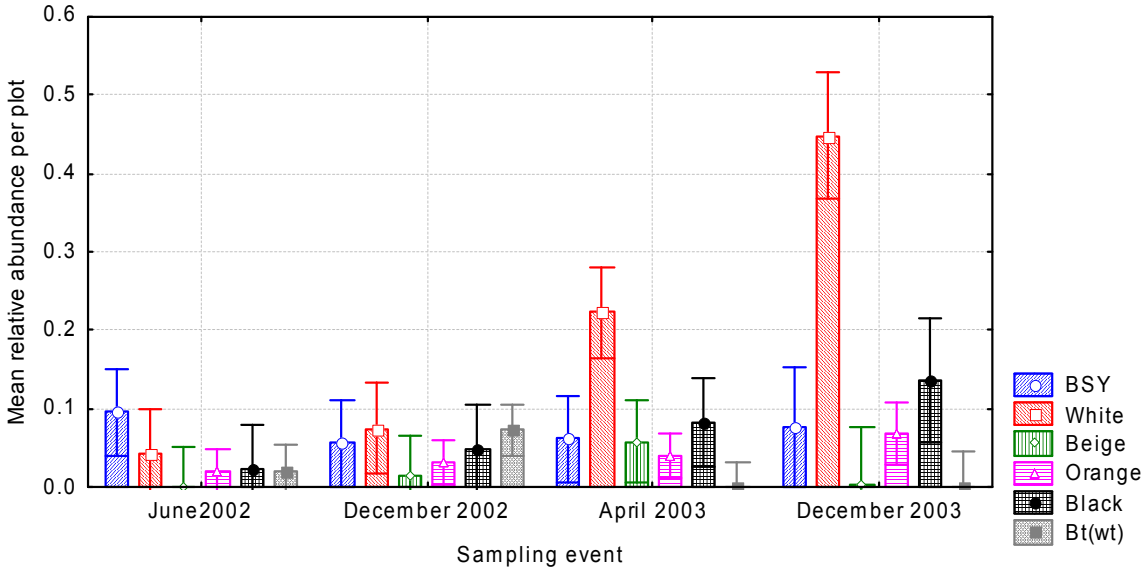


Figure 6.7 One-way ANOVA of all the dominant morphotypes present in the Brooklands study site in response to sampling event. $F_{(18,\ 20.28)} = 4.09$, $p < 0.05$, where the 95% confidence level is required for significance.

The one-way ANOVA indicated a significant overall effect of sampling event (Figure 6.7). Significant changes in relative abundance of individual morphotypes were identified by conducting a univariate analysis (Table 6.7).

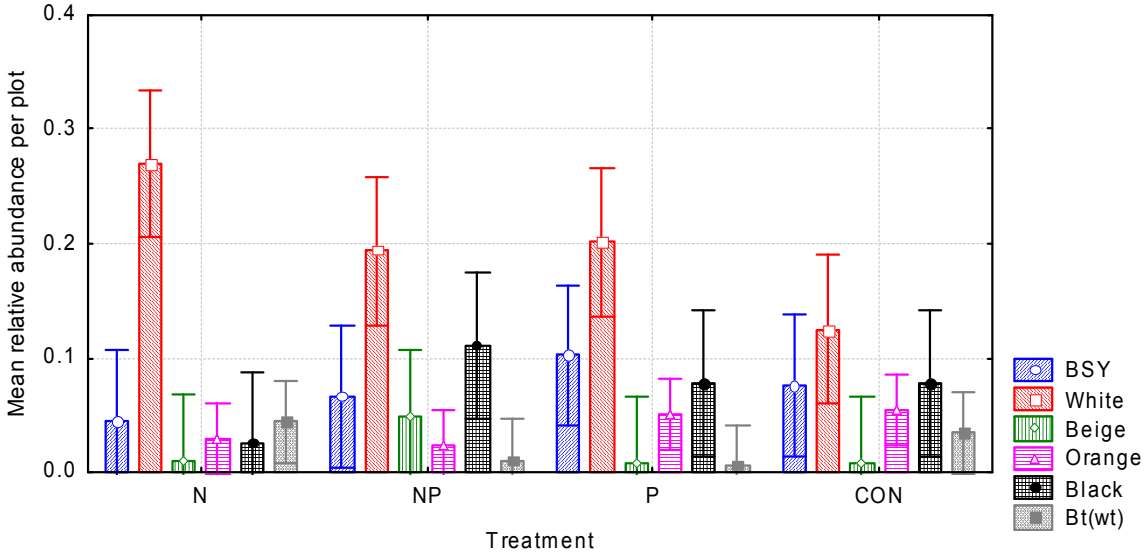


Figure 6.8 One-way ANOVA of all the dominant morphotypes present in the Brooklands study site in response to nutrient applications. $F_{(18,\ 20.28)} = 0.78$, $p = 0.69$, where the 95% confidence level is required for significance.

No significant effect of the nutrient of N, P or NP was apparent (Figure 6.8). Shifts in morphotype abundance were considered by conducting a univariate analysis of each morphotype (Table 6.7)

Table 6.7 Univariate F-values and probability values that indicate significant shifts in morphotype abundance between the Brooklands study site sampling events and between nutrient applications. * indicates significant values

Factor	**BSY**	**White***	**Beige**	**Orange**	**Black**	**Bt (wt)***
Sample event (df = 3)	F = 0.49 p = 0.69	F = 31.45 p<0.05	F = 1.13 p = 0.37	F = 1.70 p = 0.21	F = 2.38 p = 0.11	F = 4.79 p = 0.02
Nutrient effects (df = 3)	F = 0.70 p = 0.56	F = 3.98 p = 0.03	F = 0.56 p = 0.65	F = 1.11 p = 0.38	F = 1.49 p = 0.19	F = 1.25 p = 0.33

Morphotype 'White' and 'Bt(wt)' were significantly affected by sample events and/or nutrient effects (Table 6.7). The morphotype 'White' was affected by sampling event and became more dominant in April 2003 and December 2003 (Figure 6.7). Additionally, 'White' was significantly affected by nutrient application on N and was represented by an increase in abundance compared to the control plot (Figure 6.8). The morphotype 'Bt(wt)' only demonstrated a significant response to sampling event, which was represented by a decrease in relative abundance in April 2003 and December 2003 (Figure 6.7).

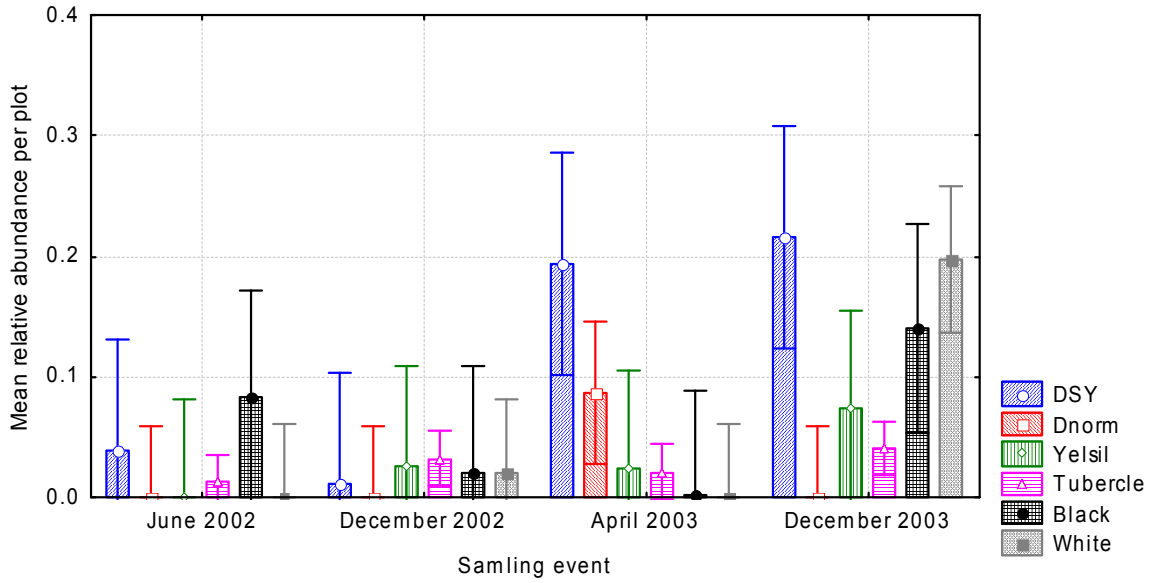


Figure 6.9 One-way ANOVA of all the dominant morphotypes present in the Driekop study site in response to sampling event. $F_{(18,\ 54.22)} = 4.23$, $p < 0.05$, where the 95% confidence level is required for significance.

Significant differences of overall relative abundance of morphotypes between the sampling events were evident (Figure 6.9). To determine sampling event effect on individual morphotypes, univariate analysis was carried out (Table 6.8).

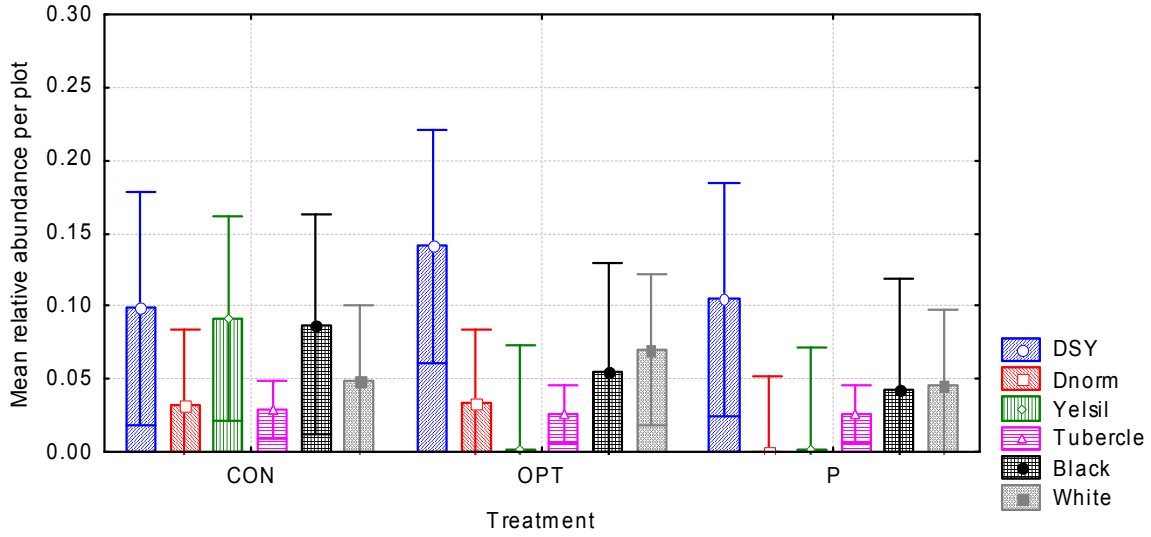


Figure 6.10 One-way ANOVA of all the dominant morphotypes present in the Driekop study site in response to nutrient applications. $F_{(18,\ 38)} = 0.76$, $p = 0.68$, where the 95% confidence level is required for significance.

No significant nutrient effect on overall ECM root tips abundance (Figure 6.10). To determine shifts of individual morphotypes in response to the nutrient applications, univariate analysis was conducted (Table 6.8).

Table 6.8 Univariate F-values and probability values that indicate significant shifts in morphotype abundance in the Driekop study site between sampling events and between nutrient applications. * indicates significant values

Factor	DSY*	Dnorm	Yelsil	Tubercle	Black	White*
Sample event (df 3)	F = 5.48 p < 0.05	F = 2.25 p = 0.10	F = 0.61 p = 0.61	F = 1.21 p = 0.32	F = 2.22 p = 0.11	F = 10.74 p < 0.05
Nutrient effect (df 2)	F = 0.35 p = 0.70	F = 0.56 p = 0.57	F = 2.30 p = 0.12	F = 0.03 p = 0.96	F = 0.39 p = 0.67	F = 0.28 p = 0.75

Morphotype 'DSY' and 'White' were significantly affected by sample event (Table 6.9). The relative abundance of 'DSY' increased in April 2003 and December 2003 (Figure 6.9), while morphotype 'White' only increased in relative abundance in December 2003. No significant response to nutrient application was experienced by any of the morphotypes analysed.

6.4 Discussion

This study did not reveal significantly low pH levels in the organic layer, compared to Dames *et al.* (1996) who found pH levels as low as pH2.17 in higher altitude stand with soil with organic layers of up to one meter.

A seasonal effect was apparent in the difference of the total % ECM abundance (Table 6.1 and Table 6.2). An increase in ECM root abundance, correlated with increased overall root tip counts, was experienced from June 2002 to December 2002 and April 2003, overlapping with the growing season (Section 2.2.1). This was observed in both the Brooklands and the Driekop study site. The December 2003 data, however, for both study sites was not congruent with December 2002, in that a lower number of ECM roots and total number of roots was encountered, but there appeared to be a higher overall ECM percentage. Fractional colonisation has been documented in South Africa in prior studies. Dames *et al*. (2002), found up to 50% ECM colonisation

of *Pinus patula* root tips in mature stands in South Africa. These relatively poor levels of colonisation, as compared to 100% colonisation found in the northern hemisphere (Taylor *et al*., 2000), are noteworthy. A few hypotheses regarding this phenomenon are put forward:

1) Plantations in South Africa do not have the appropriate fungal diversity to fulfil all the niches. As there is no importation of the natural fungal associates root niches are left empty.
2) The soil conditions in South African are not favourable environments and are not suitable for many ECM fungal species and/or may not be conducive for optimal ECM colonisation.

The distribution of ECM fungi between the different treatments during the different sampling times, confirmed a climatic effect, demonstrated as lower percentages ECM root tip abundance in June 2002 (Figure 6.1 and 6.2). In the growing season treated plots appeared to have a higher ECM abundance. Overall, the control plots appeared to have a slightly lower ECM abundance. The Driekop study site revealed more clear-cut responses (Figure 6.2). The December data (2002 and 2003) did not demonstrate any variation between the treatments, but in April 2003, a marked ECM inhibition was noted in the P treated plots.

The Simpson's index calculated for both the Brooklands and Driekop study site demonstrated high variation between measure generated between sampling dates and nutrient treatments (Table 6.3 and 6.4). No conclusive effect on ECM morphotype could be determined.

The average number of morphotypes recorded between each treatment in the Brooklands study site did not differ significantly between treatments, but demonstrated an increase in ECM morphotype richness in December 2002 and April 2002 (Figure 6.3). Similarly, the ECM morphotype richness in the Driekop study site demonstrated the same pattern (Figure 6.4).

The rank-abundance analysis demonstrated the typical distribution patterns associated with ECM root tip communities in both the Brooklands study site (Figure 6.5) and Driekop study site (Figure 6.6). The distributions are presented by the abundance of a few dominant species and many less abundant rare species (Taylor, 2002). From this distribution data the most abundant morphotypes were identified, and were highlighted as important focal points for characterisation and statistical analyses.

The analysis of the dominant ECM morphotypes in the Brooklands study site revealed a significant effect of sampling event, coinciding with climatic changes from colder, dry months (June) to cool, wet months (December). Mean morphotype abundance was significantly higher in December 2003 (Figure 6.7). Changes in the abundance of individual morphotypes in response to sampling event were also expressed (Table 6.7). Morphotype 'White' increased and 'Bt(wt)' decreased significantly in December 2003 (Figure 6.7). No change in overall morphotype abundance was observed in response to nutrient application, although the morphotype 'White' did reflect an individual response (Table 6.7) of abundance between N and control treatments. The possible reason for a lack of effect in the Brooklands study site is the extended time period since the last fertiliser application, in 1998. The results in this study indicate that fertiliser effects do not persist for long periods of time and ECM communities become stable rapidly.

The analysis of dominant morphotypes in the Driekop study site demonstrated similar responses to sample event and nutrient treatments. A significant response to sampling event was attained and was observed as an increase in morphotype abundance in December 2003 (Figure 6.9). The individual response of the morphotypes 'DSY' and 'White' were significant (Table 6.8) and are demonstrated as an increase in abundance in April 2003 and December 2003 in the case of 'DSY' and in December 2003 in the case of 'White' (Figure 6.9). No response of ECM morphotype abundance to nutrient treatment was observed (Figure 6.10, Table 6.8). This was an un-expected result as the Driekop study site has experienced fertiliser applications annually and ECM morphotype communities were expected to respond by

either change in species richness or changes in community species composition. The raw data collected was highly variable, making any realistic conclusions difficult. The natural distribution of ECM communities, (few abundant and many rare species), is such that they are prone to very high levels of variation. Further analysis of this nature should primarily utilise molecular methods for more rapid, accurate and conclusive results.

7. GENERAL DISCUSSION

7.1 ECM fungal species identification and diversity

ECM fungal diversity and the relevance to functional implications for plant growth and nutrition are still to a large degree unknown. Variation in the ability of different ECM fungal species to assimilate nutrients and render them available for plant use has been observed (Rangel-Castro *et al.*, 2002). The implications of the varying abilities of nutrient assimilation between species and isolates of the same species may have wide ranging consequences for sustainable plant health. Furthermore, a higher diversity of ECM fungi may increase the range of functional advantages to support optimal plant growth. Baxter and Dighton (2001) conducted experiments using *Betula populifolia* and levels of increasing ECM fungal species diversity. Experiments using one species, then two species and then a combination of four ECM species were conducted. Although the study did not reflect any growth promotion or increase in N levels in response to increased fungal diversity, an increase in root colonisation and a significant increase in shoot P were observed.

7.1.1 Above ground ECM species identification and diversity

From the sporocarp collections, 24 potential ECM species were recorded from the genera *Amanita*, *Boletus*, *Clavulina*, *Inocybe*, *Lactarius*, *Rhizopogon*, *Russula*, *Scleroderma*, *Suillus*, *Tricholoma*, *Thelephora*, *Tomentella* and *Xerocomus*. Although effects of fertilisation treatment on ECM species composition were not discernable, fluctuations in frequency due to climatic changes were recorded. Early autumn sampling recorded the largest number of sporocarps in the Brooklands study site of 144 sporocarps, compared to the 12 recorded in winter.

Using dual techniques of morphological identification and molecular sequencing, this study identified 24 ECM fungal species, and has contributed 11 additional species to the list of fungi thought to associate in a mycorrhizal capacity with *Pinus* species in South Africa (Table 7.1). The additional species are *Boletus aestivalis*, *Clavulina cinerea*, *Inocybe* cf. *griseolilacina*, *Lactarius*

hepaticus, *Lactarius quieticolor*, *Rhizopogon roseolus*, *Russula laricina*, *Suillus luteus*, *Tricholoma fracticum*, *Xerocomus badius* and *Xerocomus pruinatus*. The list now contains 45 species (Table 7.1). No evidence of an indigenous ECM fungal flora associating with *Pinus* was found.

Table 7.1 ECM fungi as described from sporocarp and root tip collections as found associated with *Pinus* species in South Africa.

Fungal species	Study 1a	This study	Study 2	Study 3
Albatrellus ovinus		+ (*)		
Amanita excelsa	+	+		+
Amanita muscaria	+	+	+	+
Amanita pantherina	+			+
Amanita phalloides				+
Amanita rubescens	+	+		+
Boletus (cf.) edulis	+	+	+	+
Boletus aestivalis		+		
Chalciporus piperatus	+			
Clavulina cf. cristata				+
Clavulina cinerea		+		
Hebeloma crustuliniforme	+			
Helvella lacunosa				+
Inocybe cf. griseolilacina		+		
Inocybe sp. 2		+		
Inocybe sp. 3		+		
Laccaria laccata	+			+
Lactarius hepaticus		+		
Lactarius quieticolor		+		
Lactarius deliciosus	+	+		
Lycoperdon umbrinum			+	
Lycoperdon polymorphum				+
Rhizopogon luteolus	+			+
Rhizopogon roseolus		+		
Russula caerulea	+			

Fungal species	Study 1a	This study	Study 2	Study 3
Russula capensis	+	+		
Russula laricina		+		
Russula pectinata				+
Russula sardonia	+			
Scleroderma cepa				+
Scleroderma citrinum	+	+		
Scleroderma verrucosum	+			+
Suillus bellinii	+			
Suillus bovinus	+			
Suillus granulatus		+		+
Suillus salmonicolor	+ Study 1b	+		
Suillus luteus		+		
Thelephora intybacea				+
Thelephora mollissima				+
Thelephora terrestris		+		+
Tomentella sp1	+	+		
Tricholoma fracticum		+		
Tuber rapaeodorum			+	
Xerocomus badius		+		
Xerocomus pruinatus		+		

(* ECM root tip data). Study 1a: van der West and Eicker (1987); Study 1b: Reid and Eicker (2000); Study 2: Marais and Kotzé (1977); Study 3: Lundquist (1986).

The sampling of sporocarps was sporadic, only allowing a 'snap-shot' in time within a season. More regular collections in the field would have likely yielded a higher species diversity. From the list of South African fungal species complied by Doidge (1950), it is possible that the 45 ECM fungal species found associating with *Pinus* spp. documented thus far are by no means comprehensive. Records of three other *Thelephora* species, seven *Boletus* species, four *Cantharellus* species, two other *Lactarius* species, six *Cortinarius* species, three other *Paxillus* species, three other *Rhizopogon* species, *Pisolithus tinctorius* and nine other *Scleroderma* species have been documented, but these were not necessarily collected in *Pinus* stands

(Doidge, 1950). This demonstrates the potential for higher fungal species diversity in South Africa. Additionally, species forming inconspicuous resupinate or hypogeous fruiting bodies may have been overlooked. No research in South Africa has been undertaken to determine the species diversity of resupinate or hypogeous ECM fungi. This strengthens the argument for potentially higher species diversity.

7.1.2 Below ground species identification: ECM root tip characterisation

The species diversity obtained from morphological descriptions of ECM fungi is at best only conclusive at the genus level. Without sporocarp links to ECM root tips, exact identification is not possible. Dissimilarities were noted when ECM root tips were compared with other descriptions of the same species. It may be that particular specialised hyphae, such as cystidia and lactiferous cells are associated with a particular group of fungi. The presence of such structures would facilitate species identification. In contrast, mantle and rhizomorph morphology may not always be consistent within the same species. It would be of interest to inoculate different host plants under a variety of soil conditions with a known fungal species and to compare ECM fungal morphology in order to determine whether any morphological dissimilarity is apparent in response to the factors. However, no such comparative studies have been conducted. Given that a particular ECM fungal species may form an association with several different plant host species (e.g. *Pisolithus tinctorius*), it could be considered that fungal anatomy, e.g. mantle and rhizomorph morphology, might be expected to be similar. However, this is not suggested in the Colour Atlas, where fungal species characteristics are keyed out from plant host genera.

Characterisation of the seven prominent morphotypes led to the correct genus identification of *Albatrellus* (Table 7.1), two *Amanita* species, *Rhizopogon*, *Thelephora*, *Tomentella* and *Scleroderma citrinum*. Despite these descriptions and the availability of the Colour Atlas (Agerer, 1987-2002), a definitive species determination for any of the morphotypes could not be made. The key morphological and anatomical features that characterise the morphotypes are presented in Table 7.2. As molecular data were available for most of these

morphotypes, species names were allocated them, with the exception of the morphotype 'Orange' that will remain an unidentified *Amanita* sp.

Problems associated with samples collected from the field include variation of the length of storage of ECM root material prior to analysis. This can affect the colour of the mantle and result in mis-leading morphotype allocation (e.g. 'BSY', 'Beige' and 'Grey/pink' were all a single species, *Xerocomus badius*, Table 7.4). Other associated problems are the presence of ECM root tips that have more than one species associated and the possibility of not detecting early stage colonisation that would normally go undetected. There is also a possibility that other fungi may be present and this may confuse characterisation of ECM anatomy. A possible resolution to these obstacles is ECM synthesis *in vivo* and the characterisation thereof (Taylor and Alexander 1989 & 1990). This technique can lead to accurate identification of key distinguishing features and can be used to identify field collected ECM root tips. Most characterisation of ECM root tips has been based on sporocarp-confirmed identity (Agerer 1987-2002, Kraigher *et al*., 1995). However, this is not always practical or possible in the field, especially as below ground diversity can only be characterised accurately if corresponding above ground sporocarp material is found. A number of publications (Massicotte *et al*., 1999, Taylor and Alexander, 1989, Taylor and Alexander, 1990) have characterised synthesised ECM fungi of *Rhizopogon* species on *Pinus ponderosa* and *Russula aeruginea* and *Tylospora fibrillosa* on *Picea sitchensis*, respectively. The close comparison of the *Thelephora terrestris* characterised from a synthesised association with *Pinus patula* (Mohan *et al*., 1993a) with the field collected *T. terrestris* in this study, indicates that characterisation of synthesised associations are accurate and useful for comparison with field collections for the purposes of identification.

Table 7.2 Main characteristic features of the seven primary morphotypes described from *Pinus patula*.

Features	***Scleroderma citrinum* (White)**	***Amanita excelsa* (BSY)**	***Tomentella* sp. (Black)**	***Amanita* sp. (Orange)**	***Thelephora terrestris* (Yelsil)**	***Albatrellus* (Grey) sp.**	***Rhizopogon roseolus* (Tuberculate)**
Branching	Long, straight and dichotomous	Irregularly pinnate to coralloid	Unramified to short dichotomous	Dichotomous to coralloid	Monopodial to dichotomous	Straight, unramified to dichotomous	Densely coralloid
Colour	Hydrophobic, white	Grey-yellow, smooth	Black, reticulate	Orange, smooth	Yellow, smooth	Grey, smooth to woolly	Grey to dark grey, smooth
Rhizomorphs	Differentiated, white, no cystidia or cell wall thickening	Highly differentiated, cell walls thickened and numerous cystidia	Not observed	Not observed	Not observed	Not observed	Differentiated with thickened hyphal walls
Emanating hyphae	Clamped	Unclamped	Unclamped, globular base	Unclamped	Clamped	Clamped	Unclamped
Mantle layers	2	2	1	3	3	2	1

7.1.3 Molecular identification

ITS sequencing and comparative analysis added valuable information to both the sporocarp and ECM fungal identification. Sporocarp analysis aided the clarification of two collections of *Clavulina cristata* or *C. cinerea*, as well as identifying *Lactarius hemicyaneus* and *L. semisanguifluus* as a single species (Table 5.1). The analysis indicated that *Russula capensis* has probably already been described as *R. laricina* and further research into the taxonomy of the group is necessary to confirm this. Seventeen ECM fungal species were identified from sporocarp material and sequence comparisons concurred with the morphological description at least at the genus level. Sequence analysis of the ECM roots resulted in the addition of one species to the species list (Table 7.1), namely *Albatrellus ovinus*.

The comparative analyses showed a variation of closely and poorly aligned BLAST alignments. The interpretation of the results depended on the strength of the alignment of generated sequences with those in the two databases and the concurrence between the databases. In the case of poor alignments, the absence of known species from the database probably explains poor matches. It is also worth considering that differing lengths of sequences that may have an effect on sequence alignments. Therefore, similarity trees, based on ClustalW (Chenna *et al*., 2003) alignments were generated to aid the resolution of that taxonomic position of sequences derived from this study (Figure 5.1, 5.2, 5.3, 5.4 & 5.5).

Some of the problems encountered with the molecular analysis were the low rates (30%) of successful ECM root DNA extraction, the failure to amplify DNA, especially from *Boletus* sp. and *Thelephora* sp., as well as failed sequencing. Amplification of co-habiting fungi in root tip material led to failed sequencing, even when single DNA bands were observed (Table 5.2). Additionally, the possibility of inaccurate identification of sequences deposited in Genbank database (Altschul *et al*., 1997) is a reality, and must be considered. An example of this problem was the original BLAST result for the sporocarp collection identified as *Scleroderma citrinum* based on morphology was a close match to a sequence identified as *Pisolithus tinctorius*. This

match turned out to be a mis-named deposit that was later rectified. Similarly, although this finding was not reflected in these studies, the BLAST alignments 'White C', 'Partial white A' and 'Partial white C' originally were homologous with *Hymenoscyphus ericae*. Recent BLASTs of these sequences aligned with the genus *Meliniomyces* (Hambleton and Sigler, 2005). The conclusion is that sequence data need to be frequently checked for updated and amended results. Generally, the databases were adequate when considering the diversity of sample specimens submitted for analysis. Genbank (Altschul *et al*., 1997) was more comprehensive, but not always as accurate, as discussed above. Although the deposits in UNITE (Kõljalg *et al.*, 2005) were not as comprehensive as Genbank (Altschul *et al*., 1997), and occasionally only genus level identity of specimens could be confirmed, the database is more reliable as material is verified before it is deposited.

As a result of interspecies variation within the ITS region (Horton, 2002), it may not resolve genus level taxonomy. For example, the genus phylogeny of *Amanita* is currently being re-examined based on the large subunit (LSU 28S), a more conserved DNA region. Combining sequence data of informative regions from a mosaic of conserved and non- conserved DNA regions, could better resolve all levels (from ancestral to terminal taxa) of a tree, producing a more robust phylogeny (Huelsenbeck *et al.*, 1996). This has not yet been conducted and it would be interesting to compare the resultant phylogeny with the current taxonomy.

7.2 Above ground vs. below ground ECM species diversity

Jonsson *et al*., (1999), report a remarkable percentage of non-sporulating ECM fungi in community studies associated with *Pinus sylvestris*. This study concluded that sporocarp-forming fungi formed less than 30% of the ECM fungal species accounted for in the soil. This means that over 70% of the ECM fungal diversity would go undetected by purely sporocarp observation alone, emphasising the importance of performing the below ground ECM diversity studies. It is now recognised that ECM fungal community studies should inherently involve below ground assessment Natarajan *et al.*, (1992) correlated the presence and abundance of above ground and below ground

ECM fungal species in *P. patula* stands. This study correlated the dominant sporocarp species with the dominant ECM fungal species on root tips, namely *Scleroderma citrinum*. The diversity above ground was correlated to some below ground species, but the species abundance between the two could not be compared due to poor sporocarp data. Additionally, up to 12 ECM morphotypes in the Brooklands study site and 24 ECM morphotypes in the Driekop study site were not identified morphologically or molecularly. This suggests a potential for even higher species richness than documented in this research study.

The results of this research emphasise the importance of sampling, not only the above ground but also the importance of further ECM investigation in the rhizosphere at root tip level. This was especially highlighted in the younger study site, Driekop, where sporocarp formation was hardly observed (Table 7.3), but below ground diversity was high. In the mature study site, Brooklands, the species richness was comparable (Table 7.3) between above and below ground, and although the total composition could not be compared, links between key species found above and below ground were made (Table 7.4).

Table 7.3 Comparison of the number of species found above and below ground in both the Brooklands and Driekop study sites.

Brooklands		**Driekop**	
Above ground species	**Below ground (morphotypes)**	**Above ground species**	**Below ground (morphotypes)**
20	22	1	28

The ECM diversity experienced below ground was larger than that observed above ground in the younger Driekop study site, while in the Brooklands study site, the difference between below and above ground diversity was much smaller. Whether the species of ECM fungi observed below ground correlated to the species above ground is questionable, however, identification of the most dominant ECM root tip morphotypes did correspond to sporocarp data collected (Table 7.4). Species of sporocarp-forming ECM fungi were collected

(Table5.1) and compared to the ECM root tips that were identified by both morphological and molecular techniques.

Table 7.4 Potential corresponding species above and below ground found in the Brooklands study site.

Sporocarp species	**ECM root tip species (*)**
Scleroderma citrinum	*Scleroderma citrinum* = 'White'
Amanita sp.	*Amanita* sp. = 'Orange'
Amanita excelsa	*Amanita excelsa* = 'BSY', 'white', 'Grey/pink and 'Yellow-orange'
Xerocomus badius	*Xerocomus badius* = 'Beige', 'BSY 2&6' and 'Dull orange'
Thelephora terrestris	*Thelephora terrestris* = 'Yelsil' 'Black' and 'partial white'

* Both morphological and molecular techniques were used to identify the ECM root tips in the above table.

7.3 Succession of ECM fungi:

The fungal species identified above and below ground were categorised into early or late stage species (Table 7.5) according to published literature (Smith *et al*., 2002, Jumpponen *et al*., 2002, Bigg, 2000, Lilleskov and Bruns, 2003), however, it is worth noting that between the studies, the definition of old and young plant stands differ to those in South Africa, where a 20-25 year stand is already considered mature, where as in the discussed literature this is considered a young to intermediate age. Tree growth is rapid, reaching approximately 25 metres high (Dames, 1996) in this time, already providing a substantial litter layer (Dames *et al*., 1999) and a reduced pH. It is also worth noting that the plantations examined in this study are both second rotation *Pinus patula* and due to the limits on land expansion of the forestry industry many stands are now in third rotation with very few first rotation stands.

Table 7.5 Early and late stage species derived from the Brooklands and Driekop study site.

Species found in Brooklands (mature)	**Early/Late stage fungi**	**Species found in Driekop (young)**	**Early/Late stage fungi**
Amanita excelsa	Late (1)		
A. muscaria	Late (1)		
A. rubescens	Late (1)		
Boletus cf. *edulis*	Late		
Clavulina cristata	Potentially Late (1)	*Clavulina cristata*	Potentially late (1)
Inocybe sp1	Potentially Late (1)	*Inocybe* cf. *griseolilacina*	Potentially Late stage (1)
Inocybe sp2	Potentially Late (1)		
Lactarius hepaticus	Late (1,3)		
L. deliciosus/ quieticolor	Late (1,3)		
Rhizopogon roseolus	Potentially Early (4)		
Scleroderma citrinum	Unknown	*Scleroderma* sp.	Unknown
Suillus granulatus	Late (3) Early (2)	*Suilloid* sp.	Late (3) Early (2)
S. luteus	Late (3) Early (2)		
S. salmonicolor	Late (3) Early (2)		
Tricholoma fracticum	Potentially Late (1)		
Thelephora terrestris	Early (1)	*Thelephora terrestris*	Early
Tomentella sp1	Potentially Late (4)	*Tomentella* sp2	Potentially Late (4)
Xerocomus badius	Late (1)		
X. pruinatus	Late (1)		
Albatrellus ovinus	Unknown		
Hymenoscyphus ericae	Early		

1) Peter *et al*., (2001), 2) Bigg (2000), 3) Jumpponen *et al*., (2002), 4) Lilleskov and Bruns, (2002)

The successional status of many ECM fungal species is not known and may be multi-stage species in different environments (Smith *et al*., 2002). The species composition of the Brooklands study site consisted of predominantly late stage fungi and a few persistent early stage fungi (Table 7.5). This finding is in accordance with the research conducted at the forefront of a glacial

recession by Jumpponen *et al*., (2002), who found that late stage fungi such as *Cortinarius mutabilis*, *Lactarius uvidus* var. *montanus* and *Suillus cavipes*, joined the early stage fungi, such as *Cortinarius decipiens*, *C. tenebricus*, *Inocybe lacera* and *Laccaria* cf. *montana*. Contrasting this, the young Driekop study site contained a combination of early and late stage fungi (Table 7.5), demonstrating no apparent pattern of succession. Natarajan *et al*., (1992) discussed successional ECM fungal species in *P. patula* stands in India, the findings of which are contradicted in this study. It was found that sporocarps of *Thelephora terrestris* were only found in young plantations (3-7yrs), but during sampling events conducted during this research, frequent observations of bracket sporocarps of *T. terrestris* were made in the 20-22 year old Brooklands study site and only one sporocarp was found in the 3-5 year old Driekop stand (Chapter 3). Termorshuizen (1991) suggested that successional progression from early to late stage fungi applies only to the first 10-20 years of first rotation stands. As both of these study sites are second rotation *Pinus* stands, the natural successional progression may be masked, as persistent propagules from previous rotations may be maintained in the rhizosphere and colonise the roots of successive plantations. Furthermore, expansive plantation areas in South Africa consist of a mosaic of young and old stands, where dispersal of late stage ECM fungal species from old to young stands is highly likely.

7.4 Effects of fertilisers and management strategies

No fertiliser effects on ECM sporocarp production or on the community diversity of ECM root tips were observed between N, P, NP or control plots in either the Brooklands or the Driekop study sites. What this data may suggest, particularly in the Brooklands study site, is that any nutrient effect is short-lived and that the ECM fungal community has stabilised within 4-5 years of treatment. In the Driekop study site that was fertilised annually, fertiliser effects should have been more apparent, however, the inherent variation was large and could not be used for any meaningful analysis. A significant shift of some ECM root tip morphotypes two and one morphotypes in the Brooklands and Driekop study site, respectively was observed. These results are not conclusive to infer community shifts in response to sampling date or nutrient

application, however should this pattern be demonstrated for other ECM morphotypes a more meaningful argument could be attained. The experimental sampling strategy of ECM root tip data is profoundly limited, as indicated by Taylor (2002). Sampling techniques and efforts have also come under scrutiny as ECM root tips tend to aggregate around nutrient and mineral 'hot-spots', and become localised and non-randomly distributed (Taylor, 2002). Ideally, one could extract an entire forest plantation, but limits of time, finance, and labour (not to mention practicality) mean that the present sampling methods will have to suffice until an improved method is devised. Molecular techniques using DNA extraction, PCR and RFLP analysis have proved useful in this respect (Jonsson *et al.,* 1999). These techniques allow for large samples to be processed relatively quickly and accurately. ITS-RFLP (Jonsson *et al*., 1999) and T-RFLP (Burke *et al*., 2006), as well as sequencing, of total root material, have been utilised to accurately investigate the relative abundance of ECM root tip species. These molecular techniques would prove useful in further studies of this nature in South Africa.

ECM fungal colonisation of roots in studies conducted in Europe (Taylor *et al*., 2000) report typically high percentages, greater that 95%, of successful root colonisation. This study demonstrates a relatively low colonisation of approximately 50-60%. Similarly, low levels of colonisation were reported in a study conducted by Dames *et al*., (2002) where low fractional colonisation of approximately 50% of the roots of *P. patula* was observed. These observations could be attributed to the lack of suitable ECM fungal species in South Africa resulting in empty niches that are normally filled by late stage fungi. The low ECM fungal colonisation rate may also be a result of management practices that decrease the colonisation efficiency of fungal propagules, such as pesticide, fungicide and fertiliser application. Another factor that should not be over-looked is the potential for ectendomycorrhizal (ECTENDO) associations to play a major contributing role in the colonisation of *Pinus* root tips. Torres and Honrubia (1997) demonstrated that 50-90% of *Pinus halepensis* roots were colonised by ectendomycorrhizas. The authors also note that the ECTENDO associations formed by the E-strain fungi are aggressive early stage colonisers that are particularly prevalent in disturbed

sites. Ectendomycorrhizas were not included in this study and only roots exhibiting an ECM mantle sheath were examined. Ectendomycorrhizal roots are not as conspicuous as the mantle is either rudimentary or absent (Piché *et al*., 1986) and would have been over-looked. Given the nature of the study sites examined in this study (exotic, managed plantations) it would be interesting to determine the extent of ECTENDO colonisation.

Assuming that the non-mycorrhizal root tips in this study were not colonised by inconspicuous ECTENDO fungal associates, the issue of potential empty niches arises. Although some ECM fungal species are naturalised in the forestry areas of South Africa, they are not indigenous and depend on transferral from native areas of origin. The management of the local forestry industry could therefore focus on 'missing' ECM fungal diversity that fulfils a successional progression. This may involve the importation of new, exotic ECM fungal species, which will be met with legislative complications. Additionally, ECM fungal inoculation of seedlings in the nursery with selected ECM fungal strains may improve the establishment of tree saplings at out-planting. The fractional colonisation and low diversity also implies that the full potential of ECM fungi to break down a variation of inorganic and organic nutrients otherwise locked up in the litter layer is not being realised. This should be considered when the issue of second rotation decline of tree growth is addressed. Research into this phenomenon in Australia has indicated that a decline in tree growth and/or tree quality was experienced in tree stands in second and third rotation (Evans, 2000). It was concluded that the major causal factor was slash burning after harvesting and that the organic state of the soil was of crucial importance (Evans, 2000) as a long-term nutrient source. Baar (1996) also demonstrated a decline in the ECM fungal species richness and diversity in secondary stands of *Pinus sylvestris*, which may result in changes in plant nutrition. For this reason it is important to consider the role of suitable mycorrhizal fungal associates, which by the very nature of the symbiotic association make access to these nutrients by the tree host possible.

CONCLUSION

The main objective of this study was to identify the ectomycorrhizal fungal species present in association with *Pinus* plantations in South Africa. This was achieved by collecting and identifying sporocarp and ECM root tip material. Morphological and molecular techniques were employed to meet this objective. It was evident from the data that a larger species diversity than was expected is present and it is suggested that further investigations are required of both above and below ground diversity over extended periods of time and over larger areas of plantation.

ECM root tip fungi have been identified and characterised. Results from both morphological and molecular analyses have contributed not only to the list of ECM fungal species occurring in South Africa, but also to the list of ECM fungal species associating with *Pinus patula*. In future studies, characterisation of ECM roots and the identification thereof should involve the isolation and culturing of sporocarp material and reinoculation onto host plants *in vivo* in order to confirm fungal identification and determine the most stable characteristics of the association. There are inherent problems that may hinder this research, for example not all sporocarp material will grow in culture or are maintained as viable colonisers. Failure of isolates to colonise roots and form ECM should be kept in mind, as well as the difference of colonisation potential between monokaryotic and dikaryotic mycelia (Smith and Read, 1997).

The secondary objective was to elucidate any factors affecting the abundance and diversity of ECM fungi with particular emphasis on fertiliser regimes. Effects of fertiliser additions, in terms of species abundance responses or species composition, were not determined because the variation in the data was too large to yield statistically significant results. In order to understand the contribution and behaviour of ECM fungi to forest ecosystems, further fungal community studies are essential, especially in the light of the increased acid rain pollution, nutrient addition and soil degradation. Important soil chemistry data such as soil moisture, pH and available N and P should be included in

studies assessing ECM fungal diversity in response to nutrient addition. Unfortunately, this data was unavailable for analysis and therefore not discussed. Species composition should, in future, be analysed using additional molecular methods of e.g. Restriction Fragment Length Polymorphism (RFLP), as this method allows a larger sample size that better represents communities. The analysis is faster and more reliable than morphotyping. The molecular analysis of ECM root tips may also then be extended to the extraction of DNA in soil, which would then include ECM fungal mycelia that are perhaps less dominant or go undetected.

The sub-tropical climate of South Africa and the current legislation restricting land used for forestry calls for a unique strategy for plantation management. The Water Act (36 of 1998) and land redistribution claims leave the industry with challenges to sustain economic viability. Issues of sustainable resource management will need to be addressed in terms of increased plant growth and maintenance of soil health. ECM fungal research should be an important factor pertaining to both issues in the future, especially regarding host plant nutrition and health. Identification of key ECM species on both morphological and molecular levels, within plantations serves as an important basis for the involvement of ECM fungi in management strategies within the forestry industry.

An important consideration to bear in mind is the 'non-indigenous' factor. As there are no known indigenous ECM fungi that associate with *P. patula*, it may be important to assess whether the current species freely available to colonise these hosts in the field are not only environmentally adapted, but are also functionally desirable within the symbiosis. Fungal inoculation with suitably adapted ECM species that have a set of desirable functional traits, such as the ability to use organic N and P or potential biocontrol of fungal pathogens, in particular *Fusarium* and *Heterobasidion*, should be researched. Possible importation of new species and strains may be considered, but will be met with legislative difficulty.

The present research has been instrumental in further developing the field of Ectomycorrhizal research in South Africa. The dearth of local literature has hampered the continued expansion of functional understanding of ECM symbioses in the country. ECM research based on ECM root analysis, conducted by Dames *et al*., (1999) and Carlson (1994), on characterization and fungal community diversity respectively, are noted as pioneering authors in South Africa. Other publications in this area have been limited. This is surprising as ECM fungi form such an intricate and vital element in the nutrient ecology of plant hosts (Smith and Read, 1997). Using both morphological and molecular techniques, this study has contributed towards a body of information, which is particularly relevant in a South African context, and produces a platform upon which further research on all economically important forestry species may be developed and has the potential to significantly affect management strategies.

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Appendices

Appendix 1 Voucher specimens and corresponding numbers of herbarium deposits.

Table A Voucher numbers for all the specimens deposited in the Selmar Schonland herbarium, Grahamstown.

Species list	Voucher number*	Species	Voucher number*
1. Amanita		***6. Rhizopogon***	
A. excelsa Fr.	1	*R. roseolus* (Corda) TM Fries	22
A. muscaria (L) Hook.	3	***7. Russula***	
A. rubescens (Pers.) Gray	2	*Russula* (7 specimens, consisting of 2 possible morphotypes)	***23, 24, 25, 26, 27, 28, 29***
2. Boletus		***8. Scleroderma***	
*B.*cf. *edulis* Bull.	11	*S. citrinum* Pers.	30
B. aestivalis (Paulet) Fr.	12	***9. Suillus***	
3. Clavulina		*S. granulatus* (L.) Snell	31
C. cf. *cristata* (L.) J. Schröt.	14	*S. luteus* (L.) Gray	32
C. cf. *cinerea* (Bull.) J. Schröt.	13	*S. salmonicolor* (Peck) Snell ex Slipp & Snell	33
4. Inocybe		***10.Tricholoma***	
I. cf. *griseolilacina* J.E. Lange	15	*T. albobrunneun* (Pers.) Fr.	34
Inocybe sp. 2	16	***11. Thelephora***	
Inocybe sp. 3	17	*T. terrestris* Ehrh.	35
5. Lactarius		***12. Tomentella***	
L. hepaticus Plowr.	18	*Tomentella sp1*	36
L. cf. *semisangifluus* R. Heim & Leclair	19	***13. Xerocomus***	
L. deliciosus (L.) Fr.	20	*X. badius* Pers.	37
L. cf. *hemicyaneus* Romagn	21	*X. chrysenteron* Bull.	38

- all deposits can be found under the collectors name 'Greer Hawley' followed by the corresponding number represented in this table.

Appendix 2 Protocols for DNA extraction, PCR amplification and sequencing of the ITS region.
(Modified from Zeze *et al.*, 1998)

2. 1 DNA extraction from sporocarp material

- Take a small piece of fruit body (preferably from the hymenium layer) and place it in a 1.5ml eppendorf tube
- Add 200μl 2% Cetyl Trimethyl Ammonium Bromide (CTAB) to each tube. Homogenise the sample with a micro pestle, then add another 400μl 2%CTAB and homogenise further
- Vortex and heat the sample to 65°C for 30-60 min
- Centrifuge at 13 000rpm for 10 min
- Transfer the supernatant (500μl) to a new 1.5ml eppendorf tube
- Precipitate the DNA by adding 1.5 volumes (750μl) of cold isopropanol, vortex and let sample sit in ice (or -20°C freezer) for 30 minutes. At this stage samples may be stored in the freezer. Better results were achieved by storage in -20°C freezer overnight
- Centrifuge the samples for 30 min (13 000 rpm)
- Pore out supernatant carefully
- Wash the pellet by adding approximately 200μl 70 cold Ethanol
- Centrifuge at half speed for 5 min
- Pore of the supernatant and let the pellet dry
- Re-suspend the pellet in 50μl distilled water and store in freezer (-20°C) until required

2.2 DNA extraction from ECM root tips

- Extract a single root tip using sterile technique (forceps dipped in 95% alcohol and flamed) of a mycorrhizal root and place it in a 1.5ml eppendorf tube. If the root was stored in 50% ethanol, leave the tube open for alcohol to evaporate
- Add 50μl CTAB 2% to each tube. Homogenise the sample with a micro pestle, and then add another 550μl CTAB 2%. To the homogenate add 7μl proteinase K (Sigma-Aldrich) and leave at room temperature for 10 min

- Vortex and heat the sample to 65°C for 30-60 min
- Add 250µl chloroform and gently mix
- Centrifuge at 13 000rpm for 15 min
- Transfer the upper phases to a new tube
- Precipitate the DNA by adding 1.5 volumes (600µl) of cold isopropanol, vortex and let sample sit in ice (or -20°C freezer) for 30 min. At this stage samples can be stored in the freezer
- Centrifuge the samples for 30 min (13 000rpm)
- Pore out supernatant carefully
- Wash the pellet by adding approximately 200µl 70 cold Ethanol
- Centrifuge at half speed for 5 min
- Pore of the supernatant and let the pellet dry
- Resuspend the pellet in 50µl distilled water and store in the freezer (-20°C) until required

2.3 PCR reactions

2.3.1 PCR amplification of sporocarp material

Table B Reaction mixture in volume %

Compound	**Concentration**	**Percentage volume in the reaction mixture (%)**
Water	Autoclaved milliQ	21
10Xreaction buffer	HF buffer with $MgCl_2$	10
dNTP	2mM of each base	10
$MgCl_2$	25mM	2
ITS1F	10µM	2
ITS4B	10µM	2
Red *Taq* polymerase (Sigma-Aldrich)	3.5units/µl	3
DNA template	Try different dilutions (used 100x dilution	50

Before making the reaction mixture, the number of samples and the required reaction volume, are to be determined. If a PCR program is to be tested or if a

PCR product is used to detect successful DNA extraction and amplification, 10μl total volume reaction is ample. If the PCR product is to be used for future sequencing, the volume has to be made up to 50μl.

Thaw out solutions from the freezer. The following is work is carried out on ice:

- A Master Mix (MM) is prepared by adding all solutions in Table 2. (except for the DNA template) for all reactions into an eppendorf tube. Add the solutions in the order that they appear in Table 2. The *Taq* polymerase should not be left of the ice, and returned to the freezer when it has been used
- Add calculated volume of MM to each reaction tube, followed by the DNA template
- Seal the tubes properly and place in the PCR machine
- The temperature and times of the program used:

First denaturing at	94°C for 60s
Then 35 cycles of	94°C for 15s
	50°C for 30s
	72°C for 60s

After the last completed the cycle followed an additional 7 min at 72°C

- PCR products can be stored for a considerable time in the fridge

2.3.2 PCR amplification of ECM root tips

Table C Reaction mixture in volume %

Compound	**Concentration**	**Percentage volume in the reaction mixture (%)**
Water	Autoclaved milliQ	41,75
10Xreaction buffer	HF buffer with $MgCl_2$	10
dNTP	2mM of each base	10
$MgCl_2$	25mM	6,5
ITS1F	10μM	3

Compound	Concentration	Percentage volume in the reaction mixture (%)
ITS4	10μM	3
High Fidelity polymerase (Invitrogen)	3.5units/μl	0,75
DNA template	Try different dilutions (used 5x dilution)	25

The procedure followed is the same as with sporocarp. A number of dilutions were tried and the 5x dilution showed the best results. All extractions were first amplified using a 10μl total volume, and if successful, another 50μl PCR reaction was run. The cycling programme was the same as for the sporocarp DNA.

2.4 Quantification of DNA

For a 1% agarose gel in a big tray (takes 48 samples and 1kb ladder where needed), 200ml TBE buffer is added to 2g of LE-agarose (Sigma-Aldrich) and dissolved by boiling in the microwave oven. It is important that all agarose lentils were fully dissolved. Leave to cool down to 50-60°C. Seal the gel tray with tape and apply appropriate combs. Pour gel slowly into the tray avoiding air bubbles if possible. Spray with 70% alcohol to get rid of any bubbles.

The Red Taq polymerase (Sigma) that is used to amplify the fruiting bodies already contains a loading buffer. The mycorrhizal samples where High Fidelity polymerase is used, 1-2μl of loading buffer needs to be mixed with 5-10μl of the PCR product. One sample was added into each lane and 1kb ladder in each row of combs (3μl).

For DNA separation, place the gel in the electrophoresis box. Fill with TBE-buffer so that it just covers the gel surface. Connect the electrodes and run the gel at 130-150V for 1-1,5 hours. To visualise the DNA in the gel, it is stained with Ethidium bromide (EtBr). This chemical is mutagenic. **Wear gloves and lab coat!** Move the gel into an EtBr tray and leave in an EtBr bath (one drop of concentrated EtBr into 1 litre of water, each bath may be used for

4 big gels) and let it soak for 15 mins. Rinse the gel by moving it into water bath and let it soak for another 15mins. Visualise the DNA with the UV in the geldoc. Do not contaminate the computer and discard the gel.

2.5 Sequencing

2.5.1 Sequence PCR reaction

- Run a PCR to get 50μl PCR product of each sample that you want to sequence. Use 5μl for detection and 45μl for purification
- Use the QUIGEN cleaning kit to purify the DNA

ABI Prism sequence reaction (Applied Biosystems)

Second PCR/ extension, amplification performed with only one primer, therefore a sample treated with both primers means that the sample size doubles.

Table D of reaction solutions (Applied Biosystems, Original and Version 2.0, Protocol, 2000)

Reagent	Quantity
Terminator Ready Reaction Mix	4μl
DNA template (PCR product)	3μl
Primer	0.16μl
Deionised water	2.84
Total volume	10μl

Vortex to mix. The Thermal cycles are as following:

Repeat the following for 25 cycles:

96°C for 10 seconds

Rapid thermal ramp to 50°C

50°C for 5 seconds

Rapid thermal to 60°C

60°C for 4 minutes

2.5.2 Precipitation

(ABI Prism handbook)

- Prepare a 0.5ml micro centrifuge tube containing:
  1μl of 3M sodium acetate (NaOAc), pH 4.6
  25μl of 95% ethanol (EtOH)

- Pipette the entire contents of each extension reaction into a tube of sodium acetate/ethanol mixture. Mix thoroughly (10μl)
- Vortex and leave at room temperature for 15 minutes to precipitate the extension products (no shorter than 15 minutes and no longer than 24 hours)
- Spin in centrifuge for 20 min at maximum speed
- Carefully aspirate the supernatant with a pipette tip and discard (must be removed completely)
- Rinse the pellet with 125μl of 70% ethanol
- Vortex briefly
- Spin for 5 minutes in a micro centrifuge at maximum speed. Again carefully aspirate the supernatant and discard
- Dry pellet in heating block or thermal cycler at 90°C for 1 minute (do not over dry)

2.5.3 Preparation for sequencer

Add 25μl TSR, and vortex briefly. Heat the samples (on a heating block) to 96°C for 2 minutes, and cool on ice immediately. Briefly spin, cut the tube lids off and put on septa.

Appendix 3 Sequences, sequence alignments and synonyms

Amanita group comparison

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CLUSTAL W (1.83) multiple sequence alignment


BSY5                ---CAAGGTTTCCTGTAGGTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 46
BSY                 ------------------------------------CA-TTACTG-----AAC----GA 13
BSY3                ----CAAGGTTTCCGTAGGTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 45
BSY1                ------AGGTTTCCGTAGGTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 43
Greypink            ------------------GTGAACCTGCGGAA-GGATCAATTACTG-----AAC----GA 32
Yelorange           ------------------GTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 31
Amanitarubescens1   ------AGGTTTCCGTAGGTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 43
Amanitarubescens2   ------AGGTTTCCGTAGGTGAACCTGCGGAA-GGATCA-TTACTG-----AAC----GA 43
BSY4                ------------------GTGAACCTGCGGAA-GGATCA-TTACTGGG---AAC----GA 33
Amanitaexcelsa2     GTCGCCAGGTTTCCGTAGGTGAACCTGCGGAAAGGATCA-TTACTG-----AAC----GA 50
Amanitaexcelsa1     --GCACAGGTTTCCGTAGGTGAACCTGCGGAG--GATCA-TTACTG-----AAC----GA 46
Amanitamuscaria     ------AGGTTTCCGTAGGTGAACCTGCGGAA-GGATCA-TTATTGAAAGAAACCTCAGG 52
WhiteA              ------------------------------------------------------------


BSY5                AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 103
BSY                 AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 70
BSY3                AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 102
BSY1                AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 100
Greypink            AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 89
Yelorange           AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 88
Amanitarubescens1   AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 100
Amanitarubescens2   AATGGGTGGCAAGGCTGTCGCTGGCTTAAATGAGCATGTGCAC---GTCTTTTTGTCGCT 100
BSY4                AATGGGTGGCAAGGCTGTAGCTGGCTCGAAGGAGCATGTGCAC---GTCTTTT-GCTGCT 89
Amanitaexcelsa2     AATGGGTGGCAAGGCTGTAGCTGGCTCGAAGGAGCATGTGCAC---GTCTTTT-GCTGCT 106
Amanitaexcelsa1     A-TGGGTGGCAAGGCTGTAGCTGGCTCGAAGGAGCATGTGCAC---GTCTTTT-GCTGCT 101
Amanitamuscaria     CAGGGGGGAGATGGTTGTAGCTGGCCTCTAGGGGCATGTGCACACTGTGTCTCTCTTGCT 112
WhiteA              ------------------------------------------------------------


BSY5                TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 162
BSY                 TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 129
BSY3                TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 161
BSY1                TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 159
Greypink            TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 148
Yelorange           TACTTCATTCTCNTCTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 148
Amanitarubescens1   TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 159
Amanitarubescens2   TACTTCATTCTCT-CTTCCACCTGTGCACTCTTTGTAGACACTTGGGATGTGAGAGAGGG 159
BSY4                TGCTTCATTCTCT-TTTCCACCTGTGCACTCTTTGTAGACACTCGGGATG-GGGAGAGAG 147
Amanitaexcelsa2     TGCTTCATTCTCT-TTTCCACCTGTGCACTCTTTGTAGACACTCGGGATG-GGGAGAGAG 164
Amanitaexcelsa1     TGCTTCATTCTCT--TTTCCCCTGTGCACTCTTTGTAGACACTCGGGATG-GGGAGAGAG 158
Amanitamuscaria     TGTTTC-TTCATTCTCTCCACTTGTGCACTGCTTGTAGGCAGCC---------------- 155
WhiteA              ------------------------------------------------------------


BSY5                GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 220
BSY                 GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 187
BSY3                GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 219
BSY1                GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 217
Greypink            GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 206
Yelorange           GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 206
Amanitarubescens1   GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 217
Amanitarubescens2   GTTGGCATTGATTTGTCGGACT-TCTCTTGATATTGAAAA-TCTGGGTGTTTATGTATTT 217
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BSY4                GTTGGCATTGATTTGTTGACCCCTCTCTTGATATTGAAAAGTCTGGGTGTTTATGTATTT 207
Amanitaexcelsa2     GTTGGCATTGATTTGTTGACCCCTCTCTTGATATTGAAAAGTCTGGGTGTTTATGTATTT 224
Amanitaexcelsa1     GTTGGCATTGATTTGTTGCCCC-TCTCTTGATATTGAAAAGTCTGGGTGTTTATGTATT- 216
Amanitamuscaria     -CTGGCATTGTT------------------------------CAGGCTGTCTATG-ATTT 183
WhiteA              ----------------------------------------GGTCAAGTTTATCTATTG 18
                                                             ** ***  ***

BSY5                TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 277
BSY                 TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 244
BSY3                TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 276
BSY1                TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 274
Greypink            TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 263
Yelorange           TTT-GACATACAC-GGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 263
Amanitarubescens1   TTT-GACATACACCGGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 275
Amanitarubescens2   TTT-GACATACACCGGTTGAATGTC-TATAGAATGAAATTATAGGCTCTTGTCAGCCTTT 275
BSY4                TTTTGACATACAC-AGTTGAATGTC-TATAGAATGAAAT-GTAGGCTTTTGTCAGCCTTT 264
Amanitaexcelsa2     TTTTGACATACAC-AGTTGAATGTC-TATAGAATGAAAT-GTAGGCTTTTGTCAGCCTTT 281
Amanitaexcelsa1     ----GACATACAC-AGT--GATGTC-TATAGA-TGAA--TGTAG---CTTGTCAGCCTT- 261
Amanitamuscaria     TATTTACATACAT-GAACAATTGTTGTACAGAATGTGATTAAA----------------- 225
WhiteA              TCC-AATAAAGAC-AGTTAGATGGC--AGAGGATCATG----AAGCTTCTCCTGGCAT-- 68
                         * * * *         **    * **  *        *

BSY5                AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 336
BSY                 AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 303
BSY3                AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 335
BSY1                AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 333
Greypink            AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 322
Yelorange           AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 322
Amanitarubescens1   AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 334
Amanitarubescens2   AAATGATAAAATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 334
BSY4                AAATGATAAAATACAACTTTTAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 323
Amanitaexcelsa2     AAATGATAAAATACAACTTTTAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 340
Amanitaexcelsa1     -AATGATAATACAC----TTACAC----GATCTC-T--GCTCTCGCATCGATGAG--ACG 307
Amanitamuscaria     -AATAGTA--ATACAACTTTCAACAACGGATCTC-TTGGCTCTCGCATCGATGAAGAACG 281
WhiteA              AGATAGTCTATCACACCAATAGAGTGCAAAGCTCCTCAACTAATGCTTTTCAGAAGAGCC 128
                      **  *     **     *  *      * *** *   **   ** *    **    *

BSY5                CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCAT--CGAATCTTT 393
BSY                 CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 359
BSY3                CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCAT--CGAATCTTT 392
BSY1                CAAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 390
Greypink            CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 378
Yelorange           CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 378
Amanitarubescens1   CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCAT--CGAATCTTT 391
Amanitarubescens2   CAGCCGAAATGCGATAAGTAATGTGAATTGCAGAAT-CAAGTGAATCAT--CGAATCTTT 391
BSY4                CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 379
Amanitaexcelsa2     CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCAT--CGAATCTTT 397
Amanitaexcelsa1     CA-GCGAA-TGCGATAGT--ATGTGA--TGCAGA--TCA-GTGA-TCAT--CGA---TCT 352
Amanitamuscaria     CA-GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCAT--CGAATCTTT 337
WhiteA              CA-CCACTTTATCAGCGGCCG-GCAACTCCCAATGTCCAAAAATCCCATGCCAAAAATGC 186
                    **  *    *   *        *  *    **     **       ***  * *   *

BSY5                GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 450
BSY                 GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 416
BSY3                GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 449
BSY1                GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 447
Greypink            GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 435
Yelorange           GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 435
Amanitarubescens1   GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 448
Amanitarubescens2   GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 448
BSY4                GAACGCATCTTGCGGCTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 437
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Amanitaexcelsa2     GAACGCATCTTGCGGCTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 455
Amanitaexcelsa1     TGACGCATCT-GCG----CT--CTGTATC---GAGAGCATGCT--GTTGAGTGTCAT--- 397
Amanitamuscaria     GAACGCATCTTGCG-CTCCT--TGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA 394
WhiteA              ACAAGCTTTTTGAGATATTTAATGACACTCAAACAGGCATGCTCCTCGGAATACCAAGGA 246
                      * ** * * * *     *      *         ******      ** *  **

BSY5                ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 509
BSY                 ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 475
BSY3                ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 508
BSY1                ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 506
Greypink            ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 494
Yelorange           ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 494
Amanitarubescens1   ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 507
Amanitarubescens2   ATATC-TCAAAACTCTTGTGCTTTTGTTGGCATAGGACTTTTGGACATTGGGAGTTGCCG 507
BSY4                ATATC-TCAAAAAGCTTGTGCATTT-TTGGCATGGGATTTTTGGACATTGGGAGTTGCCG 495
Amanitaexcelsa2     ATATC-TCAAAAAGCTTGTGCATTT-TTGGCATGGGATTTTTGGACATTGGGAGTTGCCG 513
Amanitaexcelsa1     ATATC-TCAAAGC---TGTGCATTT---GGCATGA------TTGACAT---GAGT----G 437
Amanitamuscaria     ATTCTGTCAAAAC----ATGCACTTG--AGTGTG----TTTTGGATTGTGGGAGT----G 440
WhiteA              GCCGC-AAGATGCGTTCAAAGATTCGATGATTCA------CTGAATTCTGCAATTCAC-- 297
                             *            *                *  *       * *

BSY5                GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 556
BSY                 GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 522
BSY3                GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 555
BSY1                GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 553
Greypink            GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 541
Yelorange           GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 541
Amanitarubescens1   GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 554
Amanitarubescens2   GCTGCTGGTAAAGT---AGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 554
BSY4                GCCGCTGATAAAGT---GGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 542
Amanitaexcelsa2     GCCGCTGATAAAGT---GGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----- 560
Amanitaexcelsa1     CGCGCTGATAA-GT---GGTGG----CTCTCTGAAGCAT-AGT-------GAGAG----- 476
Amanitamuscaria     TCTGCTGGCTTTATG--AGCCAGCTCTCCTGAAATACATTAGCTTTGGGGGGGAGGTGCC 498
WhiteA              ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCC------AAGAG----- 346
                        **       *             *    *  *   **          ***

BSY5                -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 600
BSY                 -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 566
BSY3                -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 599
BSY1                -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 597
Greypink            -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 585
Yelorange           -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 585
Amanitarubescens1   -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 598
Amanitarubescens2   -------CTTTGCACTCTTATTGGTGTGATAAAC---------TATCTATGCCAAGAGAT 598
BSY4                -------CTTTGCACTCT-ATTGGTGTGATAGAC---------TATCTATGCCAGGAGAA 585
Amanitaexcelsa2     -------CTTTGCACTCT-ATTGGTGTGATAGAC---------TATCTATGCCAGGAGAA 603
Amanitaexcelsa1     -------CTT-GCACTCT-ATTGGTGTGATAGAC---------TATCTATGC--AGAGAT 516
Amanitamuscaria     AAGTCACTTCTGCCTTTCCATTGGTGTGATAGATGAATTAACTTATCTACGCCAGGAAAG 558
WhiteA              -------ATCCGT-TGTTAAAAGTTGTATTTTAT---------CATTTAAGGCTGACAAA 389
                            *  *       *  * ***  *  *           ** ** *       *

BSY5                ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 648
BSY                 ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 614
BSY3                ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 647
BSY1                ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 645
Greypink            ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 633
Yelorange           ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 633
Amanitarubescens1   ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 646
Amanitarubescens2   ---GCTTCA--TGATCCTCT-GCCGTCTAACTGT---CTTTATAAGACA---ATATGATA 646
BSY4                ---GCTTCA--TGATCCTCT-GCCATCTAACTGT---CTTTATTGGACA---ATATGATA 633
Amanitaexcelsa2     ---GCTTCA--TGATCCTCT-GCCATCTAACTGT---CTTTATTGGACA---ATATGATA 651
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Amanitaexcelsa1     ---GCT-CA--TGATC-TCT-GCCATCTA-CTGT---CTT---ATGACA----TATGATA 557
Amanitamuscaria     CAGGCTTCAGGTGATGCACT-GTGATCTCTCTCTGCTCTCTAATTGACATTTGTCTGATA 617
WhiteA              -AGCCTACATTTCATTCTATAGACATTCAACTGTGTATGTCAAAAAATC-----ATAACA 443
                        ** **  * **    * *   *    ** *           *        * * *

BSY5                AACTTGACCTCAAATCAGGTAGGACTACCC----------------------------- 678
BSY                 AACTTGACCT------------------------------------------------- 624
BSY3                AACTTGACCTCAAATCAGGTAGGACTACCCG---------------------------- 678
BSY1                AACTTGACCTCAAATCAGGTAGGACTACCC----------------------------- 675
Greypink            AACTTGACCTC------------------------------------------------ 644
Yelorange           AACTTGACCTC------------------------------------------------ 644
Amanitarubescens1   AACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG 706
Amanitarubescens2   AACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG 706
BSY4                AACTTGACCTC------------------------------------------------ 644
Amanitaexcelsa2     AACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG 711
Amanitaexcelsa1     A--CTGACCTCAA-TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGA--G 612
Amanitamuscaria     A-CTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG 676
WhiteA              C-CCAGACTTTTCAATATCAGAGAGGGGTCACAAATCA---------------------- 480
                         *** *

BSY5                ------------------------------------------------------------
BSY                 ------------------------------------------------------------
BSY3                ------------------------------------------------------------
BSY1                ------------------------------------------------------------
Greypink            ------------------------------------------------------------
Yelorange           ------------------------------------------------------------
Amanitarubescens1   AAAAGAAACTAACAAGGATTCCCTTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTT 766
Amanitarubescens2   AAAAGAAACTAACAAGGATTCCCTTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTT 766
BSY4                ------------------------------------------------------------
Amanitaexcelsa2     AAAAGAAACTAACAAGGATTCCCTTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTT 771
Amanitaexcelsa1     AAAAGAAACTAACAAGGATTCCCTTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTT 672
Amanitamuscaria     AAAAGAAACTAACAAGGATTCCCTTAGTAACTGCGAGTGAAGAGGGAAGAGCTCAAATTT 736
WhiteA              ------------------------------------------------------------

BSY5                ------------------------------------------------------
BSY                 ------------------------------------------------------
BSY3                ------------------------------------------------------
BSY1                ------------------------------------------------------
Greypink            ------------------------------------------------------
Yelorange           ------------------------------------------------------
Amanitarubescens1   AAAATCTGGCAGTGTTTCACTGTCCGAGTTGTAA-------------------- 800
Amanitarubescens2   AAAATCTGGCAGTGTTTCACTGTCCGAGTTGTAACCTAGAGAAGTGTA------ 814
BSY4                ------------------------------------------------------
Amanitaexcelsa2     AAAATCTGGCAGTGTTTCACTGTCCGAGTTGTAACCTAGAGAAGTGA------- 818
Amanitaexcelsa1     AAAATCTGGCAGTGTTTCACTGTCCGAGTGACCTAGAGAAGTGTAACCTGG--- 723
Amanitamuscaria     AAAATCTGGCAGTGTTTTGCTGTCCGAGTTGTAATCTAGAGAAGTGCTGCCCGG 790
WhiteA              ------------------------------------------------------
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Clavulina group comparison

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DSY             ------------------------CCTGCGGAAGGATC-TTAATGAGTTGTGATGGGGTT 35
C.cristata      AGCGCA-AGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAGTTGTGATGGGGTT 59
C.cinerea       AGCGCACAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAGTTGTGATGGGGTT 60
                                ************** ********************

DSY             TGATGCTGGCAGCCAATTTTTGGATGCATGTGCTTGCCCTAACAATCATCTTCCAAACAC 95
C.cristata      TGATGCTGGCAGCCAATTTTTGGATGCATGTGCTTGCCCTAACAATCATCTTCCAAACAC 119
C.cinerea       TGATGCTGGCAGCCAATTTTTGGATGCATGTGCTTGCCCTAACAATCATCTTCCAAACAC 120
                ************************************************************

DSY             CTGTGCACATTTTTGAGGGAGTTTCGAGTTGATTGCCACTTTTGGGTGATTTTCTTGCAT 155
C.cristata      CTGTGCACATTTTTGAGGGAGTTTCGAGTTGATTGCCACTTTTGGGTGATTTTCTTGCAT 179
C.cinerea       CTGTGCACATTTTTGAGGGAGTTTCGAGTTGATTGCCACTTTTGGGTGATTTTCTTGCAT 180
                ************************************************************

DSY             TCCCTTAAATCATTATACGCCATTGACAATGCTGAACGTGCTCTGTGCCGCAAGGCCATT 215
C.cristata      TCCCTTAAATCATTATACGCCATTGACAATGCTGAACGTGCTCTGTGCCGCAAGGCCATT 239
C.cinerea       TCCCTTAAATCATTATACGCCATTGACAATGCTGAACGTGCTCTGTGCCGCA-GGCCATT 239
                ************************************************* *******

DSY             AATATAATACAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG 275
C.cristata      AATATAATACAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG 299
C.cinerea       AATATAATACAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG 299
                ************************************************************

DSY             AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACC 335
C.cristata      AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACC 359
C.cinerea       AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACC 359
                ************************************************************

DSY             TTGCGCTCCCTGGTATTCCGGGGAGCACGCCTGTTCGAGTGTCACGAAATTTCTCAAGCT 395
C.cristata      TTGCGCTCCCTGGTATTCCGGGGAGCACGCCTGTTCGAGTGTCACGAAATTTCTCAAGCT 419
C.cinerea       TTGCGCTCC-TGGTATTCCGGGGAGCACGCCTGTTCGAGTGTCACGAAATT-CTCAAGCT 417
                ********* ****************************************** ********

DSY             AGGATGGCTTTTTGTCTGTCCTTAGCCTTGGTTGTTGGGCTTTGCCGTGTCCTTCATTGG 455
C.cristata      AGGATGGCTTTTTGTCTGTC-TTAGCCTTGGTTGTTGGGCTTTGCCGTGTCCTTCATTGG 478
C.cinerea       AGGATGGCTTTTT-GTCTGTCCTTAGCCTTGGTTGTTGGGCTTTGCCGTGTCCTTCATTGG 476
                ************ ******* ************************************

DSY             GACGGCTGGCCTTAAAAGCATTAGCTGATCCTCGTGTGGCACTGGTTCTACTCAGCGTGA 515
C.cristata      -ACGGCTGGCCTTAAAAGCATTAGCTGATCCTCGTGTGGCACTGGTTCTACTCAGCGTGA 537
C.cinerea       GACGGCTGGCCTTAAAAGCATTAGCTGATCCTCGTGTGGCACTGGTTCTACTCAGCGTGA 536
                 ************************************************************

DSY             TAACAGTCTGATCGCTGAGGACATCTTTTGGGATGGCCAGTCCTCATTTGGGTTGCTTCT 575
C.cristata      TAACAGTCTGATCGCTGAGGACATCTTTTGGGATGGCCAGTCCTCATTTGGGTTGCTTCT 597
C.cinerea       TAACAGTCTGATCGCTGAGGACATCTTTTGGGATGGCCAGTCCTCATTTGGGTTGCTTCT 596
                ************************************************************

DSY             AAACTTGGTTTCGCGGATTGTTCAATCTGCGTTCCACTTTTCAGC--------------- 620
C.cristata      AAACTTGGTTTCGCGGATTGTTCAATCTGCGTTCCACTTTTCAGCTTTGACCTCGAATCA 657
C.cinerea       AAACTTGGTTTCGCGGATTGTTCAATCTGCGTTCCACTTTTCAGCTTTGACCTCGAATCA 656
                ******************************************

DSY             ------------------------------------------------------------
C.cristata      GGTGGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGG 717
C.cinerea       GGTGGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGG 716
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DSY             ------------------------------------------------------------
C.cristata      ATTCCCCTAGTAACGGCGAGTGAAGCGGGAAGAGCTCAAATTTAAAATCTGGCAATCTTT 777
C.cinerea       ATTCCCCTAGTAACGGCGAGTGAAGCGGGAAGAGCTCAAATTTAAAATCTGGCAATCTTT 776


DSY             -------------------------------------
C.cristata      GGTTGTCCGAATTGTAATACTAGAGAAGCGTTCCGCG 814
C.cinerea       GGTTGTCCGAATTGTAAT-CTAGAGAAGCG------- 805
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Inocybe cf. griseolilacina

CAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTATAAACTTGAACAGGCTGTTGCTGGC
CCTCCGGGGTGTCGTGCACGCTTGTCTCATCTTTGTCATTTCTTCCAACTGTGCACATTTTGTAGAT
CCTGGAAATGGGAGAGCTCCATGAACTTGTGTTCGAGTCGGGAATTGCTGTCGCTTTTTTCTTAGG
GCCGGCTTTTCCTTGTACCTTTCCCCCAGGTCTATGTCATTTTATCACAATCTCTCGAGTATGTTCAG
AATGTCGTTGGAATATAATATAATATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCATCTTGCGCTCTTGGTATTCCGAGGAGCATGCCTGTTGAGTGTCATTTACTTATCAACCATGTTT
CATGTCTGGAACGTGGCTTGGATGTGGGGCTCTTTTGCAGGCCTATCTATGGGTCGGCTCCCCTGA
AACGCATTAGTGGCATCTGAGCTGCTACTGTAGGTGTGATAAATTATCTATGCCTTTGGTATGCTGC
ATGAACAGATCACGCTGCTCAGAACCAAACTTGAGAAAGACTTGACAAACTTGACCTCAAATCAGGT
AGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTA
GTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTGAAATCTGGCGGTTCTCTGGGCCGTCCGAGTT
GTAATCTAGAGAAGTGCTACCTGG

Inocybe sp.2

AGTCGTACAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTATAAACTTGAACAGGCTGT
TGCTGGCCCTCCGGGGTGTCGTGCACGCTTGTCTCATCTTTGTCATTTCTTCCAACTGTGCACATTT
TGTAGATCCTGGAAATGGGAGAGCTCCATGAACTTGTGTTCGAGTCGGGAATTGCTGTCGCTTTTTT
CTTGGGCCGGCTTTTCCTTGTACCTTTCCCCCAGGTCTATGTCATTTTATCACAATCTCTCGAGTAT
GTTCAGAATGTCGTTGGAATATAATATAATATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCATCGAATC
TTTGAACGCATCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTTAACTTATC
AACCATGTTTCATGTCTGGAACGTGGCTTGGATGTGGGGCTCTTTTGCAGGCCTATCTATGGGTCG
GCTCCCCTGAAACGCATTAGTGGCATCTGAGCTGCTACTGTAGGTGTGATAAATTATCTATGCCTTT
GGTATGCTGCATGAACAGATCACGCTGCTCAGAACCAAACTTGAGAAAGACTTGACAAACTTGACC
TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAA
GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTGAAATCTGGCGGTTCTCTGGG
CCGTCCGAGTTGTAATCTAGAGAAGTGCTGACCTGG

Lactarius group comparison

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L.hemicyaneus       AGTCGCACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTACAACGTGTGTG 60
L.semisangifluus    ----GCGCCAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTACAACGTGTGTG 56
L.hepaticus         ----------------------GTGAACCTGCGGAAGGATCATTATCGTACAAAATGTGA- 38
                                          ******************************  **** 

L.hemicyaneus       TGAGGTGTGTGAGGGCTGTCGCTGACTTTTTGACACAAAAGTCGTGCACGCCGGAGCACG 120
L.semisangifluus    TGAGGTGTGTGAGGGCTGTCGCTGACTTTTTGACACAAAAGTCGTGCACGCCGGAGCACG 116
L.hepaticus         -GGGGCATGCAAGGGCTGTCGCTGACTCTAT------AAAGTCGTGCACGCCCGAGTGTG 91
                     * **  **  *************** * *      ************** ***   * 
L.hemicyaneus       TCCTCTCTCTCACATAA-AATCCATCTCACCCTTTTGTGCACCACCGCGCGGGCACCCTT 179
L.semisangifluus    TCCTCTCTCTCACATAA-AATCCATCTCACCCTTTTGTGCACCACCGCGCGGGCACCCTT 175
L.hepaticus         TCCTCTC----ACATAATAATCCATCTCACCCTTT-GTGCATCACCGCGTGGGCACCCTT 146
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                *******    ****** ***************** ***** ******* **********

L.hemicyaneus       TGGGAT-------------GCTTGCGTTTTCACACAAACCCCTT-TTAAAAAAGTGTAGA 225
L.semisangifluus    TGGGAT-------------GCTTGCGTTTTCACACAAACCCCTT-TTAAAAAAGTGTAGA 221
L.hepaticus         TGGGATCATCTCGGAGGGGGCTCGCGTTTTCACACAAACCCCCCCTTTTAAAAGTGTAGA 206
                    ******             *** ******************   **  ***********

L.hemicyaneus       ATGACCCCACTT-TGCGATGACACGCAATCAATACAACTTTCAACAACGGATCTCTTGGC 284
L.semisangifluus    ATGACCCCACTT-TGCGATGACACGCAATCAATACAACTTTCAACAACGGATCTCTTGGC 280
L.hepaticus         ATGACCTCATTTATGCGCTAACCCGCAATCAATACAACTTTCAACAACGGATCTCTTGGC 266
                    ****** ** ** **** * ** ************************************

L.hemicyaneus       TCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCA-G 343
L.semisangifluus    TCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAAG 340
L.hepaticus         TCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCA-G 325
                    ********************************************************** *

L.hemicyaneus       TGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCACACCCGT 403
L.semisangifluus    TGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCACACCCGT 400
L.hepaticus         TGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCACACCCGT 385
                    ************************************************************

L.hemicyaneus       TTGAGTGTCGTGAAAATCTCAACCTTCTCGGTTTTCTTCTGGACACCGAAGGAGGCTTGG 463
L.semisangifluus    TTGAGTGTCGTGAAAATCTCAACCTTCTCGGTTTTCTTCTGGACACCGAAGGAGGCTTGG 460
L.hepaticus         TTGAGTGTCGTGAAAACCTCAACCTCCTTGGTTT-CTTCTGGAGACCAAAGCAGGCTTGG 444
                    ***************** ******** ** ***** ******** *** *** ********

L.hemicyaneus       ACTTTGGAGGCCTTTGCTGGCGTCTCTCT----AGAGCCAGCTCCTCTTAAATGAATTAG 519
L.semisangifluus    ACTTTGGAGGCCTTTGCTGGCGTCTCTCT----AGAGCCAGCTCCTCTTAAATGAATTAG 516
L.hepaticus         ACTTTGGAGGCCTTTGCTGGCACCTCTCTTTTGAAGGCCAGCTCCTCTTAAACAAATTAG 504
                    **********************  ******    *  ****************  ******

L.hemicyaneus       CGGGGTCCTCTTTGCCGATCCTTGACATGTGATAAGATGTTTCCATGACTCGGTTTCTGG 579
L.semisangifluus    CGGGGTCCTCTTTGCCGATCCTTGACATGTGATAAGATGTTTCCATGACTCGGTT-CTGG 575
L.hepaticus         CAGGGTCCTCTTTGCCGATCCTCGACGTGTGATAAGATGTTTCCATGTCTTGGTT-CTGG 563
                    * ******************** *** ******************** ** **** ****

L.hemicyaneus       CTCTGTTGCATTT-GGGACCTGCTTCTAACCGTCTCGAC--------GAGACGACG-TTT 629
L.semisangifluus    CTCTGTTGCATTT-GGGACCTGCTTCTAACCGTCTCGAC--------GAGACGACGGTTT 626
L.hepaticus         CTCTGTCACCTTTTGGGACCCGCTTCTAACCGTCTTGACCTTGCGTCGAGACAACG-TTC 622
                    ******  * *** ****** ************** ***        ***** *** **

L.hemicyaneus       GGGCGTGTCTCCCTTCT--CGGGAGACTCTCTCGACCCCACGAACCCTTGACCTCAAATC 687
L.semisangifluus    GGGCGTGTCTCCCTTCT--CGGGAGACTCTCTCGACCCCACGAACCCTTGACCTCAAATC 684
L.hepaticus         GAGCATGTCTCCCTTTCTCCGGGAGGCTCCCTTGACCCCACGAACCCTTGACCTCAAATC 682
                    * ** **********    ****** *** ** ***************************

L.hemicyaneus       GGGTGAGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAG 747
L.semisangifluus    GGGTGAGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAG 744
L.hepaticus         GGGTGAGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAG 742
                    ************************************************************

L.hemicyaneus       GATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGTGGTCTT 807
L.semisangifluus    GATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGTGGTCTT 804
L.hepaticus         GATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCTT 802
                    ************************************************** ******

L.hemicyaneus       TGGCCATCCGAGTTGAA-TTTAGAGAAGCGTCGTCCGCG 845
L.semisangifluus    TGGCCATCCGAGTTGTA-TTTAGAGAA------------ 830
L.hepaticus         TGGCTGTCCGAGTTGTAATTTAGAGAAGCGTCGTCCGCG 841
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Scleroderma citrinum

ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCTGAAATAGAACCTCTAGGGAGGGGGA
GGGGGAGAGTCCTCGCGTCGCTCCTTCGAACCTTTCCGAGGCTTCATACCTCCCACACCCGTGTG
CACCCGCTGTAGGTCCTTAGGGACCTATGTCTTCTCAGAACTCGCATGTCTACAGAATGTCGTCCG
TGGCCTCGGCATCGTAAACCTAGTACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAA
GGACGCAGCGAATCGCGATAAGTAATGTGAATTGCAGATTTTCCGTGAATCATCGAATCTTTGAACG
CACCTTGCGCTCCTCGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATCGAAATATCAAATCGAA
GCTTTCGCCCCCGGTCGAAGCTAGGTTTGGACCATGGAAGTCTGCCGGCGGGTCCCCTCCTGTCG
GAAGGGGGACTTTCGGCTCTCCTCAAAAGCATTACCGGCGTGTGCAAGCCTGGCACGGCACGGCC
TCTTCGACGTCATAATGACCCGTCGTGGCCTGAAAGTGCGTGGATCGCGGTCCCTTTCCACCTTTG
CAAGCCGGTCTCGGACGGGCCGCGCGTCATCGATGTTTGACCTCAAATCAGGTAGGACTACCCGC
TGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACGAGGATTCCCCTAGTAACTGCGAGT
GAAGCGGGAAGAGCTCAAATTTCGAATCTGGCGGTCCTCGGCCAGCCCGAGTTGTAAATCTAGAG
AAGC

Suillus group comparison

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S.granulatus        AGTCGTACA-GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTACAATCCG 59
S.luteus            -GTCGCACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTATAATCCG 59
S.salmonicolor      ----------GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTATAATCCG 50
                            ****************************************** ******

S.granulatus        GCGAGGG-AAAGGC--GGAGAGTTGTAGCTGGCCCCT-----CCGGGCATGTGCACGCTC 111
S.luteus            GCGAGGGGAAAGGC--GGAGAGTTGTAGCTGGCCTCT-----AGGGGCATGTGCACGCTC 112
S.salmonicolor      GCGAGGG-AAAGGCTGGGAGAACTGTCGCTGGCCCGCTTAAAACGGGCATGTGCACGCTC 109
                    ******* ******  *****  *** *******          ****************

S.granulatus        TCTTCCGGAC-TTTCGCCGTATGGGCGCGGGGCGA---------CCCGCGTCTTCATATA 161
S.luteus            TCTTCCGGACCTTTCGCCGTATGGGCGCGGGGCGA---------CCCGCGTCTTCATATA 163
S.salmonicolor      TCTTTCTCGACCTTCGCCGTATGGGCGTGTGGGGGGCCAACCCCTCCGCGTCTTCATATA 169
                    **** *      *************** * ** *           ***************

S.granulatus        CCTCTTCGTGTAGAAAGTCTTTGAATGTCGAAACCATCATCGAGTCGCGACTTCTAGGAG 221
S.luteus            CCTCTTCGTGTAGAAAGTCTTTGAATGTTTTA-CCATCATCGAGTCGCGACTTCTAGGAG 222
S.salmonicolor      CCTCTTCGTGTAGAAAGTCTTTGAACGTTTTTATCATCGTCGAGTCGCGACTTCCAGGAG 229
                    ************************* **      **** *************** *****

S.granulatus        AC-GCGATTCTTTGAGACAAAA-GTTATTACAACTTTCAGCAACGGATCTCTTGGCTCTC 279
S.luteus            ACTGCGATTCTTTGAGAAAAAAAGTTATTACAACTTTCAGCAACGGATCTCTTGGCTCTC 282
S.salmonicolor      ACGCCGATTCTTCGAGACAAAA-GTTATTACAACTTTCAGCAATGGATCTCTTGGCTCTC 288
                    **  ******** **** **** ******************** ****************

S.granulatus        GCATCGATGAAGAACGCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAA 339
S.luteus            GCATCGATGAAGAACGCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAA 342
S.salmonicolor      GCATCGATGAAGAACGCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAA 348
                    ************************************************************

S.granulatus        TCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGA 399
S.luteus            TCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGA 402
S.salmonicolor      TCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTCGA 408
                    ************************************************************ **

S.granulatus        AGTGTCA-GTAAATTCTCAACCCCTCTCGATTTGCTTCGAGAGGGCGCTTGGATGGTGGA 458
S.luteus            AGTGTCAAGTAAATTCTCAACCCCTCTCGATTTGCTTCGAGCGGGTGCTTGGATGTTGGG 462
S.salmonicolor      GCCGTCA-GTAAATTCTCAACCCCTCTCGATTAGCTTCGAGCCGGGGCTTGGATCCTGGG 467
                       **** ********************** ********  ** ********  ***
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S.granulatus        GGCTGCCGGAGAC-CTGGATTCGTTCAGGACTCGGGCTCCTCTGAAATGAATCGGCTTGC 517
S.luteus            GGCTGCCGGAGACACTGGATTCGTCCAGGACTCGGGCTCCTCTTAAATGAATCGGCTCGC 522
S.salmonicolor      GGCTGCCGAA--CCCTG--TTTTTTCAGGACTCGGGCTCCTT--AAATGCATCGGCTTGC 521
                    ******** *  * ***  **  * ****************   ***** ******* **

S.granulatus        GGTCGACCTTCGACTTTGCATGACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGA 577
S.luteus            GGTCGACTTTCGACTTTGCATGACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGA 582
S.salmonicolor      GG-CGACTTTCGACTTTGCGCAACA-GGCTTTCGGCGTGATAATGATCGC-GTTCGCAGA 578
                    ** **** ***********   *** *** ** ***************** ****** **

S.granulatus        AGT-GCACGACAGAAA-ATGGTCCC--GCGCCTCTAAT-GCGTC-GA--CGCCTTCC--G 627
S.luteus            AGT-GCAAGACAGAA---TGGTCCC--GTGCCTCTAAT-GCGTCAGA--CAGCCTCTAGG 633
S.salmonicolor      AGCAGCATGAATGAACCGCTGTCCGCAGCGCCTCTAATCGCGTCAGAGCCACCTTGCAGA 638
                    **  *** **  ***     ****   * ********* ***** **  *  * *

S.granulatus        GGCGTCTTCCTCATTGAC-GTTTGACCTCAAATCAGGTAGGACTACCCGCTGA-ACTTAA 685
S.luteus            GGCGTCTTCCTCATTGACAGTTTGACCTCAAATCAGGTAGGACTACCCGCTGATACTTAA 693
S.salmonicolor      GTTGTCCTCCATATAGAC-GTTTGACCTCGAATCAGGTAGGACTACCCGCTGA-ACTTAA 696
                    *  *** ***  ** *** ********** ********************** ******

S.granulatus        GCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAA 745
S.luteus            GCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAA 753
S.salmonicolor      GCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAA 756
                    ************************************************************

S.granulatus        CCGGGAAAAGCTCAAATTTT-GAATCTGGCGGTCTTTC-GGCCGTCCGAGTTGTA----- 798
S.luteus            CCGGGAAAAGCTCAAATTTT-GAATCTGGCGGTCTTCA-GGCCGTCCGAGTTGTA----- 806
S.salmonicolor      CCGGGAAAAGCTCAAATTTTTGAATCTGGCGGTCTTTACGGTCGTCCGAGTTGAATCTAG 816
                    ******************** ***************   ** *********** *

S.granulatus        -----------------
S.luteus            -----------------
S.salmonicolor      AGAAGCGTCTGCCGCG 832
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Tricholoma albobruneum

TAGTCGCACAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAGCTTGGTTGGGTTG
TCGCTGGCTCTTCGGAGCATGTGCACGCCTAACACCAATCTTCTTACCACCTGTGCACTTTTTGTAG
ATCTGGATATCTCTCGAGGAAACTCGGTATTGAGGGCTGCTGTGCGTTAAAGCCGGCTTTCCTTAC
ATTTCCGGTCTATGTCTTTATATACACCATTTGTATGTCTACGAATGTTATTATAGGACTTTGTCCAAA
TAAACCTTATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGACGCACCTTGCGCTCCTTGG
TATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTTTTTGGCTTTTTCTTAAGTTGA
TTAGGCTTGGATGTGGGGGTTTGCGGGCTTTGTCTGAAGTCGGCTCTCCTTAAATTCATTAGTAGG
GACCTTTGTTGCCTTAGCTTTTGGTGTGATAATTATCTACGCCATTATGTGAAGCAGCTTTAAATTGG
AGGTTACTGCTCTCTAACCGTCTCTCATGGGACAATTTTCTGACAATTTGACCTCAAATCAGGTAGG
ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAA
CTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGAGGGTCTTTATGGCCTTCCGAGTTGTAAT
CTAGAGAAGTGTAGCC

Xerocomus group comparison

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BSY2                ----------------GTAGGTGAACCTGCGGAAGGATCA-TTATC-------GAGCAAG 36
Beige               --------------------GGTGAACCTGCGGA-GGAGCA-TTATC-------GAACAAG 32
Dullorange          ------CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCAATTATCC------GAACAAG 48
X.badius            GTCGCACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA-TTATC-------GAACAAG 52
X.cf.chrysenteron   GTCGCACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA-TTATCGAATTTCGAGGGGG 59
                                      ************** *** ** *****       **    *

BSY2                AAGG----GGTAAAGACTGCCTCTGGCC-------GATAGCATGTGCACGC-------TT 78
Beige               AAGG----GGGAAAGACTGTCGCTGGC--------GAAAGCATGTGCACG--------TC 72
Dullorange          AAGG----GGGAAAGACTGTCGCTGGC--------GAAAGCATGTGCACG--------TC 88
X.badius            AAGG----GGGAAAGACTGTCGCTGGC--------GAAAGCATGTGCACG--------TC 92
X.cf.chrysenteron   AAGATGGAGGGGAAGACTGTCGCTGGCTTTCACTGGGAAGCATGTGCACGCCTTTCGTTT 119
                    ***     **  ******* * *****        *  ************        *

BSY2                CAAC--CTTTTCTATTAC----ACACCCGTGCACCTTTTGTGGGTCCTCGAAAGAGGATC 132
Beige               CAAC--CTTTTCTATTAC----ACACCCGTGCACCTTTTGTAGGTCCTCGAAAGAGGATC 126
Dullorange          CAAC--CTTTTCTATTAC----ACACCCGTGCACCTTTTGTAGGTCCTCGAAAGAGGATC 142
X.badius            CAAC--CTTTTCTATTAC----ACACCCGTGCACCTTTTGTAGGTCCTCGAAAGAGGATC 146
X.cf.chrysenteron   CGTCGACCTTTCTCTTACTCTCACACCTGTGCACACATTGTAGGTCCTCGAAAGAGGATC 179
                    *  *  * ***** ****    ***** ******   **** ******************

BSY2                TATGTATTTCATCATCACACCTATCGTATGTCTAGAATGT--CATA--CGTCGACC-ACT 187
Beige               TATGTATTTCATCATCACACCTATCGTATGTCTAGAATGT--CAT---CGTCGACC-ACT 180
Dullorange          TATGTATTTCATCATCACACCTATCGTATGTCTAGAATGT--CAT---CGTCGACC-ACT 196
X.badius            TATGTATTTCATCATCACACCTATCGTATGTCTAGAATGT--CAT---CGTCGACC-ACT 200
X.cf.chrysenteron   TATGT-CTTTATCATCACACACATAGCATGTCTAGAATGTATCATGATCGTCGACCGACT 238
                    *****  ** **********  ** * *************  ***   ******** ***

BSY2                --------GGGCGGCG---AAATTAATAA-TACAACTTTCAGCAACGGATCTCTTGGCTC 235
Beige               T-------GGGCGGCG---AAATAAATAA-TACAACTTTCAGCAACGGATCTCTTGGCTC 229
Dullorange          --------GGGCGGCG---AAATAAATAA-TACAACTTTCAGCAACGGATCTCTTGGCTC 244
X.badius            --------GGGCGGCG---AAATAAATAA-TACAACTTTCAGCAACGGATCTCTTGGCTC 248
X.cf.chrysenteron   TTGCGGTCGGGCGGTGGTCAAACAAATAAATACAACTTTCAGCAACGGATCTCTTGGCTC 298
                           ****** *   ***  ***** ****************************

BSY2                TCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGATTTTCAGTG 295
Beige               TCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGATTTTCAGTG 289
Dullorange          TCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGATTTTCAGTG 304
X.badius            TCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGATTTTCAGTG 308
X.cf.chrysenteron   TCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGATTTTCAGTG 358
                    ************************************************************

BSY2                AATCATCGAATCTTTGAACG-CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT 354
Beige               AATCATCGAATCTTTGAACGGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT 349
Dullorange          AATCATCGAATCTTTGAACG-CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT 363
X.badius            AATCATCGAATCTTTGAACG-CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT 367
X.cf.chrysenteron   AATCATCGAATCTTTGAACG-CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT 417
                    ******************* ************************************

BSY2                TCGAGTGTCATTACATTCTCAACCATGACTTTGTTGA--------CATGGCTTGGAGTTG 406
Beige               T-GAGTGTCATTAAATTCTCAACCATGACTTTGTTGA--------CATGGCTTGGAGTTG 400
Dullorange          T-GAGTGTCATTAAATTCTCAACCATGACTTTGTTGA--------CATGGCTTGGAGTTG 414
X.badius            T-GAGTGTCATTAAATTCTCAACCATGACTTTGTTGA--------CATGGCTTGGAGTTG 418
X.cf.chrysenteron   T-GAGTGTCATTGAAT-CTCAACCATGTCTTGATCGATTTCGAGGCATGGCTTG-ACTTG 474
                    * **********  ** ********** ***  * **        ********* * ***
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BSY2                GAGGTTGCTGGCTTGT---TTGTCAGCTCTCCTGAAATACATTAGCCGTGGACAA----- 458
Beige               GGGGTTGCTGGCTTGT---TTGTCAGCTCTCCTGAAATACATTAGCGGTGGACAA----- 452
Dullorange          GGGGTTGCTGGCTTGT---TTGTCAGCTCTCCTGAAATACATTAGCGGTGGACAA----- 466
X.badius            GGGGTTGCTGGCTTGT---TTGTCAGCTCTCCTGAAATACATTAGCGGTGGACAA----- 470
X.cf.chrysenteron   GGGGTTGCTGCGGCGAAAGCGGTCGGCTCTCCTGAAATGCATTAGCAAAGGACAGCAAGT 534
                    * ********    *      *** ************* *******   *****

BSY2                -------GCAAGGCCTTCCCACGTGATAATGATCGTCT--GTGGGGACGGAGCATCAACG 509
Beige               -------GCAAGGCCTTCCGACGTGATAATGATCGTC---GTGGGG-CGGAGCATCAACG 501
Dullorange          -------GCAAGGCCTTCCGACGTGATAATGATCGTC---GTGGGG-CGGAGCATCAACG 515
X.badius            -------GCAAGGCCTTCCGACGTGATAATGATCGTC---GTGGGC---GAGCATCAACG 517
X.cf.chrysenteron   CTGACGTGCACGGCCTT--GACGTGATAATGATCGTCGTCGCTGGAGCGTAGGACAAGCA 592
                           *** ******   *****************   *  **     ** *  * *

BSY2                TCTGTACTGACGTCTGAGAATCCCAGCATGCCTTAGCTAGACCATCATATCGAA-AGCTT 568
Beige               TCTGT-CTG-CTTCTA--AATCCCAG-ATGCCTTAGCTAGAC-ATCATTTCGAA-CGCTT 554
Dullorange          TCTGT-CTG-CTTCTA--AATCCCAG-ATGCCTTAGCTAGAC-ATCATTTCGAACAGCTT 569
X.badius            TCTGT-CTG-CTTCTA--AATCCCAG-ATGCCTTAGCTAGAC-ATCATTTCGAA-CGCTT 570
X.cf.chrysenteron   TGAATGAGTCCGTTTG---CTTCCAA---TCCTTGACTTGGATGCTTTAGCTACTAG-TT 645
                    *   *     * * *     * ***     ****  ** *       *  * *   * **

BSY2                GACCTCGAATCAGGTAGAGA------------------------------------------ 588
Beige               GACC-------------------------------------------------------- 558
Dullorange          GACCTCAAATCAGGTAGGACTACCCG----------------------------------- 595
X.badius            GACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAG 630
X.cf.chrysenteron   GGTCGCGAGGCCGACGAACGCAGGGTTTCCCTCGTCTTCCTTTGACAACTTGACCTCAAA 705
                    *  *

BSY2                ------------------------------------------------------------
Beige               ------------------------------------------------------------
Dullorange          ------------------------------------------------------------
X.badius            AAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAGAGCTCAAATTTTGAAT 690
X.cf.chrysenteron   TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACA 765

BSY2                ------------------------------------------------------------
Beige               ------------------------------------------------------------
Dullorange          ------------------------------------------------------------
X.badius            CTGGCGGTCTTTGGCCGTCCGAGTTGAATCTAGAGAAG---------------------- 728
X.cf.chrysenteron   AGGATTCCCCTAGTAACTGCGAGTGAAGCGGGATGAGCTCAAATTTTGAATCTGGCGGTC 825

BSY2                ------------------
Beige               ------------------
Dullorange          ------------------
X.badius            ------------------
X.cf.chrysenteron   TTTGGCCGTCCGAGTTGTA 844
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Russula group comparison

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R.sp1           TTAGTCGTACA-GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTTAAACCGAGG 59
R.sp6           ---GTCGTACA-GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTTAAACCGAGG 56
R.sp4           ----------A-GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTTAAACCGAGG 49
R.sp7           ----------GGTTTCGTAGGTGAACCTGCGGAAGGATCATTATCGTTAAACCGAGG 47
R.sp3           ---GTCGCACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGTTAAACCGAGG 57
R.drimeia       ------------------CGTAGGTGAACCTGCGGA-GGATCATTATCGTACAACCGAGG 41
                                  ****************** *************  ********

R.sp1           CGCGA-GGGCTGTCGCTGACCTTCCCCGGTCGTGCACGCCCGAGCCCTCTCA--CAATCC 116
R.sp6           CGCGA-GGGCTGTCGCTGACCTTCCCCGGTCGTGCACGCCCGAGCCCTCTCA--CAATCC 113
R.sp4           CGCGA-GGGCTGTCGCTGACCTTCCCCGGTCGTGCACGCCCGAGCCCTCTCA--CAATCC 106
R.sp7           CGCGA-GGGCTGTCGCTGACCTTCCCCGGTCGTGCACGCCCGAGCCCTCTCA--CAATCC 104
R.sp3           CGCGA-GGGCTGTCGCTGACCTTCCCCGGTCGTGCACGCCCGAGCCCTCTCA--CAATCC 114
R.drimeia       CACGAAGGGCTGTCGCTGACTTTTTT--GTCGTGCACGCCCGAGTGCTCTCAAACAATCC 99
                * *** ************** **     ****************  ******  ******

R.sp1           ATCTCACCCTTTGTGCATCACCGCGTGGGTCCTCCTTT--GCC--------GGAGGGCCC 166
R.sp6           ATCTCACCCTTTGTGCATCACCGCGTGGGTCCTCCTTT--GCC--------GGAGGGCCC 163
R.sp4           ATCTCACCCTTTGTGCATCACCGCGTGGGTCCTCCTTT--GCC--------GGAGGGCCC 156
R.sp7           ATCTCACCCTTTGTGCATCACCGCGTGGGTCCTCCTTT--GCC--------GGAGGGCCC 154
R.sp3           ATCTCACCCTTTGTGCATCACCGCGTGGGTCCTCCTTT--GCC--------GGAGGGCCC 164
R.drimeia       ATCTCACCCTATGTGCACCACCGCGTGGGTCCCCCTTTTGGCTCGTTCCGAGGGGGGCTT 159
                ********** ****** ************** *****  **         ** ****

R.sp1           GCGTCTTTACACAAAACTCGATACAGTGTAGAATGTTTATTTTCGCGCTCGCACGCTGAT 226
R.sp6           GCGTCTTTACACAAAACTCGATACAGTGTAGAATGTTTATTTTCGCGCTCGCACGC-GAT 222
R.sp4           GCGTCTTTACACAAAACTCGATACAGTGTAGAATGTTTATTTTCGCGCTCGCACGCTGAT 216
R.sp7           GCGTCTTTACACAAAACTCGATACAGTGTAGAATGTTTATTTTTGCGCTCGCACGC-GAT 213
R.sp3           GCGTCTTTACACAAAACTCGATACAGTGTAGAATGTTTATTTTCGCGCTCGCACGC-GAT 223
R.drimeia       GCGTTTTCACACAAACTTAAATACAGTGTAGAATGTC---TTTTGCGATAACACGC---- 212
                **** ** *******  *  ****************    *** *** *  *****

R.sp1           CAATAATACAACTTTCAACA-CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 285
R.sp6           CAATAATACAACTTTCAACA-CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 281
R.sp4           CAATAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 276
R.sp7           CAATAATACAACTTTCAACACCGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 273
R.sp3           CAATAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 283
R.drimeia       AATTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA 272
                 * ***************** ************************************

R.sp1           AATGCGATACCGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCAC 344
R.sp6           AATGCGATACCGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCAC 340
R.sp4           AATGCGATAC-GTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCAC 334
R.sp7           AATGCGATACCGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCAC 332
R.sp3           AATGCGATACCGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCAC 342
R.drimeia       AATGCGATACCGTAATGTGAATTGCAGAATTCAAGTGAATCATCGAATCTTTGAACGCAC 332
                ********** ********************** *************************

R.sp1           CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGA-GTGTC-GTGAAATCTTCAAA 402
R.sp6           CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGA-GTGTC-GTGAAATCTTCAAA 398
R.sp4           CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGA-GTGTC-GTGAAATCTTCAAA 392
R.sp7           CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGA-GTGTC-GTGAAATCTTCAAA 390
R.sp3           CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGA-GTGTC-GTGAAATCTTCAAA 400
R.drimeia       CTTGCGCCCCTTGGCATTCCGAGGGGCACACCCGTTTGAAGTGTCCGTGAAATTCTCAAA 392
                ************************************ ***** *******  *****

R.sp1           ATCCCTCTCTTTTGAGGA-TTTTTGGACTTGGAGGTTCAATGCTCGCTTTTGCCTTTTAA 461
R.sp6           ATCCCTCTCTTTTGAGGA-TTTTTGGACTTGGAGGTTCAATGCTCGCTTTTGCCTTTTAG 457
R.sp4           ATCCCTCTCTTTTGAGGA-TTTTTGGACTTGGAGGTTCAATGCTCGCTTTTGCCTTTTAG 451
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R.sp7           ATCCCTCTCTTTTGAGGA-TTTTTGGACTTGGAGGTTCAATGCTCGCTTTTGCCTTTTAG 449
R.sp3           ATCCCTCTCTTTTGAGGAATTTTTGGACTTGAAGGTCCAATGTTGGCTTTTGCCTTTTAG 460
R.drimeia       AGCC-TTTCTTTTGAAAGGATTTTGGACATGGAGGCTTT-TGCTGGCTTC-ACCTC---- 445
                * ** * ********     ******** ** ***     ** * ****   ***

R.sp1           AAAAGCGAGCTCCTCTCAAATGAATCAGTGGGGTCTGCTTTGCCGGTCCTTGACGTGATA 521
R.sp6           AAAAGCGAGCTCCTCTCAAATGAATCAGTGGGGTCTGCTTTGCCGGTCCTTGACGTGATA 517
R.sp4           AAAAGCGAGCTCCTCTCAAATGAATCAGTGGGGTCTGCTTTGCCGGTCCTTGACGTGATA 511
R.sp7           AAA-GCGAGCTCCTCTCAAATGAATCAGTGGGGTCTGCTTTGCCGGTCCTTGACGTGATA 508
R.sp3           AAA-GCGAGCTCCTCTCAAATGAATCAGTGGGGTCTGCTTTGCCGGTCCTTGACGTGATA 519
R.drimeia       -GAAGCCAGCTCCTCTTAAACGAATTAGTGGGGTCTGCTTTGCCGATCCTCGACGTGATA 504
                  * ** ********* *** **** ****************** **** *********

R.sp1           AGATCTTTCTACGTTTTGGATTCGACATCGTC----------CCGCTTCCAATCGTCCCA 571
R.sp6           AGATCTTTCTACGTTTTGGATTCGACATCGTC----------CCGCTTCCAATCGTCCCA 567
R.sp4           AGATCTTTCTACGTTTTGGATTCGACATCGTC----------CCGCTTCCAATCGTCCCA 561
R.sp7           AG-TCTTTCTACGTTTTGGATTCGACATCGTC----------CCGCTTCCAATCGTCCCA 557
R.sp3           AGATCTTTCTACGTTT-GGATTCGACATTGTC----------CCGCTTCCAATCGTCCCA 568
R.drimeia       AGATGTTTCTACGTCTGGGGTTCGGTGATGTCTTTTGGGCACCTGCTTCTAATCGTCTCA 564
                ** * ********* * ** ****     ***          * ***** ******* **

R.sp1           CGG-ACAAAAGGATGGTGCTCCGGTCGC--------------------GGCCGGACCCAC 610
R.sp6           CGG-ACAAAAGGATGGTGCTCCGGTCGC--------------------GGCCGGACCCAC 606
R.sp4           CGG-ACAAAAGGATGGTGCTCCGGTCGC--------------------GGCCGGACCCAC 600
R.sp7           CGG-ACAAAAGGATGGTGCTCCGGTCGC--------------------GGCCGGACCCAC 596
R.sp3           CGGGACAAAAGGATGGTGCTCCGGTCGCT-------------------GGCCGGACCCAC 609
R.drimeia       TCG---ACAATGATGGCGTTCCGGTCACCGCCGTTTCATCGGTCGGTCGGCTTGACCCAC 621
                  *   * ** ***** * ******* *                    ***  *******

R.sp1           CAAAAAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 670
R.sp6           CAAAAAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 666
R.sp4           CAAAAAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 660
R.sp7           CAAAAAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 656
R.sp3           CAAAAAAAACCTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 669
R.drimeia       CAAAAAAA-CTTTGACCTCAAATCGGGTGAGACTACCCGCTGAACTTAAGCATATCAATA 680
                ******** * ********************************************

R.sp1           AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 730
R.sp6           AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 726
R.sp4           AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 720
R.sp7           AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 716
R.sp3           AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 729
R.drimeia       AGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGC 740
                ************************************************************

R.sp1           TCAAATTTAAAATCTGGTGGTCTTTGGCCGTCCGAGTTGTAATTTAGAGAAG------- 782
R.sp6           TCAAATTTAAAATCTGGTGGTCTTTGGCCGTCCGAGTTGTAATTTAGAGAA-------- 777
R.sp4           TCAAATTTAAAATCTGGTGGTCTTTGGCCGTCCGAGTTGTAATT--------------- 764
R.sp7           TCAAATTTAAAATCTGGTGGTCTTTGGCCGTCCGAGTTGTAATTTAGAGAAGCGTCTCC 775
R.sp3           TCAAATTTAAAATCTGGTGGTCTTTGGCC------------------------------ 758
R.drimeia       TCAAATTTAAAATCTGGTGGCCTTTGGCCA------------------------------ 770
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Thelephora group comparison

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BlackA             -------CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAATTGTCAA-ACG 52
PartialwhiteB      GTCGCAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAATTGTCAA-ACG 59
YelsilA            -----------------GTAGGTGAACCTGCGGA-GGATC-TTACTGAATTGTCAA-ACG 40
YelsilC            ----------------------------CAGCGGAAGGATC-TTACTGA-TTGTCAA-ACG 30
Fwhite             ------------------------------------GGATCATTACAGAGTTGTCGACACG 25
                                                  ***** **** ** ***** * ***

BlackA             GGTTGTTGCTGGCCCTCATA-GGGGGCATGTGCACGCTCTGTTTACACATCCACTCACAC 111
PartialwhiteB      GGTTGTTGCTGGCCCTCATA-GGGGGCATGTGCACGCTCTGTTTACACATCCACTCACAC 118
YelsilA            GGTTGTTGCTGGCCCTCATA-GGGGGCATGTGCACGCTCTGTTTACACATCCACTCACAC 99
YelsilC            GGTTGTTGCTGGCCCTCATA-GGGGGCATGTGCACGCTCTGTTTACACATCCACTCACAC 89
Fwhite             AGTTGTTGCTGGTCCTCAAACGGGGGCATGTGCACACTCTGTTCACACATCCACTCACAC 85
                    *********** ***** * ************** ******* ***************

BlackA             CTGTGCACCCTCTGTAGTGTCTATGGTCTGGGGGACC--CTGTCTTCCTTCTGTGGTTCT 169
PartialwhiteB      CTGTGCACCCTCTGTAGT-TCTATGGTCTGGGGGACC--CTGTCTTCCTTCTGTGGTTCT 175
YelsilA            CTGTGCACCCTCTGTAGT-TCTATGGTCTGGGGGACC--CTGTCTTCCTTCTGTGGTTCT 156
YelsilC            CTGTGCACCCTCTGTAGT-TCTATGGTCTGGGGGACC--CTGTCTTCCTTCTGTGGTTCT 146
Fwhite             CTGTGCACCCTCTGTAGT-TCTATGGTCTGGGGGACGTGCCGTCTTCCTTCCGTGGTTCT 144
                   ****************** *****************   * ********** ********

BlackA             ACGTCTTTACACACACACT-GTAATAAAGTTTTATGGAATGTACATCGCGTCTAACCGCA 228
PartialwhiteB      ACGTCTTTACACACACACT-GTAATAAAGTTTTATGGAATGTACATCGCGTCTAACCGCA 234
YelsilA            ACGTCTTTACACACACACTTGTAATAAAGTTTTATGGAATGTACATCGCGTCTAAC-GCA 215
YelsilC            ACGTCTTTACACACACACTTGTAATAAAGTTTTATGGAATGTACATCGCGTCTAAC-GCA 205
Fwhite             ATGTCTTTACACACACGCCGTAGCTGAAGTCTTATGGAATGTACTCTGCGTTTAAC-GCG 203
                   * ************** *      * **** *************   **** **** **

BlackA             ATACAATACAACTTTCAGCAACGG-ATCTCTTGGCTCTCGCATCGATGAAGAACGCA-GC 286
PartialwhiteB      ATACAATACAACTTTCAGCAACGGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA-GC 293
YelsilA            ATACAATACAACTTTCAGCAACGG-ATCTCTTGGCTCTCGCATCGATGAAGAACGCA-GC 273
YelsilC            ATACAATACAACTTTCAGCAACGG-ATCTCTTGGCTCTCGCATCGATGAAGAACGCAAGC 264
Fwhite             ATATAATACAACTTTCAGCAACGG-ATCTCTTGGCTCTCGCATCGATGAAGAACGCA-GC 261
                   *** ******************** ******************************** **

BlackA             GAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCA 345
PartialwhiteB      GAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCA 352
YelsilA            GAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCATCGAATCTTTGAACGCA 333
YelsilC            GAAATGCGATAAGTAATGTGAATTGCAGAATTCAAGTGAATCATCGAATCTTTGAACGCA 324
Fwhite             GAAATGCGATAAGTAATGTGAATTGCAGAATTCA-GTGAATCATCGAATCTTTGAACGCA 320
                   ********************************** ************************

BlackA             CCTTGCGCCCCTTGGCTATTCCGAGGGGCATGCCTGTTTGAAGTATCATGAACACCTCAA 405
PartialwhiteB      CCTTGCGCCCCTTGGCTATTCCGAGGGGCATGCCTGTTTGA-GTATCATGAACACCTCAA 411
YelsilA            CCTTGCGCCCCTTGGCTATTCCGAGGGGCATGCCTGTTTGA-GTATCATGAACACCTCAA 392
YelsilC            CCTTGCGCCCCTTGGCTATTCCGAGGGGCATGCCTGTTTGA-GTATCATGAACACCTCAA 383
Fwhite             CCTTGCGCCCCTTGGCCATTCCGAGGGGCATGCCTGTTTGA-GTATCATGAACACCTCAA 379
                   ***************** ************************ ******************

BlackA             CTCTCATGGTTTGCC-ATGATGAAGTTGGACTCTGGGGGTTTTGCTGGCCTGTGGTCGGC 464
PartialwhiteB      CTCTCATGGTTTGCC-ATGATGA-GTTGGACTCTGGGGGTTTTGCTGGCCTGTGGTCGGC 469
YelsilA            CTCTCATGGTTTGCC-ATGATGA-GTTGGACTCTGGGGGTTTTGCTGGCCTGTGGTCGGC 450
YelsilC            CTCTCATGGTTTGCC-ATGATGA-GTTGGACTCTGGGGGTTTTGCTGGCCTGTGGTCGGC 441
Fwhite             CTCTCATGGCTTGCCCGTGATGAGCTTGGACTTTGGGGGTTTTGCTGGCCTGTGGTTGGC 439
                   ********* *****  ******  ******* ********************** ***
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BlackA              TCCTCTCAAATTAATCAGCCTCCCAAGTGTTTGGTGGCATCACGGGTGTGATAAATATCT 524
PartialwhiteB       TCCTCTCAAATTAATCAGCCTCCCA-GTGTTTGGTGGCATCACGGGTGTGATAAATATCT 528
YelsilA             TCCTCTCAAATTAATCAGCCTCCCA-GTGTTTGGTGGCATCACGGGTGTGATAAATATCT 509
YelsilC             TCCTCTCAAATTAATCAGCCTCCCA-GTGTTTGGTGGCATCACGGGTGTGATAAATATCT 500
Fwhite              TCCTCTCAAATGAATCAGCTTGCCAGTGTTTTGGTGGCGTCATTGGTGTGATAACTATCT 499
                    ********** ******* * ***    ********* ***  ********** *****

BlackA              ACGCTCGCTGTT---------------------------------------------- 536
PartialwhiteB       ACGCTCGCTGTTGTCTGCCAGGTAACCTTTGGTCACAAAGGTTTGCTGGAGCTCACAGAT 588
YelsilA             ACGCTCGCTGTTGTCTGCCAGGTAACCTTTGGTCTACAAAGGTTTGCTGGAGCTCACAAG 569
YelsilC             ACGCTCGCTGTTGTCTGCCAGGTAACCTTTGGTC-ACAAAGGTTTGCTGGAGCTCACAG- 558
Fwhite              ACGCTTGTGATCGTCTGCCGAGTGACCTTCAGTGAATGGAGGTTCGCTGGAGCTTACAG- 558
                    ***** *   *

BlackA              ------------------------------------------------------------
PartialwhiteB       GTCTCTCCTCAGCGAGGACAGCTTTTTTAACGTTCTGATCTCAAATCAGGTAGGACTACC 648
YelsilA             TATGTCTCTCCTCAGCGAGGACAGCTTTTTTAACGTTCGA-------------------- 609
YelsilC             -ATGTCTCTCCTCAGCGAGGACAGCTTTTTTAACGTTCGATCT----------------- 600
Fwhite              -ATGTCTCTCCTCAGTGAAGACAGCTTTTCGAACGTTCG--------------------- 596


BlackA              -
PartialwhiteB       C 649
YelsilA             -
YelsilC             -
Fwhite              -
```

Scleroderma group comparison

```
YelsilB         ------------------------------GCGGAAGGATC-TTATC-GAAATAGAACCTC 29
S.citrinum      ------ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCTGAAATAGAACCTC 54
WhiteB          GTCGTACAAGGTTTCCTGTAGGTGAACCTGCGGAAGGATCATTATC-GAAATAGAACCTC 59
                                        *********** ***** *************

YelsilB         TAGGGAGGGGGAGGGGGAGAGTCCTCGCGTCGCTCCTTCGAACCTTTCCGAGGCTTCATA 89
S.citrinum      TAGGGAGGGGGAGGGGGAGAGTCCTCGCGTCGCTCCTTCGAACCTTTCCGAGGCTTCATA 114
WhiteB          TAGGGAGGGGGAGGGGGAGAGTCCTCGCGTCGCTCCTTCGAACCTTTCCGAGGCTTCATA 119
                ************************************************************

YelsilB         CCTCCCACACCCGTGTGCACCCGCTGTAGGTGCCTTAGGGACCTATGTCTTCTCAGAACT 149
S.citrinum      CCTCCCACACCCGTGTGCACCCGCTGTAGGT-CCTTAGGGACCTATGTCTTCTCAGAACT 173
WhiteB          CCTCCCACACCCGTGTGCACCCGCTGTAGGT-CCTTAGGGACCTATGTCTTCTCAGAACT 178
                ****************************** ****************************

YelsilB         CGCATGTCTACAGAATGTCGTCCGTGGCCTCGGCCATCGTAAACCTAGTACAACTTTCAG 209
S.citrinum      CGCATGTCTACAGAATGTCGTCCGTGGCCTCGGC-ATCGTAAACCTAGTACAACTTTCAG 232
WhiteB          CGCATGTCTACAGAATGTCGTCCGTGGCCTCGGCCATCGTAAACCTAGTACAACTTTCAG 238
                ********************************** *************************

YelsilB         CAATGGATCTCTTGGCTCTCGCATCGATGAAGGACGCAGCGAATCGCGATAAGTAATGTG 269
S.citrinum      CAATGGATCTCTTGGCTCTCGCATCGATGAAGGACGCAGCGAATCGCGATAAGTAATGTG 292
WhiteB          CAATGGATCTCTTGGCTCTCGCATCGATGAAGGACGCAGCGAATCGCGATAAGTAATGTG 298
                ************************************************************

YelsilB         AATTGCAGATTTTCCGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTATTC 329
S.citrinum      AATTGCAGATTTTCCGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTATTC 352
WhiteB          AATTGCAGATTTTCCGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTCGGTATTC 358
                ************************************************************
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YelsilB         CGAGGAGCATGCCTGTTTGAGTGTCATCGAAATATCAAATCGAAGCTTTC-GACCCCCGT 388
S.citrinum      CGAGGAGCATGCCTGTTTGAGTGTCATCGAAATATCAAATCGAAGCTTTC-GCCCCCGGT 411
WhiteB          CGAGGAGCATGCCTGTTTGAGTGTCATCGAAATATCAAATCGAAGCTTTCAGACCCCCGT 418
                ********************************************** * **** **

YelsilB         CGGAGCTAGGTTTG-ACCATGGA-GTCTGCGGGCGGATCCCT--CTGTCGGGAGGGGGAC 444
S.citrinum      CGAAGCTAGGTTTGGACCATGGAAGTCTGCCGGCGGGTCCCCTCCTGTCGGAAGGGGGAC 471
WhiteB          CGGAGCTAGGTTTGGACCATGGGAGTCTGCGGGCGGGTCCCCTCCTGTCGGGAGGGGGAC 478
                ** ********** *******  ****** ***** ****   ******* ********

YelsilB         CTTCGG---TCTCTAAAAGCATTAGCG-CGTGTGCAAGCTTGAC---GGACGGCTTTTCA 497
S.citrinum      TTTCGGCTCTCCTCAAAAGCATTACCGGCGTGTGCAAGCCTGGCACGGCACGGCCTCTTC 531
WhiteB          CTTCGGCTCTCCTCAAAAGCATTAGCGGCGTGTGCGAGCCTCGCACGGCACGGCCTCTTC 538
                 *****   **   ********** ** ******* *** *  *   * ***** * *

YelsilB         CGTAT-ATGACCGC-------------------------------------------------- 510
S.citrinum      GACGTCATAATGACCCGTCGTGGCCTGAAAGTGCGTGGATCGCGGTCCCTTTCCACCTTT 591
WhiteB          GACGTCATAATGACC-GTCGTGGGCTGGAAGTGCGTGGATCGCG-TCGCTTTCCAACTCT 596
                    * ** *   *

YelsilB         ------------------------------------------------------------
S.citrinum      GCAAGCCGGTCTCGGACGGGCCGCGCGTCATCGATGTTTGACCTCAAATCAGGTAGGACT 651
WhiteB          GCGAGCCCGTCTCGGACGGGCCGCGCGTCATCGATGTTTGACCTCAAATCAGGTA----- 651


YelsilB         ------------------------------------------------------------
S.citrinum      ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACGAGGATTCCCCTA 711
WhiteB          ------------------------------------------------------------


YelsilB         ------------------------------------------------------------
S.citrinum      GTAACTGCGAGTGAAGCGGGAAGAGCTCAAATTTCGAATCTGGCGGTCCTCGGCCAGCCC 771
WhiteB          ------------------------------------------------------------


YelsilB         ----------------------
S.citrinum      GAGTTGTAAATCTAGAGAAGC 792
WhiteB          ----------------------
```

D17A Tuberculate

CAAGGTTTCCTGTAGGTGAACCTGCGGAAGGATCATTAAACGAGTAAGAGGTCTCTGGCCCGAACC
TGCCCCCTCTGTTGTTGAAACTACCTTGCTCGCTTTGGCGGGACCGTTGGTCTCCGAGCGCGCCG
GTCTTCGGATTGGCGAGCGCCCGCAGAGTCCAACCAAACTCTTGTATTAAACCAGTCGTCTGAGTA
TAAAATTTTAATTTAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCAGATGAAGAACAGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CCGCCCCTTGGTATTCCGAGGGCATGCCTGTTACGAGCGTCATTACACCACTCAAGCACTGCTTGG
TATTGGGCATCGTCCGTCGAAAGACGGGCGTGCCTGAAGACCTCGGCGGGGCCTAACCGGCTTC
GGGCGTAGTAGAGTTAAATCGAACGTCTTATGAGTCTGGTTAGAACCCATTGCCGTAAAACCTTATT
ATTTTATAGGTTGACCTCGGACAG

Black B (Brooklands) *Phialocephala fortinii*

TACGTGAACCTGCGGAAGGATCATTACAAGTGAGGCTACCGAACGTTGGAAACAGCGGTTAGGAG
CTTACACCCACCCGTGTTTACATACTATTGGTGCTTTGGCGGGCCGTGGCCTCCACTGCGGGCTCT
GCTCGTGTGTGCCCGCAGAGAACCAAACTCTGAATGTTAGTGATGTCTGAGTACTATATAATAGTTA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCA

GGGCATGCCTGTTCGAGCGTCATTTAACCACTCACGCCTAGCGTGGTATTGGGGCACGCGGGTCT
CCGCGGCCCTCAAAATTAGTGGCGGCGCCGGTGGGCTCTAAGCGTAGTACATACTCCCGCTATAG
AGTTCCCCCGGGGCTCGCCAGAACCCCCAATTTTTACAGGTTGAC

Beige/White (*Heterobasidion annosum*)
CGCGGAAGGATCATTAACGAATATCGTGCGGGGTTGAAGCTGGCCTCTCGGGGCATGTGCTCGCC
TTGTTCATATCCATCTCACACCTGTGCACACTCGCGTGGGTCGGTCGGGCTCTTTTTTGCGCCCTT
CCGAGCCGCGTCTTCTCACAAACTCTTCGTATGTCTTCAGAATGGTATCAATGCTATAAAACGCATC
TTATACAACTTTCAACAATGGATCTCTCGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATT
CCGAAGGGCACGCCTGTTTGAAGTGTCGTGAAATTCTCAACCCTGCGCTTTCTTGTGAAAGCGCGT
GGGCTTGGACTTGGAGGCTTGCTGGTCTCGCGGATCGGTTCTCTAAATGCATTAGCAGACCTTGTG
GTGCGCCCCGGTGTGATAATTGCTACGCCG

Table E Species synonyms of ECM fungi.

Species Name as used in the current study	**Synonyms in current literature**
Amanita *excelsa*	*Amanita ampla* *Amanita cariosa* *Amanita spissa*
A. muscaria	*Amanita muscaria var. formosa* *Amanita muscaria var. puella*
A. rubescens	none
Clavulina *cf. cinerea*	*Clavaria cinerea* *Clavaria cinerea var. gracilis* *Clavaria fuliginea* *Ramaria cinerea*
C. cristata	*Clavaria coralloides* *Clavulina cristata* *Clavulina cristata f. subcinerea* *Clavulina cristata var. cineroides* *Clavulina cristata var. coralloides* *Clavulina cristata var. incarnata* *Clavulina cristata var. lappa* *Clavulina cristata var. subrugosa*
Inocybe *cf. griseolilacina*	none
Inocybe specimen 1	-
Lactarius *hemicyaneus*	*Lactarius quieticolor*
L. hepaticus	*Lactarius theiogalus*
L. semisanguifluus	none
Scleroderma *citrinum*	*Scleroderma aurantiacum* *Scleroderma aurantium* *Scleroderma vulgare*
Suillus *granulatus*	*Boletus granulatus* *Xerocomus granulatus*
S. luteus	*Boletus luteus* *Xerocomus luteus*
S. salmonicolor	*S. subluteus, S. pinorigidus*
Tricholoma *albobrunneum*	*Agaricus albobrunneus*

Species Name as used in the current study	Synonyms in current literature
Xerocomus *badius*	*Boletus badiorufus* *Xerocomus badius* *Xerocomus badiorufus* *Xerocomus badius*
X. cf. chysenteron	*Boletus chrysenteron*
X pruinatus	*Boletus pruinatus* Fr. & Hök
Russula *specimen 1* *Specimen 3* *Specimen 4* *Specimen 6* *Specimen 7*	
R. drimeia Fr.	*Russula chrysodacryon*

Appendix 4

4.1 Sporocarp-sporocarp sequence comparison

As a consequence of the results presented in Table 5.1, two particular sporocarp collections required sequence comparisons. The *Russula* group (Table 5.4) and the two collections of *Clavulina cristata* were subjected to sequence alignment on the Genbank website (Altschul *et al*., 1997). *Clavulina cristata* and *C.* cf. *cinerea* sporocarps were compared on Genbank (Altschul *et al*., 1997): the Identity was 99% (796/803) and the gaps 0%, Score bits 1436, and e-value was 0.0 indicating near identical matches.

Sequences of each *Russula* specimen were aligned systematically with each of the other specimens on Genbank (Altschul *et al*., 1997). Score bit values, e-values, sequence identity and gap values are given (the two latter values also given as a percentage). *R. drimeia* column has a number of values. This is due to the very poor alignment achieved. Genbank (Altschul *et al*., 1997) managed to align parts of the sequences and the values given are alignment for each segment.

Table F Sequence comparisons of *Russula* specimens.

	***R. laricina* Sp3**	***R. laricina* Sp4**	***R. laricina* Sp6**	***R. laricina* Sp7**	**R. drimeia**
R. laricina Sp1	S=1275 E = 0.0 I = 732/753 (97%)	S = 1404 E = 0.0 I = 754/765 (98%)	S = 1444 E = 0.0 I = 768/778 (98%)	S = 1369 E = 0.0 I = 752/766 (98%)	S = 269, E = 7e-69 I = 154/156 (98%) S = 127, E = 4e-26 I = 122/140 (87%)
R. laricina Sp3		S = 1281 E = 0.0 I = 733/753 (97%)	S = 1294 bits E = 0.0 I = 733/752 (97%)	S = 1283 E = 0.0 I = 724/744 (97%)	S = 260, E = 5e-66 I = 137/138 (99%) S = 127, E = 4e-26 I = 122/140 (87%) S = 100, E = 6e-18 I = 68/76 (89%)
R. laricina Sp4			S = 1394 E = 0.0 I = 754/765 (98%)	S = 1354 E = 0.0 I = 744/758 (98%)	S = 269, E = 7e-69 I = 154/156 (98%) S = 260, E = 5e-66 I = 137/138 (99%) S = 127, E = 4e-26 I = 122/140 (87%) S = 100, E = 6e-18 I = 72/82 (87%)
R. laricina Sp6				S = 1386 E = 0.0 I = 752/764 (98%)	S = 269, E = 7e-69 I = 154/156 (98%) S = 260, E = 5e-66 I = 137/138 (99%) S = 127, E = 4e-26 I = 122/140 (87%)
R. laricina Sp7					S = 28, E = 2e-72 I = 199/218 (91%) S = 260 bits, E = 5e-66 I = 137/138 (99%) S = 127, E = 4e-26 I = 122/140 (87%)

* S = score bit value, E = e values and I = identity. Blue highlights emphasis significant dissimilarities. Blue indicates specimen three slightly more dissimilar to all others and Pink indicates dissimilarity between *R. drimeia* and all other specimens.

4.2 Morphotype-morphotype sequence comparisons

The ECM morphotypes have been grouped according to BLAST results. This means that a number of different morphotypes with the same BLAST result (Table 5.2), may be taken as one species. To further determine the strength of this statement, ECM morphotypes within the groups were compared to each other. Pair-wise comparisons were conducted on the *Thelephora* and *Amanita* group (Table 5.5 and Table 5.6).

Table G Pair-wise Comparison of morphotype sequences that were BLASTed as *Thelephora* spp.

	Partial White	**Black (D35)**	**Yelsil A**	**Yelsil C**
Black (D35)	S = 956, E = 0.0 I = 532/537(99%)			
Yelsil A	S = 1040, E = 0.0 I = 590/597(98%)	S = 840, E = 0.0 I = 507/517 (98%)		
Yelsil C	S = 1040, E = 0.0 I = 601/611(98%)	S = 877, E = 0.0 I = 519/528 (98%)	S = 1069, E = 0.0 I = 588/594 (98%)	
Fwhite	S = 671, E = 0.0 I = 531/597(88%)	S = 610, E = e-171 I = 457/507 (90%)	S = 656, E = 0.0 I = 511/576 (88%)	S = 656, E = 0.0 I = 510/577(88%)

*S = score bit value, E = e values and I = identity.

Four different morphotypes formed part of an *Amanita spissa* 'group' based on the results from Table 5.2. The morphotypes were compared to one another to test how similar their sequences were to determine whether, in fact, they were the same species. Table 5.6 compares the morphotypes 'BSY', 'BSY1, 2, 4 & 5', 'White', 'Grey/pink' and 'yellow orange'.

Two morphotypes, 'BSY 4' and 'White', stand out against the rest of the morphotypes in that the Score bit values and identity percentages were lower in pair-wise comparison to the other morphotypes. 'BSY' and 'White' are well matched to one another (purple cell) as the other morphotypes are to each other (Blue, Yellow, Green and Brow column).

Table H Pair-wise comparisons of the sequences of "*Amanita*" morphotypes.

	Grey/pink	White A	BSY1	BSY2	BSY4	BSY5	BSY
White A	S= 660, E = 0.0 I = 442/469 (94%)						
BSY1	S = 1210, E = 0.0 I = 643/645 (99%)	S = 646, E = 0.0 I = 442/470 (94%)					
BSY2	S = 1210, E = 0.0 I = 643/645 (99%)	S = 646, E = 0.0 I = 442/470 (94%)	S = 1269, E = 0.0 I = 674/676 (99%)				
BSY4	S = 885, E = 0.0 I = 605/650 (93%)	S = 833, E = 0.0 I = 472/479 (98%)	S = 885, E = 0.0 I = 605/650 (93%)	S = 885, E = 0.0 I = 605/650 (93%)			
BSY5	S = 1210, E = 0.0 I = 643/645 (99%)	S = 646, E = 0.0 I = 442/470 (94%)	S = 1256, E = 0.0 I = 674/677 (99%)	S = 1288, E = 0.0 I = 677/678 (99%)	S = 862, E = 0.0 I = 599/650 (92%)		
BSY	S = 1198, E = 0.0 I =623/623(100%)	S = 660, E = 0.0 I = 442/469 (94%)	S = 1186, E = 0.0 I = 624/625 (99%)	S = 1186, E = 0.0 I = 624/625 (99%)	S = 842, E = 0.0 I = 573/621 (92%)	S = 1186, E = 0.0 I = 624/625 (99%)	
Yellow orange	S = 1210, E = 0.0 I = 643/645 (99%)	S = 660, E = 0.0 I = 442/469 (94%)	S = 1210, E = 0.0 I = 643/645 (99%)	S = 1210, E = 0.0 I = 643/645 (99%)	S = 887, E = 0.0 I = 605/650 (93%)	S = 1210, E = 0.0 I = 643/645 (99%)	S = 1186, E = 0.0 I = 624/625 (99%)

S = score bit value, E = e values and I = identity. All highlighted cells are significant similarities in terms of the high score bit values, low e- values and strong identities.

Appendix 5

Table I Sequence accession number from UNITE and Genbank databases.

ECM fungal species	UNITE	Genbank
Albatrellus ovinus		AY198203
Albatrellus pes-caprae	UDB000691	
Albatrellus syringae		DQ789394
Amanita rubescens	UDB 000037 UDB000038	
Amanita regalis	UDB000710	
Amanita virosa	UDB001112	
Amanita spissa	UDB000083	AF085486
Boletus edulis	UDB000759	
Boletus radicans	UDB000761	
Clavulina cinerea	UDB000074	
Clavulina cristata	UDB000007 UDB000052	
Clavulina rugosa	UDB000057	
Clavulina monodiminutiva		DQ056372
Lactarius deterrimus	UDB000297	
Lactarius hepaticus	UDB000861	
Lactarius quieticolor	UDB000321 UDB000880	
Lactarius semisanguifluus	UDB000320	
Pseudotomentella tristia	UDB000029	

Ramaria stricta		DQ367910
Rhizopogon roseolus		AM084707
Russula caeruea	UDB000335	
Russula laricina		AY061685
Russula sororia		AB211275
Russula foetens	UD000065	
Russula sardonia		AF41826
Russula drimeia		AY061672
Suillus granulatus	UDB000650	
Suillus luteus	UDB000663	
Thelephora caryophyllea	UDB000119	
Thelephora penicillata	UDB000214	
Thelephora terrestris	UDB000215	
Tomentella badia	UDB000238	
Tomentella bryophila	UDB000035	
Tomentella fuscocinerea	UDB000240	
Tomentella lateritia	UDB000267	
Xerocomus badius	UDB000050	
Xerocomus chrysenteron	UDB000439	
Xerocomus pruinatus	UDB000008	

Mycorrhizal status of indigenous tree species in a forest biome of the Eastern Cape, South Africa

Greer L. Hawley* and Joanna F. Dames*†

Mycorrhizal fungi are intimately associated with plant roots, affecting plant growth, health and increasing the plants' tolerance to environmental stress. Several mycorrhizal types are recognized based primarily on morphological characteristics within plant roots. When considering propagation and management of an indigenous plant species, it is essential to know its mycorrhizal status. Root samples from 17 tree species common to the pockets of forest in the Eastern Cape province, and representing the families Rubiaceae, Scrophulariaceae, Oleaceae, Podocarpaceae, Myrsinaceae, Anacardiaceae, Caesalpinoideae, Papilionoideae, Rutaceae, Meliaceae, Celastraceae, Flacouticeae and Ebenaceae, were sampled and examined for mycorrhizal colonization. Microscopic examination of all the species produced evidence of morphological structures indicative of endomycorrhizal associations as indicated by the presence of intercellular hyphae combined with vesicles, arbuscules or hyphal coils. Hyphal coils (also known as *Paris*-type associations) appeared to be abundant, especially within the *Cassine* genus. Arbuscules (also known as *Arum*-type associations) were scarce but sometimes present, and vesicles were prolific in *Olea capensis.* Most of the tree species examined have been assigned arbuscular mycorrhizal status. No ectomycorrhizal associations were recorded.

Introduction

The management and conservation of forest biomes is a recognized priority on a global scale. Integral to the understanding of forest ecology is the rhizosphere and in particular the mycorrhizal symbiotic associations between plant hosts and fungi. Mycorrhizal relationships are an example of mutual symbioses, involving plants and fungi, whereby both organisms benefit through an exchange of nutrients at the root–soil interface.[1] A number of different types of mycorrhiza can be identified by the hyphal structures they form, but only two types that are relevant to this study are discussed here.

Arbuscular mycorrhizas (AM), sometimes referred to as endomycorrhizas, are formed predominantly by the fungal group Glomeromycota.[2] The association is identified by intracellular dichotomously branching haustorial structures called arbuscules, found in the cortical plant root cells and hyphal coils, as well as intercellular hyphal networks and external hyphae that extend into the soil. AM are found on a wide range of host species, predominantly colonizing herbaceous shrubs and tree species. AM colonization has no visible effect on root morphology.[3] Ectomycorrhizas (ECM), on the other hand, form an outer sheath (mantle), an internal, intercellular network of hyphae (Hartig net) and extra-radial hyphal networks and rhizomorphs. ECM fungi have a visible effect on root morphology. Root tip branching often becomes dichotomous or irregular. The root tip also tends to swell and the mantle may colour the area of colonization. ECM are formed predominantly by the group Basidiomycotina, to a lesser extent by Ascomycotina and only rarely by Zygomycota,[1] and are largely associated with tree species. ECM fungi are more host specific, with associations being more dominant in temperate and tropical forest regions. AM fungi, on the other hand, are generalists, occurring in most vegetation types.[1] There are other mycorrhizal types but these are generally more specific in their host range and fungal partners and are not mentioned in this paper.

Mycorrhizal associations play important roles in the ecological functioning of ecosystems, forming an integral part of the nutrient cycle. Mycorrhizal fungi break down organic nutrients, assimilate inorganic nutrients and transport these to the plant. This is necessary for the survival of over 90% of all flowering plants.[1] Mycorrhizal associations are essential to forest biome ecology,[1] and have been shown to support the diversity of higher plants in experimental models.[4] Recent research in the rainforests and Miombo forests of central, east and southern Africa reveal a large diversity of ECM fungi.[5–9] Hogberg[5] detailed the mycorrhizal status of species representative of a number of plant families, namely, Caesalpiniaceae, Dipterocarpaceae, Euphorbiaceae, Papilionaceae, Proteaceae (*Faurea saligna*), Mimosaceae and Stilaginaceae. Such studies have been concentrated in Tanzania, Zambia and Zimbabwe, with little research carried out in South Africa. Allsopp and Stock[10] and Skinner[11] have outlined the mycorrhizal status of some Western Cape fynbos species and Eastern Cape shrubs, respectively. Most of these plant species were recorded as having AM associations. Although both studies included some tree species, most of the accounts related to herbaceous shrubs. Fungal records[12] indicate that ECM fungi, such as *Amanita, Cantherellus* and *Boletus,* have been collected in Eastern Cape forests, in particular Knysna and Tsitsikamma.

The objective of this study was to determine the mycorrhizal status of selected indigenous tree species in the Eastern Cape. Tree species that predominantly make up the Eastern Cape forest biome were chosen and identified in the field. Root samples were taken for macroscopic and microscopic examination and the assessment of mycorrhizal status.

Materials and methods

Sampling site and tree selection

Grahamstown (26°31′E, 33°18′S) is situated on the eastern border of the Cape Fynbos region, in the Eastern Cape. [13] The areas sampled around the city lie within the 'Albany Hotspot',[14] where five major African phytochoria meet. Having formed part of a conservancy, this area has retained a high plant species biodiversity.[14] The natural pockets of forest surrounding Grahamstown, and particularly in the Featherstone Kloof, are relatively young. The Grahamstown area is approximately 700 m above sea level and receives an annual rainfall of 400–600 mm. The mean summer temperature (November–May) is 22–24°C, with a mild mean winter temperature (June–October) of 12–14°C.[15] Samples were taken during the summer (December 2003 – January 2004), the growing season for the vegetation, but

*Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, P.O. Box 94, Grahamstown 6140, South Africa.
†Author for correspondence. E-mail: j.dames@ru.ac.za

this was also a relatively dry period, in which only 38 mm fell.[13]

Seventeen species representative of 16 genera from 13 families were examined in this study, each selected on the likelihood of finding ECM within certain tree families (Table 1).[16] The tree species were identified taxonomically in the field, and root samples were collected. The soil at the base of the tree was loosened using a hand fork and roots were followed from the base of the tree to the feeder roots to ensure accuracy of identification. Approximately 15–20 g of root material was collected in each case.

Processing and examination of root samples

Freshly collected roots were examined under a dissecting microscope (Leica S4E) to identify any external mycorrhizal features, such as mantles, rhizomorphs and hyphal networks. The presence of these features provides preliminary indication of ECM associations.[17] The roots were washed gently with water to remove excess soil particles. A sub-sample of the roots was then cleared and stained using the procedure described in Brundrett *et al.*[18] Ten grams of root material was heated to 90°C in 5% KOH for 30 min to soften the root, then bleached in alkaline H_2O_2 for 30–40 min. Some of the root samples had high phenolic concentrations and required longer bleaching times. The roots were then acidified with 0.1 M HCl overnight and heated to 90°C with lactoglycerol trypan blue stain for 30 min; this was followed by destaining in lactoglycerol for 24 h. The remaining roots were stored in 50% ethanol for preservation, should additional analyses become necessary.

Stained roots were examined for both external and internal hyphal structures under a compound microscope (Leica CME). Each slide examined contained 4–5 root pieces 2 cm long. For each species, five slides were prepared, such that between 20 and 25 root pieces and 40–50 cm of root were examined per tree species. All the fungal structures were recorded and tabulated, and the mycorrhizal status determined. The presence of a mantle and Hartig net would be evidence of ECM status. Roots were regarded as AM only if either vesicles and/or arbuscules were found. The presence of hyphae alone was not regarded as evidence of AM status, although hyphal networks were noted. Photographs were taken on black and white film, as well as digitally, under the DIC microscope (Olympus BX50).

The most defining morphological characteristic of AM associations is the presence of arbuscules within cortical root cells. These are regarded as the interface of nutrient exchange between plant and fungus.[1,25] Two types of arbuscules are recognized and are termed *Arum* type and *Paris* type. *Arum*-forming mycorrhizas are typical of fast-growing plants. The fungus colonizes the plant rapidly through intercellular hyphae and the intracellular arbuscules are short-lived. Coils may form, but are not common.[1] *Paris*-forming mycorrhizas have a slower infection rate, passing from cell to cell within the plant cortex, and can be characterized by extensive intracellular coiled hyphae with arbuscules forming from the coils. The coil structures are suspected of functioning as active nutrient exchange interfaces, as the surface area the coil creates is comparable to that of *Arum*-type arbuscules in a cortical cell.[1,26]

Table 1. List of families, genera and species sampled and examined for mycorrhizal status.

Family	Genus and species
Rubiaceae	*Burchellia bubaline* (Linn. F.) Sims
Scrophulariaceae	*Halleria lucida* L.
Oleaceae	*Olea capensis* L.
Podocarpaceae	*Podocarpus latifolius* (Thunb.) R. Br ex Mirb.
Myrsinaceae	*Rapanea melanophloeos* (Mez)
Anacardiaceae	*Harpephyllum caffrum* Bernh. ex Krauss; *Rhus chirindensis* Baker f.
Caesalpinoideae (Fabaceae)	*Schotia afra* (L.) Thunb.
Papilionoideae (Fabaceae)	*Psoralea pinnata* L.
Rutaceae	*Vepris lanceolata* (Lam.) G. Don
Meliaceae	*Ekebergia capensis* Sparrm.
Celastraceae	*Cassine aethiopica* Thunb.; *C. papillosa* (Hochst.) Kuntze
Flacourtiaceae	*Kiggelaria africana* L.; *Scolopia mundi* (Eckl. & Zeyh.) Warb.
Ebenaceae	*Diospyros dichrophylla* (Gand.) De Winter; *D. scabrida* (Harv. ex Hiern) De Winter

Results

Examination of roots using a dissecting microscope indicated no external structures, and therefore no obvious ECM associations. Observing the roots under the compound microscope revealed several mycorrhizal fungal structures in the root as presented in Table 2; examples of these are shown in Fig. 1a–h. The presence of nodules was recorded on *Podocarpus latifolius* (Fig. 2). Table 2 indicates that *Psoralea pinnata* roots also bore nodules that are associated with nitrogen-fixing bacteria.[19] Examples of coils representing *Paris*-type associations (Fig. 1g,h) were found in roots of *Cassine aethiopica* and *Cassine papillosa.* This AM type was

Table 2. Fungal features found in the roots of each species examined, in terms of mycorrhizal characteristics.

Species	Hyphae	Vesicles	*Arum* arbuscules	*Paris* coils	Intraradical spores	Other structures	Type of association
Burchellia bubalina	+	+	–	+	–		AM
Halleria lucida	+	+	–	+	–		AM
Olea capensis	+	++	+	+	–		AM
Podocarpus latifolius	+	+	–	+	–	Nodules	AM
Rapanea melanophloeos	+	+	+	+	+		AM
Harpephyllum caffrum	+	–	+	+	–		
Rhus chirindensis	+	+	+	+	–		AM
Schotia afra	+	–	–	+	–		
Psoralea pinnata	+	+	+	+	+	Nodules	AM
Vepris lanceolata	+	–	–	+	–		AM
Ekebergia capensis	+	–	+	+	–		AM
Cassine aethiopica	+	–	–	++	–		AM
Cassine papillosa	+	+	–	++	–		AM
Kiggelaria africana	+	+	+	+	–		AM
Scolopia mundii	+	+	+	+	–		AM
Diospyros dichrophylla	+	+	–	–	–		Endo
Diospyros scabrida	+	–	+	+	–		AM

Results are presented as features absent (–) or present (+). Species displaying large frequency of certain features are designated by (++).

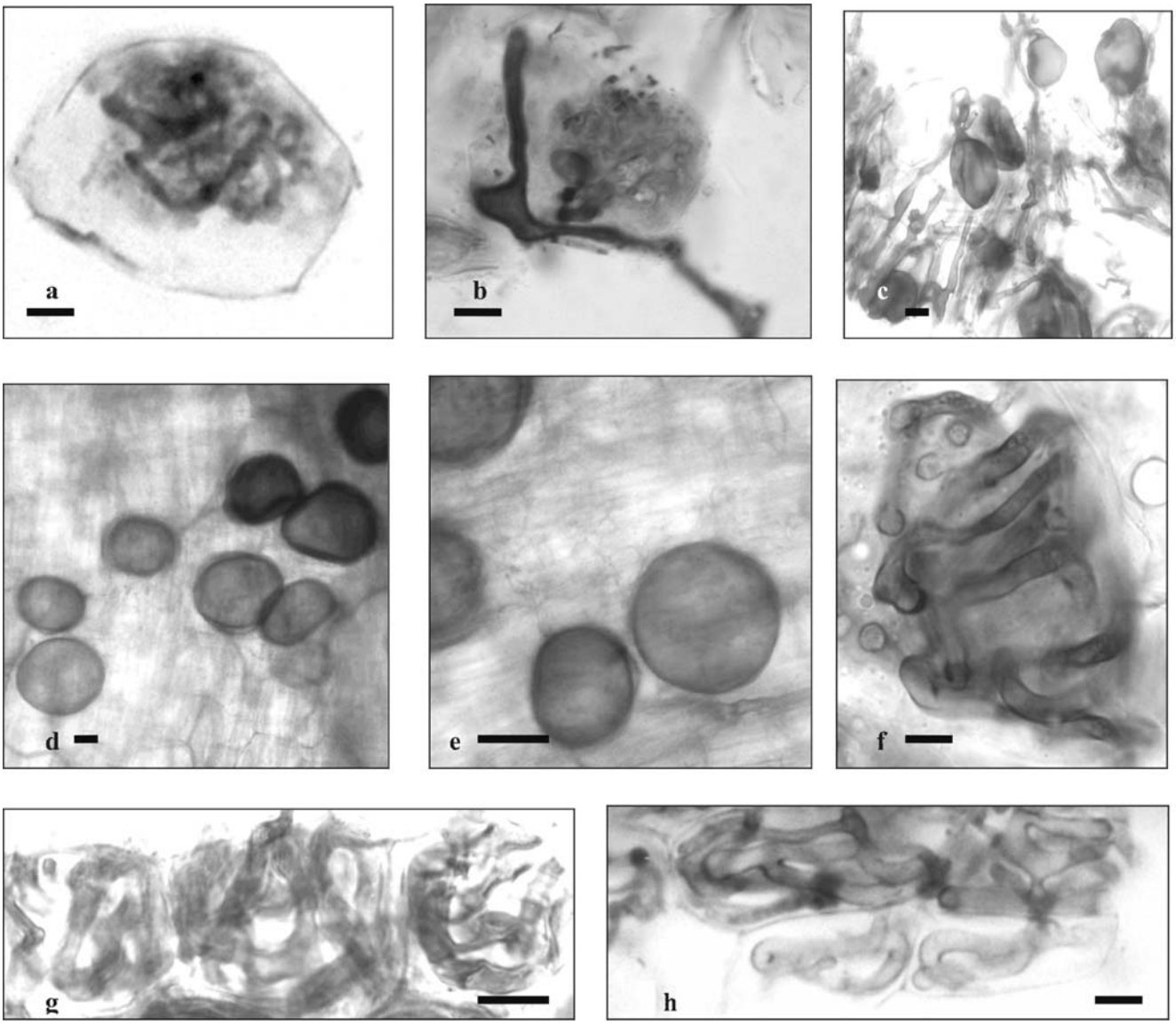

Fig. 1. **a**, **b**, *Arum*-type arbuscules found in *Psoralea pinnata*; **c**, vesicles of *P. psoralea*; **d**, **e**, vesicles of *Kiggelaria africana*; **f**, *Paris*-type coils found in *Burchellia bubaline*; **g**, **h**, *Paris*-type coils in *Cassine papillosa*. Scale bars = 1 µm.

prevalent in most species examined with the exception of *Diospyros dichrophylla* (Table 2). *Arum*-type associations (Fig. 1a,b) were less common but found together with coils in the same root. Vesicles were found within most of the species with the exception of *Vepris, Ekebergia, Cassine aethiopica* and *Diospyros scabrida* (Table 2).

Discussion

This study found that the majority of tree species examined were associated with AM fungal structures and were therefore given AM status (Table 2). Similar results have been found in other investigations, a summary of which is provided in Table 3. Within the family Rubiaceae, Skinner,[11] Allsopp and Stock,[10] Harley and Harley,[20] and Högberg[5] found that the species examined were AM, or endomycorrhizal. The exception, *Rubia peregrina*,[20] was the only ECM species out of 11 species examined.

Skinner[11] reported the family Scrophulariaceae, in the Eastern Cape, to be AM. Our study and Allsopp and Stock[10] confirm the AM status given to *Halleria lucida* (Tables 2 and 3). Harley and Harley[20] suggest that the many species they listed may either have AM fungi or be non-mycorrhizal, having no fungal association at all (Table 3). *Olea capensis* (Oleaceae), reported by Allsopp

Fig. 2. Nodules on *Podocarpus latifolius*. Scale bar = 10 µm.

Table 3. Comparative results from previous studies within the same plant families.

Family	Skinner[11]	Allsopp and Stock[10]	Harley and Harley[20]	Wubet *et al.*[21]	Högberg[5]	This study
Rubiaceae	AM	AM	1 of 11 spp. is ECM (*Ruba peregrina*); the rest are AM	–	Endo	AM
Scrophulariaceae	AM	AM	AM or absent (many spp.)	–	–	AM
Oleaceae	–	Endo	*Fraxinus excelsor* AM and ECM	AM	–	AM
Podocarpaceae	–	AM	–	AM	–	AM
Anacardiaceae	AM	AM	–	–	AM	AM
Caesalpinoideae	–	–	–	–	*Bautinia* spp. AM *Brachystegia* spp. ECM *Julbernardia globiflora* ECM	AM
Papilionoideae	–	–	–	–	AM	AM
Rutaceae	AM	–	–	–	–	AM
Meliaceae	–	–	–	AM	–	AM
Celastraceae	–	AM	–	–	AM	AM
Flacourtiaceae	–	–	–	–	AM	AM
Ebenaceae	–	AM	–	–	AM	AM

–, No data; AM, arbuscular mycorrhizal; ECM, ectomycorrhizal; Endo, endomycorrhizal.

and Stock[10] as AM, is confirmed by our study. Other species of Oleaceae examined by Wubet *et al.*[21] indicated AM colonization. Harley and Harley,[21] however, report *Fraxinus excelsor* to be either AM or ECM.

Wubet *et al.*[22] and Allsopp and Stock[10] recorded that *Podocarpus falcatus* (Podocarpaceae) associates with AM fungi (Table 3); this was confirmed for *P. latifolius* (Table 2). Nodule-like structures (Fig. 2) were observed in the roots of *Podocarpus* species. *Podocarpus henkelii* (Stapf ex Dallim & Jacks) as well as other species sampled from different regions in the country (results not presented) also displayed root nodules. These nodules were not of ECM origin, although ECM fungi such as *Scleroderma* sp. and *Amanita* sp. have been observed growing below or near *Podocarpus* species in forestry areas in Sabie, Mpumalanga, and Hogsback, Eastern Cape. These nodules have been referred to as mycorrhizal when mycorrhizal fungi are present and/or actinorrhizal when they contain N-fixing bacteria such as *Frankia*.[22] They have been recorded for a number of gymnosperm families such as Podocarpaceae[23] and angiosperm families such as Casuarinaceae.[22]

The family Myrsinaceae was not included in any of the comparative studies, but in our study the species *Rapanea melanophloeos* was found to be AM (Table 2). The *Rhus* species (Anacardiaceae) examined in this study as well as species examined by Skinner[11] and Allsopp and Stock[10] all record AM colonization (Tables 2 and 3). AM status was also assigned to *Lannea schimperii*.[5]

Several species within the family Caesalpinoideae were examined by Högberg[5] and recorded as being either AM or ECM (Table 3). He also noted that none of these species bore nitrogen-fixing nodules (although they are members of the Leguminaceae), but that all Papilionoideae species examined were nodulated. *Schotia afra* (Caesalpinoideae) did not bear any nodules, which concurs with Högberg's[5] findings, and was found to be AM. Members of genera within this family deserve more thorough examination, as the presence of ECM seems to be established.[5]

Psoralea pinnata (Papilionoideae) was found to be AM, in agreement with Högberg,[5] who recorded this status for all five species examined (Table 3). Nodules were also observed on roots (Table 2) and this was confirmed to be an association with nitrogen-fixing rhizobia.[19] Tripartite associations between the N-fixing bacteria, AM and legumes are well known[24] and indeed evidence of this was found on the roots of *P. pinnata,* where hyphae and vesicles were present inside root nodules (Table 2).

Skinner[11] examined species from the family Rutaceae and recorded that this family was AM (Table 3); this was confirmed in *Vepris lanceolata* (Table 2). This study also confirms the status of *Ekebergia capensis* (Meliaceae) as AM[21] (Tables 2 and 3). Celastraceae species were documented by Allsopp and Stock[10] and Högberg [5] as being AM (Table 3) and this was confirmed by the presence of AM structures observed in *Cassine aethiopica* and *C. papillosa* (Table 2).

Högberg[5] found *Oncoba spinosa* (Flacourtiaceae) to be AM; two other species within the same family, *Kiggelaria africana* and *Scolopia mundi,* confirmed this status. All the *Diospyros* species (Ebenaceae) examined by Allsopp and Stock[10] and Högberg[5] reveal AM colonization. *D. dichrophylla* roots that were investigated showed no evidence of arbuscule structures, but the presence of vesicles indicate that the plant is more than likely AM. More intense sampling is needed to confirm AM status convincingly.

Although a defining characteristic, arbuscules may be difficult to find under natural conditions. *Arum*-type associations were observed in low frequency and this is generally attributed to their short life span.[1] The results from this investigation show that *Paris*-type associations were more frequently encountered overall and were particularly prolific in the genus *Cassine.* Table 2 provides an indication of their presence or absence and some idea of their prolific nature. *Paris*-type coils were found in the majority of species and were generally more common (Table 2). The dominance of the *Paris* type may imply that coiled hyphae live longer and are more tolerant to environmental stress conditions such as drought, suggesting that external factors create responses that allow for the dominance of one type of mycorrhiza (*Paris* vs *Arum* type) over another. However, the arbuscular types may also be fungal species specific, with environmental conditions affecting the fungal-species composition. If arbuscular types are not fungal species specific as suggested by Kubota *et al.*,[27] then host plants may determine dominant arbuscular type, indicating that the different structures are adaptations for fungal survival. Both *Arum*- and *Paris*-type associations were observed in several plant species examined (Table 2) and this indicates that the arbuscular types are fungal-species specific, as more than one AM fungal species would be expected to colonize the host plant; this is difficult to detect when examining colonized roots. The presence of either or both types is more likely to be dictated by a combination of fungal and

host species[27] with possibly environmental influences; the relative dominance of arbuscular type is poorly understood and requires further investigation.

The family of AM fungus that has colonized the root will determine the presence (Glomineae) or absence (Gigasporineae) of vesicles and their presence is affected by the soil environment.[1] Vesicles vary from species to species in morphology and position of occurrence in the root. They contain lipids and nuclei and are generally considered to be storage organs.[1] In our study, the absence of vesicles could be attributed to either the species of fungus present, environmental factors, or simply undersampling. *Olea capensis* roots had large numbers of vesicles (Table 2), possibly ruling out the environmental factors and suggesting either an effect of host hospitality or of undersampling.

Conclusion

Allsopp and Stock[10] and Högberg[5] use the term endomycorrhiza, the former adopting it with caution. Högberg's use of the term satisfies the general definition of AM, requiring the presence of vesicle and arbuscules.

Our study provides the first account of mycorrhizal status for the following species: *Burchellia bubalina, Podocarpus latifolius, Rhus chirindensis* and *Harpephyllum caffrum, Schotia afra,* and *Vepris lanceolata.* The prevalence of AM associations reported here reflects a similar pattern to that found by Allsopp and Stock[10] and Skinner.[11] Even though these studies have not encompassed even a small percentage of the tree species of South Africa, the results confer an initial status on those investigated. No ECM associations were found in this study, although there are other species that could be considered for this possibility, for example, other genera within the Caesalpinoideae and Papilionoideae. *Afzelia quanzensis* is another promising species whose taxonomic relatives have ECM associations.[16] Special attention should be directed at the Knysna/Tsitsikamma forests and Miombo woodlands, where the presence of ECM fungi such as the species mentioned above and, in addition, species of *Clavulina* (14 species), *Lactarius* (3 species), *Cantherellus* (4 species), *Russula* (4 species), *Cortinarius* (5 species), amongst others, have been recorded.[12]

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