Arbuscular mycorrhizal community in a permanent pasture and development of species-specific primers for detection and quantification of two AM fungi

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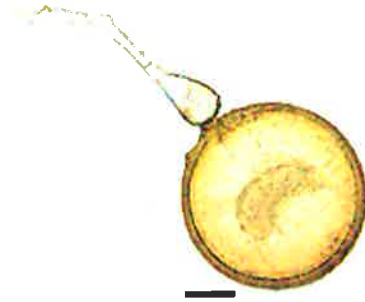In fulfilment of the requirements of

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Spores of a) *Gigaspora margarita* (Scale bar = 100 μm) and b) *Glomus mosseae* (Scale bar = 100 μm), the main species used in this thesis.

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SUMMARY

Arbuscular mycorrhizal (AM) fungi are a group of symbionts that occupy different niches during their life cycle in roots and in the rhizosphere. They occur in soil as spores, hyphae and other propagules such as colonised root fragments. During the interaction with the plant roots a bidirectional transfer of mineral nutrients and carbon occurs, frequently ensuring a positive benefit to both partners. The 152 species of mycorrhizal fungi can be difficult to identify and quantify because the taxonomy of these fungi is based on the description of spores, which are probably produced asexually. Because identification is based on spore morphology few attempts have been made to identify the species which are present in roots. Identification of AM fungal spores from field samples of spores is time consuming, requires considerable expertise and only provides information on those fungi that produce spores. It cannot be assumed that the spore population reflects the situation within the root, and therefore it is important to develop tools for identification of AM fungi during the vegetative stages. Several approaches have been tested in previous work and the development of sensitive molecular methods for identification and quantification of two species of AM fungi is described in this study.

Mycorrhizal fungal communities were sampled in both natural and agricultural ecosystems at two sites in Southern Australia, to provide a comparison between different location and cultural practices. Presence of mycorrhizal spores were used as indicators of fungal occurrence, while species composition was determined by identification of the spores. It was shown that mycorrhizal associations were common and that there was considerable fungal diversity in agro-ecosystems within direct drill and permanent pasture systems. However, in the adjacent natural or semi-natural vegetation, 80% of plant species appeared to be non-mycorrhizal and the fungal diversity and numbers of spores were very low. Based on the diversity of AM fungal populations the permanent rotation trial at the Waite Campus, South Australia was chosen for a more thorough investigation of the mycorrhizal spore population. Using the permanent pasture sampling in July and December 1996 and July and December 1997 recovered spores of thirteen species of AM fungi were recovered. Trap cultures were set up at each collection time using *Lolium perenne, Plantago lanceolata,*

Sorghum sp. and *Trifolium subterraneum* which also recovered thirteen species. Nine species were recovered by both methods; *Acaulospora* sp., *Glomus aggregatum*, *G. macrocarpum* were only recovered in field-collected soil and *Entrophospora sp.*, *G. clarum*, *G. coronatum* and *G. etunicatum* only in trap cultures. In both field-collected soil and trap cultures the dominant species was *G. mosseae*. The community diversity was not significantly different in field-collected soil at different sampling times, but it was significantly different between sampling times in trap cultures. Single spore pot cultures of *G. mosseae, G. constrictum, Glomus* sp. and *Gigaspora margarita* were established. The combination of spore identification from trap culture and field-collected soil promises to be an effective means to study diversity of AM fungi in a particular system, but more study is necessary to obtain clear picture of the activity of the AM fungal population particularly during vegetative growth.

The internal transcribed spacer (ITS) region was chosen for designing species-specific PCR primers. This required a knowledge of the genetic variability in this region in *G. mosseae* and *Gi. margarita*. To study the genetic variability in *G. mosseae* and *Gi. margarita*, sequence similarity of the ITS regions of ribosomal DNA was analysed in spores collected from the permanent pasture and from pot cultures. PCR amplification with the primers ITS1 and ITS4 was performed and products were cloned and sequenced. The sequences from single spores of *G. mosseae* and *Gi. margarita* confirmed that there is variation in the ITS region in single spores. Phenetic analysis of sequences from both species supported the morphological identification, and placed the species into two separate groups as expected. Through the analysis of these sequences an estimate of genetic diversity was derived which clearly showed that the three field spores of *G. mosseae* were at least 2 - 5 times more genetically diverse than that one single spore (field) and a pool of spores (pot culture) of *Gi. margarita*. This demonstrates that a high degree of variation exists in this natural population of *G. mosseae*.

PCR primers for *G. mosseae* and *Gi. margarita* were designed from the ITS sequences of field-collected spores, with the aim of providing tools well suited to field diagnostics. The specificity of the primers was assessed by PCR amplification of genomic DNA extracted from spores of 12 species of Glomalean (AM) fungi, from mycorrhizal roots of *Allium porrum, L. perenne, P. lanceolata, Sorghum* sp., and *T.*

subterraneum and from several non-mycorrhizal, root inhabiting fungi. Primers designed from *G. mosseae* were highly specific for spores of *G. mosseae* and roots colonised by this fungus. The primers designed form *Gi. margarita* were specific with respect to other AM fungi tested, but they amplified a fragment of a different size from *Rhizoctonia*, which is a commonly occurring root pathogen. Quantification using a DNA slot-blot hybridisation assay with synthetic oligonucleotides showed that 100 ng of total genomic DNA from 95% colonised roots contained 0.78 ng DNA of *G. mosseae*. This DNA-based quantification method could be used to estimate the amount of DNA of particular fungal species in colonised roots. The ability to differentiate between species and isolates of AM fungal symbionts using synthetic oligonucleotides has great potential for investigating fungal ecology, studying competition between species, and in field experiments where plants are inoculated with specific fungal species.

Publications from the thesis

Journals Articles:

1. Antoniolli, Z. I., Schachtman, D. P., Ophel-Keller, K. and Smith, S. E. Variation in ribosomal DNA internal transcribed spacer sequences in *Glomus mosseae* and *Gigaspora margarita* spores from a permanent pasture. *Mycological Research* (in press) (Results of Chapter 5).

2. Antoniolli, Z. I., Schachtman, D. P., Ophel-Keller, K., Herdina and Smith, S. E. Species - specific PCR primers for detection and quantification of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora margarita*. Submitted to *New Phytologist*. (Results of Chapter 6).

Conference Articles:

Antoniolli, Z. I., Smith, S. E., Ophel-Keller, K., Schachtman, D. P., and Zézé, A. (1998). Assessment of variation in sequences of ribosomal DNA internal transcribed spacers in *Glomus mosseae* and *Gigaspora margarita* from a pasture community. *In* "Second International Conference on Mycorrhiza" (U. Ahonen-Jonnarth, E. Danell, I. Fransson, O. Karen, B. Lindahl, I. Rangel and R. Finlay, eds.), Uppsala.

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of author's knowledge and belief, this thesis contains no material previously published or written by another person, except where due references is made in the text of the thesis.

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Zaida Inês Antoniolli   Date

Acknowledgments

To my husband, Silverio;

To my ten month old son, Jonas;

To my father *in memoriam;* Severino.

Chapter 1

Introduction

1.1 General Introduction

Arbuscular-mycorrhizal (AM) fungi form symbiotic associations with most land plants and are found worldwide in virtually all habitats (Harley, 1989). The positive effects of AM fungi in promoting the growth and survival of host plants under nutrient limiting conditions, as well as health and soil stability are well documented (Pfleger and Linderman, 1994; Smith and Gianinazzi-Pearson, 1988; Smith and Read, 1997). The obligate biotrophic nature of the fungi, and the difficulties in the identification of spores and vegetative structures represent a major challenge in accurately describing the ecological role of specific fungal species. Some studies have shown that the diversity of plants in a given ecosystem is dependent on the diversity of fungal symbionts (Van der Heijden *et al.*, 1998), and the relationship between fungal species and host plants (Abbott and Robson, 1991).

Understanding the significance of AM fungal diversity in natural ecosystems has been a great challenge. The fact that many plants can be colonised by most AM fungi indicates an absence of host specificity, and this could contribute to maintaining the fungal diversity in soils. Studies of AM fungal diversity and the ecology of the symbiosis have relied on data for the occurrence and frequency of different spore types and morphological methods of classification which are limited by the relatively small amount of morphological diversity between species (Dodd *et al.*, 1996; Rosendahl *et al.*, 1994). The fungi are classified on the basis of spore morphology and development. It is frequently difficult to identify the taxa in isolates field material because of the variable preservation and condition of the spores. The use of spore data alone could give rise to misleading conclusions regarding the diversity of fungal populations because spore numbers do not reflect the presence of non-sporulating fungi nor the amount of infection by particular species in plant roots. Furthermore, although the spores show low morphological diversity they are highly diverse genetically (see below). Molecular biological techniques offer the possibility of a more reliable,

sensitive and rapid method for the identification of fungal isolates, both as components of the spore populations and as vegetative structures in roots or soil. They also have the potential to provide quantitative information on the extent of vegetative development.

In recent years, the development of molecular techniques based on the polymerase chain reaction (PCR) has provided a valuable and alternative approach to morphological identification. These molecular techniques include a wide range of protocols, among which the most commonly used are: amplification of variable regions such as in the ribosomal genes Internal transcribed spacers (ITS) or Intergenic spacers (IGS), restriction fragment length polymorphism (RFLP) of PCR-generated fragments, amplification of short repeated sequences (microsatellites) and random amplification of polymorphic DNA (RAPD). Although these methods have been successfully applied to studies of arbuscular mycorrhizal fungi (Sanders *et al.*, 1996), the potential use of the ITS region to design primers to be used for species identification and quantification has not been well explored. The use of the ITS region has advantages because the genes occur in multiple copies and the regions are sufficiently variable to allow the clear discrimination between closely related species.

The morphological and genetic variability of AM fungal species, such as *G. mosseae,* is still poorly understood because most studies have focused on spores derived from pot cultures (Sanders *et al.*, 1995). In *Gi. margarita* genetic variation has been reported between spores using minisatellite PCR primers (Zézé *et al.*, 1996) and ITS regions (Lanfranco, 1999). Thus, it is important to study inter- and intra-specific variation of the fungi in natural communities and to develop primers which could improve our ability to identify and quantify these species in field material.

The extent of fungal colonisation of plant roots is also important in understanding the role of mycorrhizal fungi in ecosystem processes. A number of techniques have been used to quantify fungal colonisation in plant roots such as biochemical analysis (Frey *et al.*, 1994), isoenzymes (Hepper, 1988b; Rosendahl *et al.*, 1989), microscopy (McGonigle *et al.*, 1990) and molecular tools (Edwards *et al.*, 1997). Biochemical analysis has only been used in laboratory experimentation where

the ergosterol or chitin content has been determined in colonised root. These two biochemical compounds are not produced only by AM fungi. Isoenzyme patterns can detect the presence of single and mixed species of AM fungi in a single host plant. Microscopy can be used on field as well as laboratory material but it is time consuming and intensive work and only appropriate where the fungi have been extremely well characterised. PCR and competitive PCR reactions can be used to quantify the AM fungal DNA, but it is difficult to determine the initial concentration of fungal target DNA. The use of a specific oligonucleotide DNA probe and slot-blot hybridisation methods for the detection and quantification of AM fungi may provide a useful tool for studying the symbioses of these fungi.

Molecular tools should complement the use of morphologically based detection and identification methods and assist in understanding AM fungal biodiversity in agricultural and soil systems. This project focussed on a particular field site at which *Glomus mosseae* was the most abundant AM fungal species. *Gigaspora margarita* also occurred, but at a lower frequency (see chapter 4). These fungi were chosen as the target species for the investigation as there is a considerable amount of information about their biology and their relative frequencies in the spore communitys offered a potentially interesting comparison. *G. mosseae* is a frequently reported species in surveys around the globe and consequently, has been widely used in experimentation. Another species extensively used in experimentation is *Gi. margarita*.

1.2 The aims of this project

The broad aim of the project was to gain an understanding of the composition of AM fungal communities based on spore populations and to choose a site for fungal isolation for studies at the molecular level. The project was divided into four sections, with the following specific aims.

1) Selection of a site suitable for fungal spore isolation and the study of fungal species composition at that site based on spore populations and trap cultures. The fungal species chosen for molecular studies were *G. mosseae* and *Gi. margarita*.

2) Evaluation of the temporal variation of AM fungal species in field material and trap cultures with *Lolium perenne* L., *Trifolium subterraneum* L., *Plantago lanceolata* L. and *Sorghum* sp Moench.

3) Analysis of the inter- and intra-specific genetic diversity of *G. mosseae* and *Gi. margarita* in spores from natural ecosystems.

4) The design of species-specific PCR primers for identification of *G. mosseae* and *Gi. margarita* during their sporal and symbiotic phases and for quantification of fungal colonisation in roots.

Chapter 2

Literature Review

2.1 Introduction

Most plants in natural ecosystems form symbiotic associations with arbuscular mycorrhizal (AM) fungi, which are the most abundant zygomycete fungi in the soil. In the soil the resting spores germinate, developing a vegetative mycelium that produces appressoria in contact with host root surfaces. The appressorium produces hyphae which initiate the colonisation of the root, forming intercellular hyphae, coils, vesicles and arbuscules (Bonfante-Fasolo and Perotto, 1992). After colonisation in the root, external hyphae grow out of the root and colonise the rhizosphere in the soil. External hyphae are important for nutrient uptake from the soil solution and for the transport of nutrients to the root. Auxiliary cells and spores are formed on external mycelium originating from the root.

The entire life cycle of AM fungi is understood in general terms, but few studies have been done in relation to the way the host plant, the soil and environmental conditions may affect the timing and the extent of sporulation. AM mycorrhizal fungi life cycle is based on a symbiotic model of growth, in which the host has important effects on the hyphal growth of the fungus from a germinating spore. The presence of roots induces the fungus to stimulate hyphal growth in the roots and to develop arbuscules. These structures give the fungus the ability to utilise the roots as a nutritional source (Bécard and Piché, 1989). The life cycle is not well understood in terms of the nuclear cycle or possible occurrence of sexual stages. Furthermore, only a few studies have been done to date on nuclear division in vegetative hyphae associated with roots (Bianciotto and Bonfante, 1992) or during *in vitro* development (Bécard and Pfeffer, 1993).

The symbiotic association between plant and AM fungus has the potential to improve acquisition of nutrients such as P, and Zn by plants, increase crop yield, protect against certain root pathogens and increase tolerance to environmental stress, thus it has key significance in sustainable soil-plant systems (Smith and Read, 1997). The beneficial effects of these fungi on plant growth in the greenhouse have been well

reported (eg. Smith and Gianinazzi-Pearson, 1988).Arbuscular mycorrhizal fungi are known to have an important role in crop growth in the field. For example, cassava seems to be dependent on mycorrhizal colonisation when grown under field conditions (Sieverding, 1991). However, most studies on mycorrhizal associations have been undertaken in controlled conditions and usually with a single species of AM fungus. Relatively few studies have been carried out either in mixed plant communities such as grassland (Read *et al.*, 1976), corn and soybean cropping (Johnson *et al.*, 1991) or in natural ecosystems (Johnson, 1993; Read, 1993; Helgason *et al.*, 1998). Information on the occurrence, population dynamics and significance of AM mycorrhizal fungi in natural plant communities is still lacking.

A better understanding of the ecology of AM fungal species is needed to allow manipulation of the mycorrhizal symbiosis in crops and to understand the role of the AM fungi in community dynamics in natural systems. Previous studies have assessed changes in total spore populations in different ecosystems such as sand dunes (Nicolson, 1959), savannas, continuous corn and soybean crops (Johnson *et al.*, 1992a; Johnson *et al.*, 1991; Dodd *et al.*, 1990a), cacao plantation (Cuenca and Meneses, 1996), cropping production systems (Hendrix *et al.*, 1995), fescue and tobacco plantation (An *et al.*, 1993), natural and agroecosystems (Sieverding, 1991; Siqueira *et al.*, 1989), monoculture systems (Schenck and Kinloch, 1980), with emphasis on studying the seasonal variation in spore formation by different fungus specie. Few investigations have attempted to follow an individual AM fungal species through its life cycle, including formation of an association with the roots of the plants. However, the few studies of populations in natural communities that have been attempted, used microscopic identification (Abbott, 1982; Merryweather and Fitter, 1998a), which is difficult and time consuming. Information is required on how individual fungal species affect plants under local edaphic and climatic conditions, and what factors control their populations in natural communities.

Many studies have examined the effects of cropping sequence on total mycorrhizal colonisation and spore populations (eg. Black and Tinker, 1979; Dodd *et al.*, 1990a; Johnson *et al.*,1992b), but relatively little is known about plant/fungus interactions and species composition in natural communities. Although spore numbers

may reflect the relative abundance of individual species within the community, this does not necessarily relate to their infectivity or effectiveness in stimulating plant growth. There is still doubt about the relationship between species of fungi and plants in symbiotic associations. Given the great diversity of AM fungi in soil, the known differences between species in colonising ability and spore production (eg. Abbott and Robson, 1984a; Gazey *et al.*, 1992), and the effects of different fungal species on nutrient uptake and growth, it is likely that some species will form more effective associations with host plants than others.

This literature review covers aspects of the biology, spore populations and development of tools, specifically internal transcribed spacer (ITS) regions of AM fungi, as they relate to the general theme of understanding the population dynamics of the fungi and their role in mixed plant communities.

2.2 Classification of AM fungi

Classification can be used either as a basis for identification of a population or as a theoretical framework for understanding phylogenetic relationships between taxonomic units (Bentivenga and Morton, 1994). All classifications are composed of the same Linnaean taxonomic categories (for example, species, genera). However, the underlying kinds and causes of diversity are less universal, and these may differ greatly within each group of organisms.

Spores were the sole focus of attention of the early taxonomy of AM fungi, which was not formalised using traditional nomenclature until 1974 (Gerdemann and Trappe, 1974). Few areas of the world have been extensively sampled for indigenous species of AM mycorrhizal fungi and taxonomic research still includes a significant exploration and description component (Morton, 1993).

Until now the spore has been the most important structures used for identification. The resting spores of AM fungi in the soil usually range from about 50 to 600 µm in diameter and are some of the largest known fungal spores (Mosse *et al.*, 1981).

The first approach to classifying endomycorrhizal fungi was made in the 1960s. All species were put into the genus *Endogone (sensu lato)* of the Zygomycetes based on the production of very large, multinucleate spores. Later the genera *Acaulospora*, *Gigaspora, Glaziella, Modicella* and *Sclerocystis* were grouped together with *Endogone* in Endogonaceae, Zygomycetes (Gerdemann and Trappe, 1974). At that time few characteristics were known, but as more species were described, new characters were discovered and the information base increased. *Glaziella* and *Modicella* were transferred to the Ascomycetes (Gibson *et al.*, 1986). Ames and Schneider (1979) erected a new genus *Entrophospora*, and Walker and Sanders (1986) placed species of *Gigaspora sensu lato* (with inner walls and a germination shield) into a new genus *Scutellospora*.

Between 1982 and 1990, the number of AM fungi described increased markedly. Trappe (1982), in his synoptic key to the Endogonaceae, described 77 species excluding *Endogone,* and Hall (1984) in his dichotomous key to the Endogonaceae listed 67 species, again excluding *Endogone*. Later, Schenck & Pérez catalogued 120 described species (1987) and subsequently 147 species (1990) in the genera of fungi producing mycorrhizal associations. Walker (1986) and others (Berch and Koske, 1986; Morton, 1986; Spain *et al.*, 1989) defined phenotypically different and separable types of walls, represented by murographs. A murograph is a graphical representation of the types and relative positions of walls. The present classification of AM fungi is based on morphological characteristics of spores (including murographs) because the vegetative characters of the fungi do not vary much between taxa and are also influenced by species of host plant (Smith and Smith, 1996).

2.3 The life cycle of AM fungi

In the soil, AM fungi are found as spores or as living hyphae within root segments. These propagules germinate to produce hyphae, the growth of which is influenced by the presence of root exudates (Harley and Smith, 1983). Upon contact with the host root surface, the colonising hyphae form an appressorium, from which infection peg(s)

penetrates the cells, initiating colonisation of the root. In some cases there is no initial penetration of plant cell, but only intercellular Subsequently, the fungus grows extensively and forms intercellular hyphae, coils, vesicles and arbuscules (Bonfante-Fasolo and Perotto, 1992). AM fungi improve the mineral nutrient supply to the host by absorption through hyphae which extend into the soil beyond the root zone. The fungus obtains photosynthetically derived carbon compounds from the plant. The interaction between plant and fungus is mutualistic. It does not involve high levels of plant defence gene expression or disease symptoms (Smith and Read, 1997).

Reproduction in AM fungi is normally asexual, with the exception that *Gigaspora decipiens* may have some capacity for sexual reproduction (Tommerup and Sivasithamparam, 1990). During asexual reproduction somatic reassortment of nuclei, exchange of mitochondria, of extranuclear DNA, RNA and protein molecules occurs, following anastomosis between compatible fungi (Tommerup and Malajczuk, 1993).

Only a few studies have been undertaken of nuclear division in AM fungi, during *in vitro* development. The current definition of the life cycle of these fungi is based on a symbiotic model of growth as outlined by Bécard and Piché (1989) and Bonfante-Fasolo and Perotto (1992). It begins at spore germination and finishes when the first root is colonised and arbuscules are formed. However, this definition must be regarded as incomplete because it is necessary to consider all steps in development until new spores are formed. Little is known of the conditions that trigger spore formation, but processes involved must include transfer of carbohydrate from the host and synthesis of lipid reserves in the spores as well as nuclear division, to account for the large number of nuclei (as many as 20,000 per spore) (Burggraaf and Beringer, 1989). However, this study conflicts with more recent data showing nuclear division during germination (Bécard and Pfeffer, 1993). Recent studies of nuclear DNA content have focused on germination rather than spore formation and have shown that after spore germination in *Gigaspora margarita*, nuclei divide and replicate nuclear DNA in the absence of the host (Bécard and Pfeffer, 1993; Bianciotto and Bonfante, 1993). The quantity of nuclear DNA per nucleus was the same at three steps of their life cycle: spores, extramatrical and intraradical mycelium in *Gi. margarita* and *Glomus*

versiforme (Bianciotto and Bonfante, 1992). The assumption appears to be that if nuclear fusion and meiosis occur they are so transitory that changes in nuclear DNA content could not be detected by the methods used. The Amplified Fragment Length Polymorphism (AFLP) technique used in seven different *Glomus* species generated a large number of fragments and no evidence for recombination was found, which suggest that the AM fungi reproduce clonally (Rosendahl and Taylor, 1997).

2.4 Developmental stages of AM fungi in plant/soil systems

Arbuscular mycorrhizal fungi are obligate biotrophic symbionts with a life cycle divided into two distinct stages. On the one hand, the resting and reproductive stages (spores, sporocarps and possibly also vesicles) are independent of the plant. On the other hand, vegetative stages are involved in complex interactions with plant which include recognition, colonisation and nutrient exchange. These stages are represented by development of external hyphae in soil and hyphae and coils, arbuscules and vesicles within the root.

2.4 1 Spore

The spore are produced rapidly in the presence of a host plant, so that within four to six months, thousands of new spores of the same kind are produced. The spores are formed on the extraradical mycelium or aggregated into more or less well-defined structures called sporocarps. Although in some species sporocarp characteristics are important, the features of individual spores are mainly used for identification. The spores differ in shape, structure, cytoplasmic content, colour, size, number of walls, manner of germination, morphology of secondary spores and presence or absence of sporocarps (Mosse *et al.*, 1981; Gerdemann and Trappe, 1974; Morton, 1990). The spore phenotype is the result of developmental processes completely different from those in the vegetative thallus, so the spore is considered by some to be autonomous in form and function. However, the germ tube of a spore can originate from a filamentous hyphal network, arbuscules, vesicles or auxiliary cells (Morton *et al.*, 1995b). The involvement of the highly modified arbuscular branch hyphae in colonisation seems very unlikely. Morton (1993) considers that a fungal individual is represented by a single

identifiable spore which consists of a multinucleate single cell. This cell can certainly give rise to a new fungal colony with mycelial components within a root and in the soil. This vegetative colony will eventually give rise to new individuals in the form of new spores.

Spores are morphologically specialised cells which do not directly contribute to or support activities in mycorrhizal development and host-fungus interactions. The function of the spore is to carry the genetic information to new habitats and initiate new individuals spatially separated from the parent organism. In the absence of information about the nuclear cycle or existence of sexual stages it is not possible to determine whether spores actually represent new generations of new individuals. If the organisms are clonal, then it is doubtful that spores should really be regarded as separate individuals. However, there is considerable genetic variation between spores within a single species and even originating from a single-spore culture (Lloyd-MacGilp *et al.*, 1996; Rosendahl and Taylor, 1997; Sanders *et al.*, 1995; Wyss and Bonfante, 1993; Zézé *et al.*, 1997). This complicates the issues and highlights the need for research on the life cycle and genetics of the fungi.

2.4.2 Spore germination

Spore germination is an integral part of the life cycle of arbuscular mycorrhizal fungi as it represents the initiation of the vegetative stage of growth. Germination characters are important for taxonomy since they are used to distinguish between the two genera in the Gigasporineae, *Gigaspora* and *Scutellospora*. In the *Gigaspora*, germination takes place directly through the spore wall, while in the *Scutellospora* it occurs from a germination shield formed upon or within an inner wall layer (Walker and Sanders, 1986).

Although AM fungi have not been cultured in artificial media, isolated spores will germinate on nutrient or water agar (Hepper, 1981; Mosse, 1962). The ability to germinate, the pattern of germination and the quantity of mycelium produced are characters that can all show a high degree of variation within or between species (Hepper and Smith, 1976; Giovannetti *et al.*, 1991).

Spore dormancy may range from two weeks to several months in *Acaulospora* species, *G. intraradices* and *Gigaspora gigantea* (Gazey *et al.*, 1992; Tommerup, 1983). Spore germination can also be influenced by pH, moisture, host root exudates and other factors (Tommerup, 1983; Daniels Hetrick, 1984; Hepper, 1984; Siqueira *et al.*, 1985). For example, *Glomus mosseae* spores germinate more rapidly when stored at low temperatures (Hepper and Smith, 1976). Many soil bacteria may affect spore germination. Some can be inhibitors, while others promote germination and mycorrhizal formation (Fitter and Garbaye, 1994).

In the presence of host root factors, hyphal growth from spores can continue for about 24 days without the intervention of a symbiotic interaction. This indicates that the non-symbiotic phase is able to respond to the presence of roots, although a symbiotic stage is essential for the fungi to complete their life cycles (Bécard and Piché, 1989; Giovannetti *et al.*, 1993).

The reasons for slow hyphal growth from spores are still not well understood. It was originally proposed that lack of DNA synthesis and nuclear proliferation could be the cause (Burggraaf and Beringer, 1989), but observations of nuclear migration and replication of nuclear DNA in hyphae growing out from spores (Bianciotto and Bonfante, 1992; Bécard and Pfeffer, 1993), have led to rejection of this hypothesis, and other mechanisms must be responsible for the lack of growth. These could include ineffective membrane transport systems, so that ability to absorb nutrients is limited (Smith and Smith, 1986).

The soil-borne spores of AM fungi are considered the most important reproductive structures, but their numbers in soil are often poorly correlated with mycorrhiza formation in roots (Abbott and Robson, 1984b; Ebbers *et al.*, 1987, McGee, 1989). For some species, spore production only occurs after a threshold level of colonisation is reached (Gazey *et al.*, 1992). Spore production is influenced by many factors including the host plant and soil type.

Few useful generalisations can be made about conditions leading to spore formation other than the need for several months to elapse from initial host colonisation. In relation to other AM mycorrhizal propagules, spores are generally

considered to be more resistant to adverse conditions (Abbott and Robson, 1982b) than either colonised root fragments or hyphae and can act as long-term survival structures, with some capacity for dispersion by water and wind (Koske and Gemma, 1990; Friese and Allen, 1991).

2..4.3 Colonisation

The process and rate of colonisation determines the effectiveness of an AM fungus or a mycorrhizal association. The colonisation can originate from spores, infected root fragments or hyphae (Smith and Read, 1997). The hyphal network and root fragments are likely to be the main source by which plants become colonised (Smith and Walker, 1981; Jasper *et al.*, 1992; Hepper, 1981). At the same time as infection spreads within the cortical cells of the host root, a mycelium of extraradical hyphae grows out into the soil. The extraradical hyphae have an important role in nutrient acquisition and form a source of secondary colonisation along and between roots (Harley and Smith, 1983; Smith and Gianinazzi-Pearson, 1988; Smith *et al.*, 1992).

Within the cortex, hyphae grow longitudinally between the cells and intracellularly to form arbuscules. Using *G. mosseae* as a model, Giovannetti *et al.*, (1993) demonstrated that the first appressoria were formed within 36 hours after the beginning of the interaction with *Ocimum basilicum* and *Helianthus annuus* and the first arbuscules were formed between 42 and 48 hours after inoculation of *H. annuus*. These observations agree with earlier observations (Cox and Sanders, 1974; Brundrett *et al.*, 1985). Using a nurse pot system with *Glomus intraradices* and *Lycopersicon esculentum* , Rosewarne *et al.*, (1997) showed that after only 4 days approximately 60% of root length was associated with hyphae and the peak in arbuscule numbers occurred 4 days after intercellular hyphal growth reached a maximum. Arbuscules are assumed by many researchers to be the main interface for uptake of sugars by the fungus and transfer of ions from the fungus to the cortical cells of roots of the host plants, although some evidence for spatial separation of the functions of carbon and

phosphorus transfer a hyphal and arbuscular interface is available (Gianinazzi-Pearson, 1991; Smith and Read, 1997).

Vesicles, which are storage organs containing large amounts of lipid and many nuclei, are formed by members of the Glominaceae later when the infection unit has matured. The vesicles produced by many AM fungi are considered to function as temporary storage organs. However they often have elaborate multilayered walls like spores and can function as propagules when isolated from roots (Biermann and Linderman, 1983).

2.5 Populations of AM fungi in natural ecosystems

There is very little information on AM fungi in natural ecosystems, although evidence is emerging that the diversity is greater than has been inferred from morphological studies of spores. The populations in different ecosystems, on the basis of spore counts, vary between 5 to 25 different species. This number depends upon the plant host species involved (Table 2.1). Spore numbers are not always well correlated with the degree of mycorrhizal formation (Brundrett, 1991) and their percentage of germination varies at different times of the year (Tommerup, 1983; Gemma *et al.*, 1989).

The composition of an AM fungal species can be explained as a response to the changes in the plant community, since the obligate nature of AM fungal symbiosis links growth and reproduction of both the plant host and the fungus to soil conditions (Adelman and Morton, 1986; Hendrix *et al.*, 1995; Sanders and Fitter, 1992a). In field studies, crop sequence has been shown to modify the AM fungal community and species composition. Predominance of only one AM fungal species to each crop developed in continuous sequences of corn or soybean (Johnson *et al.*, 1991), favouring species beneficial to the crop and reducing the population of less beneficial AM fungal species (Johnson *et al.*, 1992a). Management practices such as tillage (Evans and Miller, 1988), rate and method of phosphorus application, pesticide application or liming have also been shown to influence sporulation and colonisation by AM fungi (Duke *et al.*, 1994; Medeiros *et al.*, 1994; Ryan *et al.*, 1994; Wang *et al.*,

1993). In some cases the diversity of AM fungi community has been increased in management systems utilising crop rotation and reduced herbicide inputs (Douds *et al.*, 1993).

To study the mycorrhizal contribution to an early successional plant community in the field, Gange *et al.* (1990) used a fungicide to reduce AM colonisation during the first year of establishment of plants on bare soil. Fewer plant species established in communities treated with fungicide, supporting the idea that AM fungi promote plant species diversity (Helgason *et al.*, 1998; Van der Heijden *et al.*, 1998). Mycorrhizal fungi may also affect the pattern of species diversity and relative abundance, once the mycorrhizal status of a plant community has been restored in a previously disturbed ecosystem (Barea and Jeffries, 1995). The AM fungi may reduce plant diversity if the symbiosis is of relatively greater benefit to the dominant species within the community (Hetrick *et al.*, 1994).

When ecosystems are disturbed, for example by crop monoculture or by use of pesticides, the community dynamic is disrupted and a bias can develop toward a few or even one dominant fungus (Johnson *et al.*, 1992a). For instance, in some environments tillage and fertiliser use have led to fewer species of AM fungi being found in the soil (Daniels Hetrick and Bloom, 1983; Schenck and Kinloch, 1980) while in others agricultural use may promote greater diversity (Abbott and Robson, 1977).

Although population studies of AM mycorrhizal fungi are based on morphological characters of fruiting bodies, spores, vegetative mycelia or symbiotic structures, the approach is still limited because the factors influencing sporulation of an individual species are poorly understood and cannot be extrapolated to the extent of vegetative colonisation of different plant species. Spore populations provide only a relative indication of the abundance and species composition of AM fungal populations. However, molecular studies of diversity of the fungi (Van Tuinen *et al.*, 1994) in natural ecosystems can offer a better means of identification for information about populations, especially when it is difficult to gather a sufficient number of morphological features.

The occurrence of several AM fungal species in soils or within roots suggests, that interspecific competition is possible. Variations in spore production between coexisting endophytes has been observed (Gemma *et al.*, 1989) and abundant spore production by one AM fungi was usually correlated with lower levels of spore productions by others. This may be due to antagonism between species. In pot culture experiments, where several isolates of AM fungi are inoculated together, some have proven to be better competitors than others (Wilson, 1984; Lopez-Aguillon and Mosse, 1987).

2.6 Host plant and production of spores

Almost 90% of species of vascular plants so far examined can be colonised by AM fungi (Harley and Smith, 1983) and are normally mycorrhizal in the field. Host specificity is apparently very low. Under experimental conditions a single fungal isolate can form associations with taxonomically diverse host plants (Gerdemann, 1975; Smith and Read, 1997) and single host plant species associate with many fungal isolates, leading to a widely held view that mycorrhizal associations lack specificity. However there is increasing evidence that some degree of specificity does exist between AM fungi and plants, particularly in natural ecosystems (Rosendahl *et al.*, 1994; Sanders and Fitter, 1992b; Smith and Read, 1997).

There is no doubt that associations including different plant and fungal species exhibit functional variability. Some host plants provide more benefit to AM mycorrhizal fungi than others, which is reflected in differences in the quantity of spore production. In most cases spore formation is closely related to the total length of mycorrhizal roots produced by a given host (Giovannetti *et al.*, 1988; Daniels Hetrick and Bloom, 1988; Howeler *et al.*, 1987; Simpson and Daft, 1990; Gazey *et al.*, 1992). Thus the proportion of different species at a site will depend on the extent to which they colonise the root systems of the plants. Any degree of specificity or difference in effectiveness may therefore be reflected in the spore populations.

There is evidence to support this idea. In an investigation of perennial plants of 19 families, in a southern California desert, the majority of plants were potential hosts

for endomycorrhizal fungi and the diversity of these fungi was shown to be directly related to plant diversity (Bethlenfalvay *et al.*, 1984). Again the situation is complex because in some studies of plant communities, data has been presented showing that the full diversity of fungi can be supported by a large number of related species (ie. in the same genus or family) whereas single plant species only supported some of the fungi (Brundrett and Kendrick, 1988; Trappe *et al.*, 1984). Furthermore, in a study of the fungi present in bluebell woods, *Scutellospora, Acaulospora* and *Glomus* only occurred when *Hyacinthoides non-scripta* plant was present (Clapp *et al.*, 1995), again implying a level of host specificity. This result confirms the studies by Molina *et al.* (1978), which showed that two or more species can colonise an individual plant. The data in this area are not extensive and therefore methods that allow us to compare extent of colonisation of roots by individual species with spore production will be essential, to fully understand the competition between fungal species and host plant specificity.

Mycorrhizal associations are normally beneficial (mutualistic) to plants (Johnson *et al.*, 1997; Smith and Read, 1997). Mycorrhizas increase plant growth rate through an increase in nutrient uptake, especially phosphorus under controlled conditions (Fitter, 1989; Smith *et al.*, 1994; Jackson *et al.*, 1972), however, under natural conditions, there is not enough evidence showing increases in plant growth due to mycorrhizas (Fitter, 1989; Newsham *et al.*, 1995a; West *et al.*, 1993). Low growth responses could be associated with the differences in the effectiveness of mycorrhizal species (eg. Abbott and Robson, 1981a; Abbott and Robson, 1981b; Bevege and Bowen, 1975; Jakobsen *et al.* 1992). For example the fine endophyte *Glomus tenue* does not produce a growth response, even in soil with low fertility (Powell, 1979), *G. intraradices* and *G.* City Beach WUM 16 increased plant growth and P uptake, but *G. etunicatum* and *G. mosseae* had no effect on plant growth and P uptake in different level of soil compaction (Nadian *et al.*, 1998). The relative contribution of the roots in total P uptake varied greatly between plants colonised by *S. calospora, Glomus* sp. and *G. caledonium* in cucumber (Pearson and Jakobsen, 1993). Therefore, the differences among AM fungi in their apparent requirements for either carbon or nutrient uptake efficiency should be evaluated for AM fungi from field collected species. Moreover, in

this situation, molecular probes will make it easier distinguish the different fungal species that are actually present in roots.

The effects of arbuscular mycorrhizal fungi on plants are dependant on host plant and environmental conditions (Sieverding and Howeler, 1985; Smith and Read, 1997; Smith, 1993; Newsham *et al.*, 1995b). The spore numbers of individual AM fungi in a native savanna ecosystem in Colombia changed as a result of different management practices (Dodd *et al.*, 1990). In the original soils, twelve spore types were identified and it was observed that populations of different spore types changed rapidly under different crop regimes. For example, spores of *Glomus occultum* and *Acaulospora myriocarpa* increased in subplots of sorghum, while those of *Entrophospora colombiana, A. melleae* and *A. morrowiae* dominated subplots where cowpea (*Vigna unguilata*) was grown following a crop of kudzu (*Pueraria phaseoloides* (Dodd *et al.*, 1990b). This study further supports the idea that different plant hosts can produce different populations of AM mycorrhizal fungi in the soil around the root system.

In naturally revegetated strip mine sites many different spore types were found and 13 of these were identified to the species level (Kiernam *et al.*, 1983). Each sampled plant had from one to eight mycorrhizal species associated with them. More recently, an analysis of spore populations under a natural tall grass prairie (Bentivenga and Hetrick, 1992) showed the presence of 14 species of AM fungi, with spores of *Glomus ambisporum* dominating numerically.

It is not clear whether the species that becomes dominant with each host plant is also the species that was most beneficial to that particular crop or to the stability of the soil (Barea and Jeffries, 1995). Unfortunately, there are not enough data to fully understand whether plants control the diversity of AM fungi in order to form beneficial symbioses (Van der Heijden *et al.*, 1998).

Table 2.1: Summary of some AM mycorrhizal populations in different field conditions

Field condition	Number of AM fungal - species recovered	Genera present	Reference
Corn-soybean rotation	15	*Glomus, Gigaspora, Acaulospora, Entrophospora*	Kurle and Pfleger (1996)
Mown grassland	23	*Acaulospora, Gigaspora, Glomus, Scutellospora*	Bever *et al.* (1996)
Grasslands	2-5	*Acaulospora, Glomus, Gigaspora, Sclerocystis*	Molina *et al.* (1978)
Wheat fields	3-6	*Acaulospora, Entrophospora, Gigaspora , Glomus*	Daniels Hetrick and Bloom (1983)
Pasture; Crop or native	3-5	*Acaulospora, Glomus, Gigaspora*	Abbott and Robson (1982)
Arid grassland	4	*Glomus, Entrophospora*	Henkel *et al.* (1989)
Mediterranean scrubland	6	*Acaulospora, Entrophospora, Glomus, Scutellospora*	McGee (1989)
Agro-forestry	10-12	*Acaulospora, Gigaspora, Glomus*	Walker *et al.* (1982)

2.7 Molecular approaches to study AM fungi

Most molecular research investigating AM fungal identification and detection at species and isolate level has used techniques based on PCR, which allow the characterisation of nucleic acids from small amounts of fungal DNA (White *et al.*, 1990). A first study of AM fungal DNA using PCR techniques involved amplification and sequencing of the 18S rRNA gene (Simon *et al.*, 1992). The DNA was amplified from small numbers of spores using universal primers NS1, NS2, SS38 and NS21. Using VANS1 primer it was possible to amplify and sequence the 18S rRNA gene from several different species of AM fungi representing all the genera of the Glomales (Simon *et al.*, 1993). This led to the design of primers which have some taxonomic specificity at the level of genus (VAGIGA, VAGLO and VAACAU) and these have been used to detect different genera of AM fungi in colonised roots (Clapp *et al.*, 1995; Sulistyowati, 1995). Bonito *et al.* (1995) used the primer pair VANS1-NS21 to detect *Glomus intraradices* in many different roots. During the application of these methods a number of problems have been identified. Specificity is limited to the level of genus and may not be absolute, because Sulistyowati, (1995) showed amplification of *Glomus* DNA with the VAGIGA primer.

Lanfranco *et al.* (1995) used RAPD-PCR to generate a specific primer for the identification of *G. mosseae* that could distinguish some isolates of this fungus. Using PCR-RFLP and universal primers ITS1 and ITS4, Sanders *et al.* (1995) obtained fragments of different lengths from spores of the same species obtained from a natural community. Use of these primers allowed Sanders *et al* (1995) to sequence the highly conserved 5.8S rRNA and thus revealed considerable differences between species. These techniques clearly have potential for identification of spores and vegetative stages, but are currently limited by the lack of basic knowledge of genetic diversity in the fungi and appropriate protocols for dealing with DNA from single spores. Methods are improving all the time and several mycorrhiza groups are adapting them for specific applications.

Primers that differentiate taxa at the species level have also been generated using random amplified polymorphic DNA (RAPD) (Abbas *et al.*, 1996; Lanfranco *et*

al., 1995). The primers designed by Lanfranco *et al.* (1995) were reasonably specific for *G. mosseae*, but also produced amplification products with *Scutellospora* species. Abbas *et al.* (1996) designed primers which were isolate-specific and were not useful for identification of isolates of *Glomus mosseae* or *Gigaspora margarita* from places other than the sites of origin. Such highly specific primers could be useful for the detection of individual mycorrhizal fungal isolates and for monitoring the outcome of inoculation, but would have limitations as a general diagnostic tools for ecological or field based studies of fungi at species level. Primers specific for *S. castanea* have also been designed from highly repetitive DNA sequences (Abbas *et al.*, 1996; Zézé *et al.*, 1996). Differences in the sequences of internal transcribed spacer (ITS) regions of rDNA determined by RFLP-PCR have proven useful in differentiating several AM fungi (Redecker *et al.*, 1997) and for designing diagnostic primers for *Glomus mosseae* (Millner *et al.*, 1998). These methods have their limitations for quantifying AM fungi in roots or soil, but their potential is such that further work is clearly warranted.

2.7.1 Detection of AM fungi in roots

Arbuscular mycorrhizal fungi are of fundamental importance for plant growth and they may be crucial for the development of sustainable agricultural systems. In order to understand their role it is necessary to develop tools for detection and identification of vegetative stages as well as spores in different soils during the development of these fungi. Because they cannot grow in the absence of a host plant and are very difficult (almost impossible) to identify in the vegetative stage, the use of DNA technology offers the possibility for detection and identification and eventually quantification especially in colonised roots.

Different AM fungi can colonise the root system of single plants under field conditions (Rosendahl *et al.*, 1990). It is possible to identify some species of AM fungi by comparing their colonisation patterns within roots (Abbott, 1982; Brundrett *et al.*, 1996; Merryweather and Fitter, 1998a; Merryweather and Fitter, 1998b) or by using isoenzymes and antibodies (Hepper *et al.*, 1988b; Rosendahl *et al.*, 1989; Morton, 1987; Rosendahl and Sen, 1992; Sanders *et al.*, 1992). However, vegetative structures

within colonised roots are of limited use because of the similarities between fungal species and the effects of the host plant on fungal morphology (Smith and Smith, 1996). These techniques cannot identify a single individual species within a root and therefore there is a need to develop sensitive detection and quantification techniques.

The use of polymerase chain reaction (PCR) (Mullis and Falooma, 1987), associated with other molecular techniques such as RFLP, and RAPD, have the possibility of identify AM fungi precisely. Techniques such RFLP have been used on genomic DNA extracted from infected roots to detect the fungus *Scutellospora castanea* (Zézé *et al.*, 1996), and RAPD-PCR was used to design *Glomus mosseae*-specific primers (Lanfranco *et al.*, 1995). Other techniques such as PCR-RFLP, M13 minisatellite primed PCR (Zézé *et al.*, 1997) can be used for studying variation among and between species as well as to design primers. Quantification of AM fungi using competitive PCR is possible (Edwards *et al.*, 1997), but it is difficult to determine the initial target template DNA concentration. PCR primers designed from 25S rDNA were used to detect the frequency of colonisation of roots by four species of AM fungi with nested PCR (Van Tuinen *et al.*, (1998a). These methods could be used to identify the fungi accurately but they cannot quantify fungal biomass within roots. DNA-based methods to quantify fungi, such as *Rhizoctonia solani* AG8 (Whisson *et al.*, 1995) and *Gaeumannomyces graminis* var. *tritici* (Ggt) (Herdina *et al.*, 1996; 1997), in soil have been successful developed using DNA hybridisation assay and similar methods have potential application to AM fungi.

It is important to select and adapt the most efficient and easy tool in relation to the question. Identification of AM fungal species *in planta* is essential because the fungal community within roots determines the efficiency of the symbiotic relationship with the host plant and indirectly the composition of AM fungal community in the soil.

2.7.2 Internal transcribed spacers (ITS) rDNA region

Ribosomal genes and their spacers are popular targets for fungal identification. The nuclear ribosomal DNA encoding the highly conserved 5.8S rDNA with the two flanking internal transcribed spacers has been used for studying the phylogenetic relationships between fungal groups and for designing PCR primers. Ribosomal genes are among the most promising target DNA sequences because in many organisms they are found in multiple copies per genome, and have both conserved and variable sequences allowing potentially high levels of specificity (Cahill, 1999). The ITS regions of rDNA have been shown to be generally conserved at the species level but variable in higher taxa (Bruns *et al.*, 1991), making them particularly useful for species identification. The ITS DNA sequences were shown to be highly variable in several fungi (Carbone and Kohn, 1993; Kim, 1992; Lee and Taylor, 1992; Levesque *et al.*, 1994), as well as *G. mosseae* (Lloyd-MacGilp *et al.*, 1996) and *Gi. margarita* (Lanfranco *et al.*, 1999; Antoniolli *et al.*, 1998). However, the ITS sequences may not be sufficiently variable for distinction at the species level in every case (Turner *et al.*, 1998). In Glomales, when the ITS regions were analysed with restriction enzymes different isolates from the same species could not be differentiated (Lanfranco *et al.*, 1998). Thus, it is important to have a complete understanding of ITS sequences for studying genetic variability and for designing molecular probes of AM fungi.

There has been considerable interest in the development of DNA identification techniques for fungi using rDNA ITS sequences, especially with PCR primers. Ribosomal DNA ITS sequences were used by Lee *et al.* (1993) to distinguish four *Phytophthora* species in a dot blot assay and by Levesque *et al.*, (1998) for identification of several *Pythium* species and *Phytophthora cinnamomi*. In Glomales, taxon-specific oligonucleotide probes were designed for *G. mosseae* (Millner *et al.*, 1998) and *Gi. margarita* (Lanfranco *et al.*, 1999) using the ITS regions sequences. However, the genetic variation reported in ITS regions for *G. mosseae* (Lloyd-MacGilp *et al.*, 1996; Sanders *et al.*, 1995) show that a robust species-specific primer would be needed or primers for identification of different strains of *G. mosseae*.

PCR technology based on the ITS sequences could be used to identify AM fungi at the level of species and to differentiate between isolates of the same species. Furthermore, PCR primers from these regions could allow the investigation of diversity within the roots, in the rhizosphere and quantification of these fungi.

2.8 Conclusion

The study of AM fungi has been impeded by their obligate biotrophic nature and by difficulties in the identification of spores, especially in field material. Therefore, molecular techniques have enormous potential, for detection, identification and quantification of these fungi in natural communities.

For a given natural plant community it is necessary to understand the fungal populations and the diversity within an AM fungal community, which plant roots are colonised and if there are seasonal patterns of colonisation. Although identification of AM fungi in roots based on morphological observations has been successful in pot experiments (Abbott, 1982), the difficulty of identifying AM fungi in the roots of plants has always been an obstacle to their study in natural communities. The development of molecular probes will be a powerful tool not only for species identification but also for studying important functional responses in the symbiosis.

Chapter 3

Mycorrhizal associations, types and spore populations under different crop management regimes in Southern Australia

The assessment of *Arum-* and *Paris*-type mycorrhizal associations in the root samples was done by Sally Smith.

3.1 Introduction

Previous studies on AM fungal populations in different ecosystems have been discussed in Chapter 2 section 2.5. In summary, conventional crop rotations and tillage have been shown to alter the diversity of fungal communities (Sieverding, 1991; Kurle, 1996). However, little is known about the diversity of mycorrhizal fungi populations in other agroecosystems. Therefore it is important to determine how the species composition and abundance of AM fungi populations vary, as a prerequisite to understanding their potential contribution to plant productivity in natural and agroecosystems under different crop management regimes. Studies on the fungal communities also need to take into account how the vegetative activity of the fungi affects the growth of different host species, and how the plant species can in turn influence the diversity of fungal species. This cannot be done evaluating spore populations alone and additional methods are required. Consequently, genera, species spore number, and vegetative activity of AM fungi should be quantified to provide a complete picture of the diversity of "native" or "indigenous" AM populations.

Despite the need to consider the functional aspects of mycorrhizal associations in the field, most studies of field populations of AM fungi have been based on spore counts and identification. The few studies that have assessed percent colonisation of roots have made broad distinctions between fungal types eg. 'fine' and 'coarse' endophytes or colonisation patterns (Abbott, 1982; Sanders and Fitter, 1992a). Several investigations have shown that the number of fungal species present may decrease after long periods of continuous cultivation (Allen and Boosalis, 1983; Sieverding, 1991). Cultivated agroecosystems often have relatively high spore densities compared to

natural agroecosytems (Dodd *et al.,* 1990a). This may relate to the fact that in cultivated systems the fungal species probably depend on robust propagules like spores, as compared with natural ecosystems where colonisation from living plant roots or a network of mycorrhizal hyphae may be more important.

The relationship between spore density and colonisation of roots is variable and may reflect both production of spores as the fungi grow in association with roots, as well as the activity of spores in initiating colonisation (see Smith and Read 1997). Furthermore, it appears that some fungi which are active vegetatively may not form spores or form them only in association with some host species (eg. Clapp *et al.* 1995). These findings highlight the need to develop methods of studying both vegetative and reproductive fungal structures and to link the information with patterns of colonisation in different host species and ultimately to effects of colonisation on plant growth and populations. Prior to the advent of molecular techniques, it was not possible to identify individual AM fungal species precisely in mixed populations during vegetative growth. Recent advances have made it possible to extract DNA from fungal material and accurately identify the fungus to genus or species level (eg. Clapp *et al.*, 1995; Sanders *et al.*, 1996; Dodd, 1996), although the precise quantification of fungal biomass is still very difficult.

The overall aim of this section of the thesis was to gain a picture of the composition of AM fungal communities based on spore populations and to choose a site suitable for fungal isolation and future studies of the effects of management practices. This chapter describes two geographically distinct sites that were chosen to provide a comparison of mycorrhizal fungal populations in semi-natural and in agricultural ecosystems. In chapters 5 and 6, I present the results of my efforts to develop molecular probes to follow individual fungal species in both vegetative and reproductive stages in association with different plant species.

The AM communities at the two sites including four different tillage systems (conventional cultivation, direct drill, permanent pasture and natural vegetation) are described in terms of

1) total number of spores found in soils undergoing different tillage treatments,

2) the occurrence of different mycorrhizal fungal genera,

3) the percentage of colonisation in volunteer plant species,

4) the occurrence of *Arum-* and *Paris*-type structures in roots of the volunteer plant species.

3.2 Materials and Methods

3.2.1 Sites

One long-term tillage and rotation trial in South Australia and one in western Victoria were selected as study sites. The climate at these sites is mediterranean with cool, wet winters and hot dry summers. They were situated at Walpeup, Victoria and on the Waite Campus, South Australia. Details of each site are set out in Table 3.1. At Walpeup, the treatments chosen were seven: fallow-wheat (conventional cultivation - CC), fallow-wheat (direct drill - DD), wheat-pasture(CC), wheat-pasture (DD), pasture-wheat (CC) and pasture-wheat (DD), in plots with 4 m per 29 m, with three repetitions of each. Conventional cultivation consists of 4-5 cultivations in autumn, prior to sowing, to maintain a weed free fallow. Under DD there was no tillage of the soil prior to sowing. Weeds were controlled with broad spectrum, knockdown herbicides prior to sowing. Soil disturbance was minimised at sowing, under direct drill, by the use of a narrow sowing point. Soil and plants were taken from all these plots and an area of natural vegetation adjacent to the trial site. Thus, each cultivation treatment was replicated three times. At the Waite site the rotations chosen were permanent pasture, wheat-fallow and continuous wheat (Grace *et al.*, 1995). Samples of soils and plants were taken from the trial plots and from a relatively undisturbed, seminatural arboretum. The wheat-fallow and continuous wheat rotations were cultivated conventionally. The permanent pasture and arboretum received no cultivation.

Table 3.1. Characteristics of Walpeup and Waite sites.

SITE	Location	Mean annual temp. °C	Soil[A]	pH	Average[B] annual rainfall (mm)
Walpeup	Mallee Research Station, Victoria	5 - 30	red brown earth	7.5- 9.5	342
Waite	Waite Campus Urrbrae, South Australia	12 -21	red brown earth	5.9	495

[A] Soil description from Stace *et al.* (1968
[B] Bureau of Meteorology

3.2.2 Soil and plant sampling

At Walpeup and Waite, samples were collected in May 1996 (late autumn). At this time, there had been no cultivation since the plots were sown the previous year. The first autumn rains had occurred and a few of volunteer pasture, crop and weed species were growing on the trial plots. Plant species were identified as far as possible from vegetative material. The small number of plants of each species meant that samples were too small for satisfactory statistical analysis.

Soil samples were collected by taking 32 cores (10 cm diameter x 20 cm deep) from each plot to a depth of 15 cm. Where possible, the cores included the root systems of plants growing on the plots, usually only one plant per core. The soil was shaken from the roots. The 32 cores from each plot were pooled to produce one sample per plot. Subsamples of this soil were used for the enumeration and identification of spores and for setting up trap-cultures for AM fungi with different host plants (see Chapter 4). The plant roots were assessed for percentage of AM colonisation, colonisation type and frequency of occurrence of AM fungi with course and fine hyphae (coarse and fine endophytes).

3.2.3 Assessment of percentage of root length colonised and colonisation type of AM fungi

The roots were washed thoroughly with water, cut into 1.0 cm long pieces, fixed in ethanol (50% v/v) and stored until they could be processed. Fixed roots were cleared in 10% KOH (Koske, 1989), acidified with 0.1 N HCl and stained with trypan blue

(0.01% in lactoglycerol), a modification of the method of Phillips and Hayman (1970) omitting phenol. Roots that remained dark after clearing were bleached with H_2O_2 before staining. After the roots had been stained they were rinsed in tap water and stored in 1:1 (v/v) glycerol: water. The stained roots were examined microscopically at between 10 and 100x magnification to observe the AM fungal structures. Percentage colonisation was assessed with the grid line intersect method of Giovannetti and Mosse (1980). The development of different fungal structures, *Arum*-type or *Paris*-type in the plants was assessed in the same root samples, using a compound microscope at magnifications between x100 and x1000 (oil immersion) in stained root samples. The incidence of features characteristic of the different types was recorded as shown in Table 3.2.

Table 3.2. Features of colonisation in plant species having Arum- and Paris-type patterns of fungal development (Gallaud, 1905; Smith and Smith, 1997).

Mycorrhizal type	Mycorrhizal features				
	intercellular hyphae	cell to cell spread of hyphae	arbuscules subtended by:	coils in hypodermal cells	coils in cortical cells (± arbuscules)
Arum	present	absent	intercellular hyphae	present	absent
Paris	usually absent	present	intracellular coils	present	present

3.2.4 Identification and enumeration of spores

Estimation of spore numbers in samples from Walpeup and Waite was achieved by recovering spores from triplicate 10g soil samples (Gerdemann and Nicolson, 1963). Each sample was dispersed in one litre of water and the suspension was left undisturbed for 20 minutes to allow the soil particles to settle. The suspension was then decanted through 710 µm, 250 µm, 150 µm, 90 µm, 70 µm and 25 µm sieves. More water was then added to the sample, stirred to resuspend spores and allowed to stand for 15 s, to allow sand sized particles to settle. The resultant supernatant was decanted

into the stack of sieves. This was repeated three more times (until the supernatant was clear).

The material remaining on the sieves was washed into beakers. The contents of each was then transferred to a nematode dish and intact AM fungal spores were counted under a dissecting microscope at 10 - 100x magnification. The spores were identified to genus according to the keys of Schenck and Pérez (1990), Hall (1979), INVAM Web (http://invam.caf.wvu.edu/) and CD-ROM Demo Version - The BEG - Expert System Arbuscular Mycorrhizal Fungi by Rosendahl and Dodd.

3.2.5 Statistical analysis

The spore population data for each site was analysed by analysis of variance to determine if there were significant differences between treatments.

3.3 Results

3.3.1 Walpeup

Both spore populations and percent colonisation were generally low and spore counts were not significantly different between treatments (Tables 3.3, 3.4 and 3.5). There was no clear relationship between colonisation and spore numbers for any treatment. The highest colonisation (26% in *Triticum* sp.) occurred in the pasture-wheat (conventional) rotation, where the spore count was low (0.5 spores/g). *Glomus* and *Acaulospora* were found in the natural vegetation, but only *Glomus* was recovered from the agricultural treatments.

3.3.2 Waite

Spore densities at this site were slightly higher than at Walpeup and there were significant differences (P<0.05) between treatments. Values ranged from 0.4 to 6.8 spores/g, with the highest count occurring in the permanent pasture (Table 3.5). The highest level of root colonisation (56.5% in *Trifolium* sp.) was also observed in the pasture treatment as at Walpeup. The number of fungal genera recovered was high in uncultivated soils with *Gigaspora, Acaulospora* and *Glomus* being found in both

arboretum and permanent pasture soils. *Scutellospora* was also present in the permanent pasture. Fewer different fungal genera were recovered from other rotations.

Table 3.3: Occurrence of mycorrhizas in the volunteer plants sampled at seven different sites at Walpeup , in July, 1996.

SITE/ TREATMENT	SPECIES	FAMILY	AM colonisation (%)
WALPEUP NATURAL VEGETATION	*Vitadenia*	*Asteraceae*	0.0
	Encylaena tomentosa	*Brassicaceae*	0.0
	Danthonia tenuier	*Poaceae*	22.3
	Oxalis corniculata	*Oxalidaceae*	0.0
	Rhagodia sp	*Chenopodiaceae*	0.0
	Raphanus raphanistrum	*Brassicaceae*	0.0
FALLOW-WHEAT Conventional	*Carduns*	*Asteraceae*	0.0
	Dactylus glomerata	*Poaceae*	2.6
	Hypochaeris radicata	*Asteraceae*	16.7
	Tragus australians	*Poaceae*	6.7
	Trifolium sp	*Fabaceae*	15.6
	Triticum sp	*Poaceae*	15.0
FALLOW-WHEAT Direct drill	*Dactylis glomerata*	*Poaceae*	0.0
	Hypochaeris radicata	*Asteraceae*	0.0
	Oxalis pes-capra	*Oxalidaceae*	7.2
	Plantago lanceolata	*Plantaginaceae*	17.4
	Pennisetum cladestinum	*Poaceae*	20.0
	Rumex brasnis	*Polygonaceae*	17.4
	Tragus australianus	*Poaceae*	8.3
	Triticum sp	*Poaceae*	16.0
WHEAT-PASTURE Conventional	*Trifolium sp*	*Fabaceae*	4.2
WHEAT-PASTURE Direct drill	*Hypochaeris radicata*	*Asteraceae*	6.3
	Trifolium sp	*Fabaceae*	17.3
PASTURE-WHEAT Conventional	*Danthonia sp*	*Poaceae*	8.6
	Hypochaeris radicata	*Asteraceae*	3.9
	Medicago sp	*Fabaceae*	6.3
	Trifolium sp	*Fabaceae*	3.3
	Triticum sp	*Poaceae*	26.5
PASTURE-WHEAT Direct drill	*Danthonia sp*	*Poaceae*	15.0
	Hypochaeris radicata	*Asteraceae*	9.1
	Medicago sp	*Fabaceae*	0.0
	Tragus australianus	*Poaceae*	4.5
	Trifolium sp	*Fabaceae*	2.0
	Triticum sp	*Poaceae*	0.0

Table 3.4: Occurrence of mycorrhizas in the volunteer plants sample data from four different sites at Waite Campus, in July, 1996.

SITE/TREATMENT	SPECIES	FAMILY	AM colonisation (%)
ARBORETUM	*Danthonia tenuier*	*Poaceae*	5.8
	Hypochaeris radicata	*Asteraceae*	0.0
	Trifolium sp.	*Fabaceae*	18.9
	Triticum aestivum	*Poaceae*	7.4
PERMANENT PASTURE	*Dactylus glomerata*	*Poaceae*	8.7
	Trifolium sp.	*Fabaceae*	56.5
	Plantago lanceolata	*Plantaginaceae*	21.4
	Rumex sp.	*Polygonaceae*	11.5
WHEAT-FALLOW Conventional	*Labiata sp.*	*Labiatae*	6.3
	Polygonum avicularce	*Polygonaceae*	0.0
CONTINUOUS WHEAT	*Trifolium sp.*	*Fabaceae*	14.0
	Polygonum avicularce	*Polygonaceae*	27.8

Table 3.5: Number of spores/g of soil, number of mycorrhizal/non-mycorrhizal (M/NM) association in potential hosts and genera of fungi at Walpeup and the Waite Campus.

SITES	spores/g	M/NM	Genera of fungi
Walpeup			
Natural vegetation	1.1 a*	1/3	*Acaulospora, Glomus*
Fallow-wheat C	0.1 a	5/1	*Glomus*
Fallow-wheat DD	0.3 a	6/2	*Glomus*
Wheat-pasture C	0.3 a	2/0	*Glomus*
Wheat-pasture DD	1.0 a	1/0	*Glomus*
Pasture-wheat C	0.5 a	6/0	*Glomus*
Pasture-wheat DD	0.7 a	4/2	*Glomus*
Waite			
Arboretum	1.5 bc	3/1	*Acaulospora, Glomus, Gigaspora*
Permanent pasture	6.8 a	2/0	*Acaulospora, Glomus, Gigaspora, Scutellospora*
Wheat-fallow	3.2 b	1/1	*Glomus*
Continuous wheat	0.4 c	2/0	*Glomus, Scutellospora*

C = conventional   DD= direct drill

* Means followed by same letters are not significantly different (P> 0.05).

3.3.3 Fungal occurrence and mycorrhizal types

Tables 3.3 and 3.4 show the occurrence of the volunteer weeds and percent colonisation of the roots at Waite and Walpeup. The highest consistent colonisation was observed in the pasture at Waite. At Walpeup and other treatments at Waite, colonisation was low and variable. Data relating to colonisation patterns in different species was combined for all sites (Table 3.6). 'Fine endophyte' (presumed to be *Glomus tenuis*) was observed in almost all roots samples, together with 'coarse' hyphae, more typical of other mycorrhizal species. The way in which *G. tenuis* colonises roots appears different from coarse endophytes. This fungus rarely formed coils in hypodermal cells, even when the same root segments contained coils formed by other fungi. Hyphal swellings were frequently observed, not only as appressoria on the epidermis, but also in deeper cell layers. It was frequently not possible to determine whether the colonisation pattern was *Arum-* or *Paris*-type and information from infection units of *G. tenuis* was not used in this aspect of the assessment (see below).

The incidence of characters thought to be typical of *Arum-* and *Paris-* type patterns of colonisation in the different plant species (see Table 3.2) was determined from infection units formed by 'coarse endophytes' (Table 3.5). Unequivocal *Arum*-type colonisation was observed in *Trifolium* and *Medicago* (Fabaceae), *Plantago lanceolata* (Plantaginaceae) and *Oxalis pes-caprae* (Oxalidaceae). Members of the Poaceae showed characteristics of both types, with some apparently intermediate colonisation patterns. In *Tragus australianus,* hyphal coils were very well developed and occurred in small groups of adjacent cells in the outer cortical or hypodermal cells of the root. Thus, in young infection units it appeared as though the colonisation was *Paris*-type. However, in older units the inner cortex had both typical *Arum*-type colonisation patterns and patches where the fungal hyphae grew from cell to cell, typical of *Paris*-type mycorrhizas. Members of the Asteraceae also had 'mixed' colonisation patterns.

Table 3.6. Incidence of Arum (A) and Paris (P)-type mycorrhizas in different species at the Walpeup and Waite sites.

Species	Intercellular hyphae	cell -cell hyphae	Hyphal coils in cortex	Hyphae coils in hypodermal cells	arbuscules	A or P
Asteraceae						
Hypochaeris radicata	variable	occasional	rare	yes	small	(intermediate) *Arum*
Poaceae						
Dactylis glomerata	yes	no	no	no	yes	*Arum*
Tragus australians	variable	no	yes	yes	occasional	(intermediate) *Paris*
Triticum sp.	variable	rare	yes	yes	yes	*Paris*
Fabaceae						
Trifolium sp.	yes	no	rare	yes	yes	*Arum*
Medicago sp.	yes	no	rare	yes	yes	*Arum*
Oxalidaceae						
Oxalis pes-caprae	yes	no	no	yes	yes	*Arum*
Polygonaceae						
Rumex sp.	no	yes	no	yes	no	*Paris*
Plantaginaceae						
Plantago lanceolata	yes	no	no	yes	yes	*Arum*

3.4 Discussion

There were variations in spore densities between Walpeup and Waite sites. In general, the results agree with previous work which indicates that the number of genera decreases and that *Glomus* often dominates in spore populations under cultivation. *Glomus* can also be important in undisturbed systems like the permanent pasture at Waite, where *Glomus mosseae* was the predominant species (see Chapter 4), although other *Glomus* species and members of three other genera were also present. The results agree with those of Abbott and Robson (1982) who found that in 20 different soils which had been sown in the previous year with wheat, pasture, oats or natural vegetation, the spore densities ranged from 0 to 15 spores/g of soil, with *Glomus* as the most common genus recovered. Relatively high spore densities and high diversity at the level of genus in pasture may reflect diversity of host species, the permanence of plant cover, high density of colonised roots and lack of disturbance compared with the cropped treatments. However, high spore populations are not always found in undisturbed systems. McGee (1986) found spore populations to be extremely low in native bushland where the plants were highly mycorrhizal and Hayman and Stovold (1979) found a higher spore density after wheat than in native grassland or bush soils. In agricultural systems, Smith (1978) found no difference in spore density after permanent pasture or wheat, either under conventional or direct drilled cultivation.

The data presented here describe the genera of endophytes present during a single season (autumn), but provide no information about the variation between seasons or in different years. Therefore findings in this study are likely to represent the pool of infective propagules that have survived since the crop was removed, rather than variations in spore production in existing vegetation, as plants were absent or inactive during the long dry summer. The absence of any effect of cultivation (conventional or direct drill) at Walpeup, which agrees with the findings of Smith (1978) in Australia and Henkel *et al.* (1989) in the Red Desert, Wyoming, may reflect the fact that samples were collected before autumn tillage for conventional cultivation, minimising the differences between this treatment and direct drill.

In a field experiment, McGonigle and Miller (1993) found that for maize grown in soil that had been disturbed (mouldboard plough), shoot P levels and mycorrhizal colonisation were significantly lower immediately after planting and in the early part of the season compared to reduced tillage and no-till treatments. These differences were transient, and by the end of the growing period, there was no significant difference in mycorrhizal colonisation, shoot P or final grain yield between tillage treatments. This suggests that the greatest differences in soil infectivity would occur immediately after sowing, soon after disturbance by tillage treatment. By autumn, it is likely that any differences in levels of colonisation between tillage treatments would have disappeared and that inoculum levels would have been restored.

Despite low spore densities at Walpeup and Waite, volunteer weeds became colonised in all systems, indicating that infective propagules were present. *Glomus tenuis* contributed to the colonisation in most samples, but would not have been detected in the spore populations. Most of the plant species that have been reported in the literature to be mycorrhizal (Harley and Harley, 1987) were found to be colonised in this study, though not necessarily at all sites (Table 3.3 and 3.4). Two members of the Asteraceae, a family which is normally mycorrhizal, were not colonised. At Walpeup, *Hypochaeris radicata* (Asteraceae) was not mycorrhizal in the fallow-wheat, direct drill treatment, but had 16% of root length colonised in fallow-wheat, conventional cultivation. Similarly, colonisation in *Triticum* species ranged from zero to 26% and in *Dactylis glomerata* from zero to 8.7%. *Pennisetum clandestinum* (Poaceae) was not colonised. These results agree with those of Miller (1979) who investigated AM fungal occurrences in natural and disturbed ecosystems where the majority of plants were potentially arbuscular mycorrhizal. It seems likely that the patchy occurrence of mycorrhizas reflects low and patchy distribution of inoculum, but this result may be due to the absence of plant roots at the autumn sampling time. At other times of the year when plant roots are present we might have expected higher and less patchy mycorrhizal spore populations (Bethlenfalvay *et al.*, 1984; Brundrett and Kendrick, 1988). The lack of a general correlation between the size of spore populations and mycorrhizal colonisation at Walpeup confirms that counting spores, particularly when densities are very low, may not be a good indicator of mycorrhizal

infectivity and hence percent colonisation. It seems likely that hyphae and colonised root fragments may have contributed significantly to the pool of propagules at all sites.

Mycorrhizal colonisation was not observed in members of the Brassicaceae or Chenopodiaceae, generally regarded as non-host taxa. However, it was observed that mycorrhizal structures of the *Paris*-type occurred in *Rumex brasnis* (Polygonaceae) and *Arum*-type mycorrhizas in *Oxalis pes-caprae* (Oxalidaceae). *Rumex* species are normally recorded as non mycorrhizal (Read *et al.*, 1976). More work is required on this genus, to determine if arbuscules are formed at any stage and whether colonisation has any influence on growth or fitness of the plants. The other member of the Polygonaceae sampled (*Polygonum aviculare*) was not colonised.

The patterns of mycorrhizal colonisation were in general agreement with previous observations in different plant taxa and the data provide two new records of the occurrence of *Arum*-type colonisation, in *Plantago lanceolata* (Plantaginaceae) and *O. pes-caprae* (Oxalidaceae) (see Smith and Smith 1997). Members of the Asteraceae have previously been recorded as having both *Arum*- and *Paris*-type mycorrhizas. The work has identified a feature of colonisation in *Tragus australianus* which may have led to confused descriptions in the past. Groups of cells containing hyphal coils in outer cortical or hypodermal cell layers appear *Paris*-like, although the inner cortex has typical *Arum*-type mycorrhizas. Thus plants with young infection units could be mistakenly classed as having *Paris*-type mycorrhizas. Time-course studies of mycorrhiza development are needed to confirm that observations. Similarly, both *Arum*- and *Paris*-type patterns of colonisation have been previously recorded in the Poaceae. This is a large and diverse family and it may be that, as with the Liliaceae, more careful attention to taxonomic groups within the family will clarify the picture (Smith and Smith, 1997). More data are required for a range of species in different subfamilies.

3.5 Conclusion

The results confirm in general terms previous work on the effects of rotation and cultivation on spore populations of AM fungi. The pasture phase of the permanent rotation trial at the Waite Campus, with relatively high spore numbers, diversity of fungal and plant genera and levels of mycorrhizal colonisation appears to be the most suitable site for ongoing studies. In the adjacent natural vegetation most plant species appeared to be non-mycorrhizal and the fungal diversity and number of spores was low.

The incidence of *Arum-* and *Paris*-type arbuscular mycorrhizas generally confirmed previous information (Smith and Smith, 1997) that showed both types were found in plants from the genus Asteraceae and Poaceae.

Based on this data the permanent pasture at Waite site was chosen for a study of the seasonal variation of AM fungal populations (Chapter 4) and was used for isolating *Glomus mosseae* and *Gigaspora margarita* spores for molecular work described in Chapter 5 and 6.

Chapter 4

Community and species diversity of AM fungi under pasture regime in the permanent rotation trial, at the Waite Campus

4.1 Introduction

The aim of the work described in this chapter was to study the composition of a diverse AM fungal community in a permanent pasture system, at the Waite campus (Chapter 3).

Symbiotic arbuscular mycorrhizal fungi are often the most abundant fungi in soil (Gerdemann and Nicolson, 1963). The fungi are a group of over 152 described species classified on the basis of spore morphological characteristics (Schenck and Pérez, 1990). Glomalean spores are rarely attached to roots, but are produced on mycelium which can grow some distance from roots (Merryweather and Fitter, 1998b). The symbiosis is composed of host roots and fungal mycelium (intraradical and extraradical) and spores. In consequences, these spores are the means of survival of AM fungi in the absence of living host roots (Gerdemann, 1968). The spore populations recovered at the field site may not reflect the vegetative fungal populations in the natural community and therefore trap cultures may be used to possibly recover more fungal species than detected by the direct analysis of spores from field soils.

Mycorrhizal communities have been characterised based on their spore populations (Bentivenga and Hetrick, 1992) and colonised roots (Giovannetti, 1985; Merryweather and Fitter, 1998a, 1998b; Abbott, 1982). The composition of the AM fungal community depends upon the host species present, (Hetrick and Bloom, 1986; Koomen et al., 1987) which may also in part determine fungal species diversity (Gemma et al., 1989). Conversely, the response of the plant species to colonisation also depends on the identity of the fungal species colonising its roots. The interdependence of AM fungal species diversity and host plants is poorly understood (Morton et al., 1995c). Diverse assemblages of mycorrhizal species can occur in roots of any plant community (Brundrett, 1991; Morton et al., 1995c; Rosendahl et al., 1989), but spores are the only propagules that can be identified to species level with

any degree of certainty (Smith and Read, 1997). A detailed knowledge of the species composition of AM fungi that are present in different plant communities is important for understanding the relationship between AM fungi and the factors affecting the composition and success of the ecosystem.

Mycorrhizal populations are influenced by many factors such as plant species (eg. Giovannetti *et al.*, 1988; Johnson, 1991; An *et al.*, 1993; Schenck and Kinloch, 1980), soil nutrition (eg. Abbott and Robson, 1982b; Smith *et al.*, 1994), soil pH (Abbott and Robson, 1985; Wang *et al.*, 1993), soil compaction (Nadian *et al.*, 1998), soil microorganisms (Kitt *et al.*, 1987), cultural practices (Bethlenfalvay, 1992; Jasper *et al.*, 1991; McGonigle and Miller, 1993; Smith, 1978), and season (An *et al.*, 1993). Thus, it is important to know the AM indigenous fungi present in a determined system to evaluate the infectivity and effectiveness of these species.

The mycorrhizal fungal communities of agricultural soils are complex, composed of several species, some widespread and in high population densities, others found only in patches and/or at low density (Schenck and Kinloch, 1980; An *et al.*, 1993). In a tall fescue pasture, populations of viable spores declined from spring through mid-summer then rose in the fall to densities not different from those in the spring, but declined again over the winter (An *et al.*, 1993). Furthermore, diversity of AM fungal spore populations is lower in disturbed (arable) soils compared with undisturbed soils (Helgason *et al.*, 1998; Abbott and Robson, 1982a). In cotton crops the density of viable propagules of AM fungi in soil declined over time and was reduced by disturbed soil (McGee *et al.*, 1997). For example a reduction in AM fungi diversity from 11 to one species occurred after disturbance in an alpine area (Allen *et al.*, 1987).

Arbuscular mycorrhizal fungi have been reported to be present in many soils in Australia (Samuel, 1926; Mosse and Bowen, 1968; Abbott and Robson, 1977; McGee, 1986; Michelsen, 1994), however their occurrence has not been examined in a permanent pasture in South Australia. In a survey of the AM associations in arid and semi-arid ecosystems in South Australia, 54% of plants were colonised by AM fungi (O'Connor, personal communication) but no fungal identifications based on spore

populations or morphological characteristics were carried out. In Western Australia the seasonal variation in formation of mycorrhizas in two pasture soils, analysed with undisturbed soil and *Trifolium subterraneum* in a glasshouse, showed no seasonal variation and similar species were found at both sites. The spores of *Acaulospora laevis* were the most abundant (Scheltema *et al.*, 1987). *A. laevis* and *Glomus monosporus* were also common in soil under wheat and pasture (Smith, 1978). The twenty soil samples from different land uses collected in Western Australia showed that similar species of AM fungi were found in all soils (Abbott and Robson, 1982a). In 73 soil samples collected in New South Wales *A. laevis* and *G. mosseae* were the most common species (Hayman and Stovold, 1979). It is important to know the species that comprise AM fungi populations in order understand the role of these fungi in plant communities and to select indigenous strains of AM fungi under appropriate edaphic conditions for use in agricultural, physiological and molecular studies.

The AM fungal populations and diversity in the permanent pasture, at Waite Campus, South Australia, are described in terms of:

1) the temporal variation of AM fungal species in field-collected soil and trap cultures with *Lolium perenne, Plantago lanceolata, Sorghum* sp, and *T. subterraneum*;

2) spore production, richness and diversity in both field and trap culture soils;

In addition single-species spore cultures were set up from field collected spores to provide material for molecular studies (Chapter 6).

4.2 Material and methods

4.2.1 Site and sampling soil

The study site was located in the Waite Campus, South Australia. For details of the site see section 3.2, Chapter 3 and Grace *et al.* (1995). Soil samples were collected from a permanent pasture plot (0.05 ha) in July (winter) and December (summer) 1996, and July and December 1997. A range of volunteer pasture, crop and weed species (Chapter 3) were growing on the trial plot at those times. Soil samples were collected at random by taking 43 cores (10 cm diameter x 20 cm deep) including the root systems of plants growing in the plot. The soil was separated from the roots. The 43

cores were pooled to produce one composite soil sample which was air dried, mixed and sieved through a two mm sieve before being used as inoculum for trap cultures and for enumeration of AM fungal spores. The sample was stored in a sealed plastic bag at 4°C until spores could be counted.

4.2.1.1 Meteorological records

Data for monthly rainfall and temperature were extracted from records kept by Bureau of Meteorology - South Australia beginning in 1996 (Table 4.1), in order to relate climatic changes with seasonal changes in AM fungi diversity during the period of survey.

Table 4.1 Total monthly precipitation (mm), mean daily maximum temperature (°C) and mean daily minimum temperature (°C) between 1995 and 1997, Adelaide, South Australia.

		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
1996	Precipitation	42.2	16.0	22.4	35.6	15.2	119.8	115.0*	105.0	83.8	28.0	1.0	15.4*	599.4
	Maximum temperature	27.2	28.1	27.2	19.7	19.3	16.7	15.1*	15.7	18.1	22.0	22.5	25.7*	
	Minimum temperature	14.6	16.4	15.4	11.6	9.3	9.0	7.9*	8.2	9.3	11.4	12.5	14.6*	
1997	Precipitation	36.0	29.0	8.6	9.2	65.6	33.0	22.2*	90.4	74.2	60.8	44.8	24.8*	498.6
	Maximum temperature	29.2	32.7	23.2	22.6	17.5	16.0	14.6*	15.4	18.3	22.1	26.3	26.7*	
	Minimum temperature	17.8	19.9	13.1	11.8	9.7	8.3	5.7*	7.3	10.3	12.0	15.0	15.3*	

* Time that field soil samples were collected.

4.2.1.2 Spore occurrence in field-collected soil

Direct counts of spores were made from field-collected soil. Estimation of spore numbers and identification was achieved by recovering spores from triplicate 25 g samples of the composite soil sample (see section 4.2.1). Spores were extracted by wet sieving and centrifugation (Dr. C. Walker, personal communication). Each sample was dispersed in one litre H_2O and the suspension was left undisturbed for 30 minutes to allow the soil particles to settle. The suspension was decanted through 710 μm and 25 μm sieves. More water was then added to the sample, which was stirred to resuspend spores and allowed to stand for 15 sec, to allow sand sized particles to settle. The resultant supernatant was decanted through the two sieves. This was repeated four more times until the supernatant became clear. The material from 25 μm sieve was then centrifuged in water, debris discarded and then the spores were floated on 75% w/v sucrose by centrifuging for 20 sec and then washed with water. Spores were collected in a small Petri dish. Spores were counted and collected under a dissecting microscope and the total number per sample recorded. Only spores which appeared to be unparasitized and cytoplasm-filled were counted. The spores from 710 μm sieves were collected and counted direct under a dissecting microscope.

Spores were then mounted on microscope slides in polyvinyl-lactic acid-glycerol (PVLG), covered with a thin cover slip and the water was allowed to evaporate for two days. The cover slips were then sealed. Spores were examined under a compound microscope. After measuring, the spore was opened by crushing. Once open the spore was crushed with lateral movements to dissociate inner walls. Some groups of spores were mounted in a 1:1 mixture of Melzer's reagent and PVLG before crushing (Morton, 1986). Species identification was based on spore colour, size, surface ornamentation and wall structure (see section 3.2.4). Permanent slide vouchers were made of all fungi and some fungi were established in single species cultures and retained in the Soil and Water Department, The University of Adelaide. Authorities of AM fungal species cited in this thesis are in the Appendix 1.

4.2.2 Trap cultures

4.2.2.1 Plants

Plants used were *L. perenne, T. subterraneum, P. lanceolata* and *Sorghum* sp. Plants of *L. perenne, T. subterraneum* and *P. lanceolata* were chosen as hosts because they were present at the field site. Seed of *P. lanceolata* was collected from Waite Campus in November of 1996 and stored. Sorghum was used as a host plant because it a suitable host for a wide variety of AM fungi (Morton *et al.*, 1993). Three plants per pot were used with four replicate pots per host plant. Seeds were surface sterilised with 0.5% NaOCl for 10 min followed by a rinse in sterile distilled water and germinated on moist filter paper at 25°C in an incubator. Seven day-old seedlings were transplanted into 1 kg pots containing unsterilised field-soil from the permanent pasture trial and autoclaved sand (1:1). Seedlings of *T. subterraneum* were inoculated with 1 ml of *Rhizobium trifolii* suspended in a 1% sucrose solution at transplanting. All plants were grown under the same conditions as follows: 12h, photoperiod, 18-25°C temperature, 60 - 75% relative humidity, 240 μE m^{-2} s^{-1} irradiance; and received 15 ml of Long Ashton nutrient solution without phosphate weekly (Hewitt, 1966). The plants were grown in a growth chamber for five months.

4.2.2.2 Harvesting

Four cores (1.5 cm diameter x 12 cm deep) per pot were collected. The soil was used to assess spore populations (see section 4.2.1.2) and the roots to determine the percentage of roots colonised by AM fungi.

4.2.2.3 Mycorrhizal assessment

The colonisation of roots was determined by using the roots with little pigmentation captured on the sieve, or retrieved during spore extraction. The roots were washed thoroughly with water, fixed in ethanol (50%) and stored until they could be processed. Fixed roots were cleared in 10% KOH (Koske and Gemma, 1989), acidified with 0.1 N HCl and stained with trypan blue (0.01% in lactoglycerol) (a modification of the method of (Phillips and Hayman, 1970). The stained roots were examined

microscopically between 10 and 100x magnification to observe AM fungal structures. Percentage colonisation was assessed with the grid line intersect method (Giovannetti and Mosse, 1980).

4.2.2.4 Community indices

Richness, diversity and abundance of AM fungi spores were calculated according to the following equations:

Richness: number of species found in a sample

Abundance: total number of spores found in a sample

Diversity: Simpson's index (Begon *et al.*, 1990)

$$D = 1/\sum_{i=1}P_i^2$$

Where:

D is the diversity,

$\sum_i$ is sum of species,

P_i is the proportion of total spore number in the ith species.

4.2.2.5 Statistical analysis

Abundance, richness and diversity in soils collected from the field at four times were compared by one-way ANOVA, followed by Tukey's (HSD) test ($P<0.05$).

An analysis of variance by GLM (General Linear Models Procedure, SAS, 1986) was used to investigate the effects of sampling time and host plants on richness, diversity, production of spores and percentage colonisation in trap cultures because factor combinations had different numbers of host plants (four sampling times, three host plants for July 1996 and four host plants for December 1996, July 1997 and December 1997). The data were tested for normal distribution and transformed when necessary to improve normality. The Tukey (HSD) test was used to compare means (SAS, 1986).

4.2.3 Single spore isolation

Fungi recovered from the composite field sample were used for single spore isolation. Spores of *Acaulospora* sp., *Gigaspora* spp., *G. constrictum, G. invermaium, G. mosseae, Glomus* spp., and *Scutellospora* spp. were used in attempts to initiate single-spore cultures. The spore isolation was as described in 4.2.1.2. Spores were put on filter paper on wet autoclaved sand in a Petri dish (Brundrett *et al.*, 1996). Seeds of *L. perenne, T. subterraneum* and *P. lanceolata* were surface sterilised and germinated as described in section 4.2.2.1. The seedlings were transplanted into pots (500 ml) containing autoclaved field soil-sand mixture (1:1). The mix was autoclaved at 110°C for 1 h on each of three consecutive days. At transplanting, a germinated single spore of each AM fungus was put on a branching point of the root under a dissecting microscope and covered with the soil . Five pots were treated in exactly the same manner as the other pots, except that no spore was added, and five pots were inoculated with *Scutellospora calospora* as a positive control. Sixty four open pots were placed directly in a greenhouse and 90 pots were enclosed in transparent plastic bags (Sunbag, Sigma) to prevent cross-contamination (Walker and Vestberg, 1994). The plastic bags were sealed by double folding the top and fixing them with paper clips. These pots were placed in a growth chamber in a completely randomised design, and maintained at temperature 18-25°C, relative humidity 60 - 75%, photoperiod 12h, irradiance 240 μE m^{-2} s^{-1}. No nutrients were added during the first three weeks and sterile water was supplied when required. Each pot received 10 ml of Long Ashton nutrient solution without phosphate each two weeks thereafter. Spore formation and mycorrhizas were examined after eight months.

Success was determined by examination of roots for mycorrhizal colonisation and soil for presence of spores as described by Walker & Vestberg (1994) . The soil sample, one core (1.5 cm diameter x 12 cm deep) per pot was placed in a 100 ml beaker containing tapwater and mixed. The heavier particles were allowed to settle for a few seconds before the supernatant was decanted into a 25 μm sieve, and washed to remove fine particles. The material was washed into a small Petri dish for examination

under a dissecting microscope. The mycorrhizal colonisation was assessed as described in section 4.2.2.3.

4.3 Results

4.3.1 Glomalean spores in field-collected soil and trap cultures

Temporal variation in spore populations of AM fungi in the permanent pasture was studied in field-collected soil and trap cultures. Sixteen species of AM fungi, plus an unidentified spore type, were observed in this study (Table 4.2). The composition of the spore communities differed between field-collected soil and trap cultures. Nine species were the same in both. However, *Acaulospora* sp., *Glomus aggregatum*, and *G. macrocarpum* were only recovered in field-collected soil and *Entrophospora* sp., *G. clarum, G. coronatum* and *G. etunicatum* only in trap cultures (Table 4.2). No spores or sporocarps of *Sclerocystis* were recovered by either method. Most of species observed belong to the genus *Glomus*.

At the four sampling times in the field the predominant fungal species recovered was *G. mosseae* followed by *Glomus* spp., *G. microaggregatum*, *G. invermaium* and *Gigaspora* sp. In most cases spores of *G. microaggregatum* were found inside *Gigaspora*, *Scutellospora* and *Glomus* spores. Species richness was much higher in July 1996 than December 1996, 1997 or July 1997. Spore numbers were also the highest in July 1996 (as high as 5 spores/g soil). Species diversity did not differ between sampling times. Spore numbers were higher in the season when plants were growing (July) and decreased at the end of the season (Table 4.3).

Table 4.2 Mycorrhizal fungal spores present in field collected soil or obtained in trap cultures.

AM fungal species	Field collected soil	Trap culture
Acaulospora sp.	+	-
Entrophospora sp.	-	+
Gigaspora margarita	+	+
Gigaspora sp.	+	+
Glomus aggregatum	+	-
Glomus clarum	-	+
Glomus constrictum	+	+
Glomus coronatum	-	+
Glomus etunicatum	-	+
Glomus sp1.	+	+
Glomus macrocarpum	+	-
Glomus microaggregatum	+	+
Glomus mosseae	+	+
Glomus spp.	+	+
Scutellospora sp.	+	+
Scutellospora heterogama	+	+
Not identified	+	-

Table 4.3 Spores of AM fungal species recovered from field-collected soil at the Permanent Pasture Rotation trial, in July and December/1996; July and December/1997. Species are listed in order of spore abundance per 25 g.

Species of AM fungi	Jul-1996	Dec-1996	Jul-1997	Dec-1997
Glomus mosseae	53	20	28	23
Glomus sp.	7	5	8	1
Glomus microaggregatum	0	0	7	8
Glomus sp1	8	1	3	2
Gigaspora sp.	6	0	2	1
Gigaspora margarita	6	0	1	1
Scutellospora sp.	5	0	1	1
Glomus aggregatum	4	0	0	0
Acaulospora sp.	0	1	1	0
Scutellospora heterogama	1	1	0	0
Glomus constrictum	2	0	0	0
Not identified	4	1	2	1
Total	96	29	53	38
Community indices				
Richness	9.67a	5.33b	6.33b	4.67b
Diversity	3.04a	2.04a	3.02a	2.17a
Abundance	133a	47c	81b	68c

Means for community indices followed by the same letter are not different ($P<0.05$)

The study with *L. perenne, P. lanceolata, Sorghum* sp and *T. subterraneum* as host plants for trap cultures also indicated that *G. mosseae* was the predominant species, followed by *G. invermaium* and *Glomus* spp. (Table 4.4). In general all hosts produced more *G. mosseae* spores from the field-collected soil samples in 1997 than 1996. Spores of *G. etunicatum* were recovered only with *L. perenne* and *Sorghum* sp. as hosts, *Entrophospora* sp. and *G. constrictum* were recovered with *T. subterraneum,* and *S. heterogama* and *G. coronatum* with *Sorghum* sp.

Arbuscular mycorrhizal spore community indices (richness, diversity and abundance) changed with sampling time and host. The index for richness was significantly higher in cultures of *L. perenne, T. subterraneum* July 1996, *T. subterraneum* December 1996, of all host in July 1997 and of *L. perenne, Sorghum* sp. and *T. subterraneum* in December 1997, compared to other sampling times and hosts. Cultures with all hosts in July 1996, 1997 and *T. subterraneum* December 1996 were characterised by significantly higher species diversity (Table 4.4). *T. subterraneum* grown on soil sampled in July 1997 and in December 1996 and *Sorghum* sp. grown in soil sampled in July 1997 produced the highest numbers of spores (p<0.0001). Most of those spores were immature. *L. perenne* grown in soil collected in December 1996 showed the lowest species richness, diversity and abundance of associated fungi. The Simpson's diversity index in this study with field collected material ranged from 2 to 3 and in trap cultures ranged from 1.5 to 3.5. (Table 4.4).

Sorghum sp. and *T. subterraneum* showed higher mycorrhizal colonisation in soil from all sampling times and *L. perenne* the lowest. *P. lanceolata* showed higher mycorrhizal colonisation in December 1996 than in any other collection time.

Table 4.4 Influence of *Lolium perenne* (L), *Plantago lanceolata* (P), *Sorghum sp.* (S) and *Trifolium subterraneum* (T) as hosts for trap cultures on spore populations of mycorrhizal fungi and on mycorrhizal fungal community indices. Spore number per 25 g soil.

Species of AM fungi	July/1996			December/1996				July/1997				December/1997			
	L	S	T	L	P	S	T	L	P	S	T	L	P	S	T
G. mosseae	7	3	11	4	7	6	8	20	11	17	20	22	17	12	18
G. invermaium	3	0	2	0	1	0	1	10	9	9	13	4	4	4	8
Glomus spp.	1	5	5	1	2	3	2	3	1	4	4	0	2	2	3
G. microaggregatum	2	0	8	0	0	0	6	2	0	2	3	0	0	1	1
Gi. margarita	3	0	1	0	1	0	1	1	2	4	3	1	0	1	1
Gigaspora spp	1	1	0	0	0	1	2	1	1	0	1	3	1	1	2
Scutellospora spp	1	0	1	0	1	0	0	2	0	2	2	1	3	0	0
G. etunicatum	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Entrophospora sp.	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
S. heterogama	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
G. coronatum	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
G. constrictum	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Community indices															
Richness	5.0ab	3.7bc	6.7a	1.7c	3.3bc	3.3bc	5.3ab	6.3a	4.3ab	5.3ab	5.7ab	5.3ab	3.7bc	4.7ab	4.3ab
Diversity	3.5ab	2.8abc	4.2a	1.5c	2.4bc	2.5bc	3.5ab	3.0abc	2.8abc	3.4ab	3.2ab	2.2bc	2.1bc	2.6bc	2.6bc
Abundance	22.3ed	22.7ed	34.3bcd	11.7e	31.0bcd	27.0cde	63.0a	44.7b	44.7b	69.0a	67.7a	37.0bcd	35.3bcd	20.1ed	40.0bc
Colonisation (%)	59.0cd	83.7a	75.0ab	43.9ef	78.4ab	83.7a	81.0a	20.3g	56.9ed	79.9a	83.3a	32.3fg	64.8bcd	70.9abc	79.8a

Means for community indices followed by the same letter are not different (P< 0.05).

4.3.2 Single spore isolation

The method where pots were enclosed in transparent plastic bags was more successful for establishment of single spore cultures of AM fungi from field-collected spores than open pots (Table 4.5). Using the Sunbag system *P. lanceolata* was the most successful host, which established mycorrhizas in 31.4% of the pots and sporulation in 11.4%. *G. mosseae, G. constrictum, Glomus sp.* and *Gi. margarita* all produced successful cultures in *P. lanceolata*, and mycorrhizal associations were produced in 11 pots. The identification of these species was confirmed by Dr. S. Rosendahl. The Sunbag cultures with *P. lanceolata* allowed single spore production of a higher number of fungal species than other plant species. *T. subterraneum* did not develop very well in Sunbags even though *G. mosseae* sporulated in one pot and nine of 27 pots showed colonisation. The trap plants *L. perenne* and *Sorghum* sp. failed to establish single spore cultures (a smaller number of pots was used) although there were some pots with mycorrhizal colonisation. These plants also did not grow well in Sunbags.

The open pot method showed success in producing mycorrhizas and spores only with *T. subterraneum* as host. *G. mosseae* was the only successful species in producing spores. *L. perenne, P. lanceolata* and *Sorghum* sp. had colonised roots but did not sporulate (Table 4.5). In the open system three of the 64 pots were contaminated. The pots showed more than one species of AM fungus and a lot of larvae of insects. The positive control with *Scutellospora calospora* had good production of spores. None of the uninoculated controls became colonised.

Table 4.5 Single spore pot culture initiated from spores collected from field soil between July/1996 and December/1997 from the pasture plot, at the Permanent Rotation Trial at The Waite Campus.

Method	Hosts	Attempts at inoculation	Number of pots with colonisation	Number of pots with contamination	Number of pots with spore production (%)	Fungi obtained as spores in single spore culture
A) Sunbag - Growth chamber						
	L. perenne	15	3	0	0	none
	P. lanceolata	35	11	0	4 (11.4)	*G. mosseae*
						G. constrictum
						Glomus sp.
						Gi. margarita
	Sorghum sp.	13	3	0	0	none
	T. subterraneum	27	9	0	1 (3.7)	*G. mosseae*
B) Open pot - greenhouse						
	L. perenne	12	4	0	0	none
	P. lanceolata	18	5	1	0	none
	Sorghum sp.	14	5	0	0	none
	T. subterraneum	20	8	2	1 (0.05)	*G. mosseae*

4.4 Discussion

4.4.1 Community and species diversity of AM fungi in a permanent pasture

This study was designed to determine the community structure of AM fungi in a permanent pasture by identification and quantification of spores found in field soil and in trap cultures. The high richness index in the July samples could reflect the large number of spores, rather than seasonal differences. The increase in the species richness index concomitant with increased sporulation that has been reported in three cycles of successive trap cultures associated with *Prosopis* spp. in arid ecosystems, suggests that *G. mosseae, G. microaggregatum* and *G. intraradices* inhibit the sporulation of associated species (Stutz and Morton, 1996). The lower number of spores in July 1997 compared to July 1996, which coincided with maturation of the pasture plant community, may be due to the presence of nematodes, collembolans, acarians and other organisms observed in the field collected soil (data not shown) that was not observed in July 1996.. Predation and hyperparasitism of mycorrhizal fungi can be caused by many organisms found in soil and can result in degradation of spores (Fitter and Garbaye, 1994; Lee and Koske, 1994). Another explanation for the low number of spore in July 1997 could be that the precipitation for the first five months was lower than the same period of 1996 (Table 4.1) resulting in less mycorrhizas and just the robust propagules could survive.

Occurence of some species of AM fungi in the field depends on the cultural practices. In the permanent pasture studied in this thesis *G. mosseae* spores were the most abundant species in field-collected soil. In Western Australia and New Zealand, in both virgin and agricultural soils a single species of mycorrhizal fungus also dominates, but the dominant species is *A. laevis* (Abbott and Robson, 1977; Crush, 1975; Crush, 1973; Mosse and Bowen, 1968). In other situations single mycorrhizal species have been shown to be dominant in terms of spore number, such as in a continuous soybean crop where *Gigaspora* spp. dominated and in a soybean rotation with corn (*Zea mays* L.) , milo (*Sorghum bicolor* (L.) Moench), or fescue (*Festuca arundinaceae* Schreb cv. Johnstone), where *Glomus* spp dominated (Hendrix *et al.*, 1995). It may be that these particular fungal species have effective mycorrhizal associations with the plant hosts that are present in the different

systems, leading to high spore production. Alternatively, the presence of large amounts of infective propagules (hyphae) in the soil may lead to rapid colonisation and promote spore production whenever conditions are suitable for plant growth (Jasper *et al.*, 1987, McGee, 1989; McGee *et al.*, 1997). In our studies the presence of a susceptible host in the field that could increase the sporulation and number of hyphae may have kept the number of *G. mosseae* spores constant over the year. This will require further investigation. The reason for the dominance of one species over another could be due to antagonism between species. Support for this suggestion comes from a study in which the abundant spore production by one AM fungus was correlated with lower levels of spores production by other AM fungi (Gemma *et al.*, 1989). The distribution of AM fungi may also be correlated with soil pH where species such as *G. tenue* have been found to be more abundant in acidic soils (Porter *et al.*, 1987). At the Waite site the pH was around 5.9.

Spore numbers may also vary with time of year. In July 1996 the number of *G. mosseae* spores at the Waite permanent pasture was almost twice that measured at the three other sampling times. In another study on perennial grasses where *Uniola paniculata* (Sylvia, 1986) was the predominant plant species, spore density varied with AM fungal species. *G. globiferum* increased more than 500% from May to August, while *G. aggregatum* spore numbers increased less than 30%. The predominance of a single AM fungal species could be due to the constant structure and composition of the plant community in the pasture system which results in a stable AM fungal community that favours a single species. Many studies have suggested that spore numbers increase when plants are in the mature stage of development (eg. Hayman, 1970; Sutton and Barron, 1972). The highest abundance in July 1996 and 1997 could be explained by the presence of host plants. However this sporulation of AM fungi could be influenced by many other factors, such as temperature (Schenck and Smith, 1982), nutrients (Sylvia and Schenck, 1983) edaphic and other environmental factors (Daniels Hetrick and Bloom, 1986; Kurle and Pfleger, 1996). Since variation in spore numbers was observed at different times of the year it appears that more than one year is required to determine how the AM fungal community is affected by temporal changes and climatic conditions. Spore abundance in the field collected soil cannot be related to

the trap cultures because growth environments for the fungi are different (Stutz and Morton, 1996).

Different species were recovered by trap culture studies as compared to collection from the field. In the trap cultures the recovery of *Entrophospora* sp., *G. clarum, G. coronatum* and *G. etunicatum* could be due to the presence of root fragments containing mycelia or some other form of propagules when the soil was sieved and mixed for the assay. This procedure may have exposed propagules present inside the roots which colonised the roots of the trap plants, followed by sporulation (An *et al.*, 1993). Another possible explanation is that the number of field-collected soil samples analysed may not be high enough to recover these species. The trap cultures may also have increased the opportunity for spore production of certain species. The differences in spore production and species recovered in trap cultures indicated that AM fungi respond differently to the sampling times (field samples) and host species (growth chamber). Differential responses in spore production of AM fungi in relation to host species were also reported by Sanders and Fitter (1992), where the number of spores of *G. constrictum* increased with *P. lanceolata* and spores of *A. laevis* increased with *Holcus lanatus* or *Rumex acetosa*. In almost all trap cultures spore production seems to show that high diversity and abundance of AM fungi are associated with particular hosts. Mycorrhizal fungal diversity and plant diversity (Van der Heijden *et al.*, 1998) may be related because a reduction of AM fungal diversity from four to one AM fungal taxon leads to a decrease in biomass of several plant species and to a change in AM fungal species richness. Therefore the composition and richness of AM fungal communities is an important contributor to plant species composition, abundance and diversity in artificial microcosms and macrocosms.

Some specificity between hosts and AM fungi seems to occur, but it is clear from the hosts that they have effects on the Glomales community which cannot be explained simply by host specificity. *P. lanceolata, Sorghum* sp. and *T. subterraneum* as hosts for trap culture did not show different influences on richness and diversity of AM fungi, however *L. perenne* gave the lowest richness and diversity in December 1996. This implies that it is difficult to analyse the specificity because of the interactions between different fungi in the same root system or the

presence of potential host plant species (Newman *et al.*, 1994; Smith and Read, 1997).

Using The Shannon-Weiner index, Hendrix *et al.* (1995) reported values around 1.0 in different cropping systems for AM fungal diversity. In a cacao plantation Cuenca and Meneses (1996) obtained an average value of 4.3 using Whittaker index. In this study (permanent pasture) the average of diversity index was 2.7. There are no published Simpson's index values for pastures to compare with the permanent pasture fungal community studied.

The high percentage of colonisation in trap cultures of *P. lanceolata*, *T. subterraneum* and *Sorghum* sp. was not related to the total number of spores in the field soils that were used. The lack of a correlation between total spore numbers and colonised roots has been reported previously (Abbott and Robson, 1982a; Hayman and Stovold, 1979; Scheltema *et al.*, 1987). Also differences in effectiveness of propagules has been shown by two isolates of the AM fungi *G. etunicatum* (O'Connor, 1994). Individual species in the mycorrhizal fungal community seem to vary in their occurrence on different host plants and a knowledge of the relative importance of different kinds of propagules of individual species is needed for a complete understanding of the mycorrhizal community dynamics in a particular system.

4.4.2 Single spore pot cultures

Arbuscular mycorrhizal fungi have not yet been cultured axenically and normally their isolation and maintenance are in open (Gilmore, 1968) or closed pot cultures - Sunbags (Walker and Vestberg, 1994). The success of *P. lanceolata* as a trap plant could be because this host was one of the predominant plant species in the permanent pasture. Alternatively this species may have particular characteristics that are favourable for the recovery of mycorrhizal fungi. Sanders and Fitter (1992b) reported that *P. lanceolata* promoted an increased density of spore of *G. constrictum* whereas *T. pratense* and *Festuca rubra* did not increase spore density of this AM fungal species.

Trap plants *L. perenne*, *Sorghum* sp. and *T. subterraneum* did not develop well in Sunbags. Establishment of optimum conditions for each host in Sunbags

such as light, temperature and substrate would be necessary for better production of AM fungi (Walker, 1994) using this system.

The successful isolation *G. mosseae*, *G. constrictum*. *Glomus* sp. and *Gi. margarita* from field soils provides new strains of AM fungi that could be used in future studies on infectivity, effectively, competition, nutrient uptake and genetic variation.

4.5 Conclusion

In the permanent pasture fungal community at the four sampling times between 1996 and 1997, *G. mosseae* was the predominant species.

The community diversity was not significantly different in field-collected soil at different sampling times, but it was significantly different between sampling times in trap cultures. The combination of spore identification from field-collected soil and trap cultures promises to be an effective tool to study populations and diversity of AM fungi, because higher numbers of species could be recovered by both methods together.

Successful production of single spore cultures of *G. mosseae*, *G. constrictum*. *Glomus* sp. and *Gi. margarita* with *P. lanceolata* and *T. subterraneum* as hosts will now allow these indigenous strains of AM fungi to be used in studies to analyse their ability to stimulate nutrient uptake and growth by different plant species.

Chapter 5

Variation in ribosomal DNA internal transcribed spacer sequences in *Glomus mosseae* and *Gigaspora margarita* spores from a permanent pasture

5.1 Introduction

In recent years much research has been done to elucidate the genetic diversity in Glomales (see Section 2.7). A major problem in studying AM fungi is that these obligatory biotrophic fungi do not grow in pure cultures in the absence of host roots and cannot easily be identified from vegetative structure. The taxonomy of these fungi has been based mainly on the morphological characteristics of the resting spores, including spore cell wall appearance and development (Morton, 1990; Walker, 1992). DNA sequences are powerful tools to confirm phylogenies based on morphology and to understand new aspects of the genetic variation of these organisms.

To develop molecular tools it is essential to first understand the variation between and within species at the molecular level. The nuclear ribosomal genes (rDNA) in all eukaryotes contain highly conserved regions (18S, 5.8S and 28S) (Simon *et al.*, 1992; White *et al.*, 1990). These conserved regions are separated by internal transcribed spacer (ITS) and the intergenic spacer (IGS) sequences which vary in length and contain sequence polymorphisms. Non-coding rDNA such as the ITS region is a good taxonomic indicator and has been used to detect variation between species in many fungi including *Pythium* spp. (Chen, 1992), some ectomycorrhizal fungi (Erland *et al.*, 1994; Henrion *et al.*, 1992; Martin *et al.*, 1998; Morton *et al.*, 1995a), and the AM fungus *Glomus mosseae* (Lloyd-MacGilp *et al.*, 1996; Sanders *et al.*, 1995).

Molecular studies have shown that *Glomus mosseae* is a highly variable species, based on ITS sequences. Restriction enzyme analysis of ITS DNA from ten morphologically identical spores of *Glomus* sp. from a field soil showed different polymorphisms (Sanders *et al.*, 1995). In a similar study, the analysis of the ITS region from one spore of *G. mosseae* (BEG 12 - European Bank of Glomales - pot culture) revealed that two out of the three sequences obtained were identical. A

higher level of diversity was reported within and between spores from different isolates of *G. mosseae, G. dimorphicum, G. fasciculatum and G. coronatum* spores (Lloyd-MacGilp *et al.*, 1996). These results demonstrated that *G. mosseae* ITS sequences from single spores grown in laboratory pot cultures were different from each other. Because AM fungi reproduce asexually, the genetic variability of ribosomal genes in single spores could occur both within and among nuclei in a spore (Sanders *et al.*, 1996). Most of the previous work on diversity within species has been conducted on spores from pot cultures maintained over different periods of time, a fact which could influence genetic diversity in the spore populations. If we are to understand the roles of these fungi in ecosystem processes it will also be important to understand the molecular diversity within natural populations. Furthermore, development of molecular tools for monitoring the fungal populations must take this diversity into account.

This chapter describes the inter- and intra-specific genetic variability of *G. mosseae* and *Gi. margarita* in spores collected from a permanent pasture at the Waite Campus, South Australia. The ITS1, ITS2 and 5.8S sequences of these species are described in terms of

1) inter- and intra-specific genetic diversity of these fungi in a natural ecosystem,

2) their relation to *G. mosseae* and *Gi. margarita* sequences deposited in GenBank,

3) the genetic divergence and genetic distances between *G. mosseae* and *Gi. margarita.*

5.2 Material and Methods

5.2.1 Fungal spores

Spores of *G. mosseae* and *Gi. margarita* were obtained from the permanent rotation trials at The Waite Campus of The University of Adelaide at Urrbrae, South Australia. The details of the trial have been described by (Grace *et al.*, 1995). The site selected has been under permanent pasture since 1950.

Spores were extracted from field soil collected in July 1996 and separated by repeated washing and resuspension in water and collected using a 38 µm sieve and sorted under a dissecting microscope. Spores from the field soil were grouped,

based on similar external features such as spore colour, size, shape, visible contents and shape of the subtending hypha (Schenk & Pérez, 1990). Greater than 90% of all spores were identified as *G. mosseae* (see chapter 4). The external morphological analysis was made first using a dissecting microscope and later a compound microscope. A group of matching spores was separated under the dissecting microscope and a more detailed identification was completed on 25 out of the 100 spores under the compound microscope for *G. mosseae* and 10 out of the 30 spores for *Gi. margarita*. Only spores with visible contents, clear characteristics cited above and that were apparently alive were selected for DNA extraction. Voucher specimens are retained at the Department of Soil and Water, The University of Adelaide. Identifications were kindly confirmed by Dr. C. Walker and Dr. S. Rosendhal. *G. mosseae* was the most common AM fungus in the pasture soil (see Chapter 4) and was chosen as the main focus for the work. *Gi. margarita* (BEG 34) grown in pot cultures with *Trifolium subterraneum* L. (clover) as described by Zézé (1997) was also used in this study.

5.2.2 DNA extraction

DNA from single spores of *G. mosseae* and *Gi. margarita* collected from field soil and DNA from single spores and pools of 15 spores of *Gi. margarita* from pot culture were analysed. DNA was extracted according to the method of Zézé *et al.* (1997). Groups of spores were cleaned three times by 20 sec sonication, followed each time by a sterile deionised water rinse. Water was removed by transferring the spore suspension onto filter paper. Spores were transferred to 1.5 ml microfuge tubes and DNA was extracted with minipestle over ice in 50 µl of extraction buffer containing 100 mM Tris, 100 mM NaCl, 2mM $MgCl_2$ and 2% Triton-X 100, pH 8.0. The samples were kept on ice for 2 - 3 min and centrifuged at 6000 rpm for 2 min. The supernatant containing DNA was transferred into a fresh microcentrifuge tube and stored at -20°C. This extracted DNA was used as template for the PCR reactions.

5.2.3 Amplification of rDNA

The internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) were

amplified by polymerase chain reaction (PCR) using primers ITS1 (5'

TTCCGTAGGTGAACCTGCGG 3') and ITS4 (5'

TCCTCCGCTTATTGATATGC 3') (White *et al.*, 1990). These primers are

specific for the region between the 3' end of the 18 s rDNA gene and the 5' end of

the 28s rDNA gene. The region amplified included the 5.8s gene and the ITS1 and

ITS2 regions (Fig. 5.1).
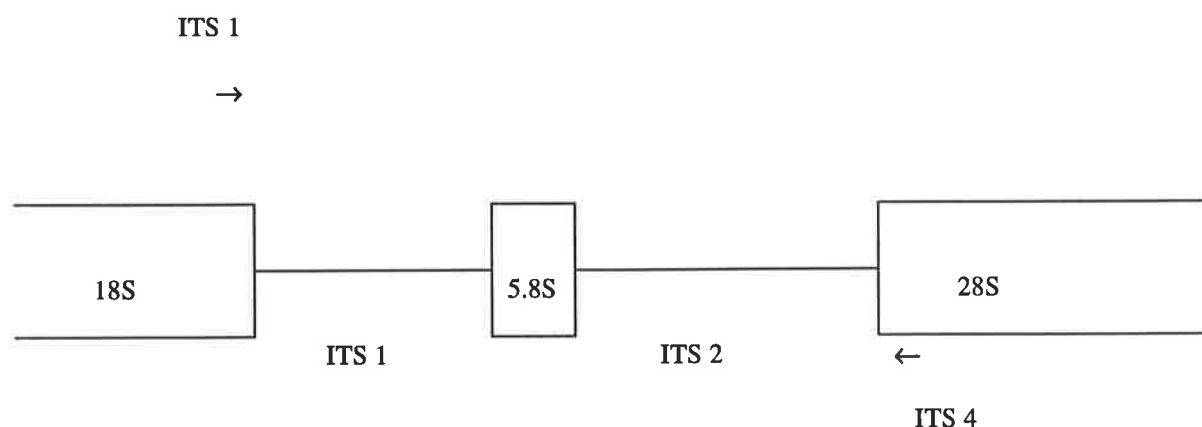
Figure 5.1. Schematic organisation of the region of rDNA used in this study.

Amplification of fungal DNA was carried out in a volume of 25 µl, containing 5 µl
of the diluted DNA from a single spore or pool of spores, 0.5 units of Expand™
High Fidelity PCR System (Boehringer Mannheim, Germany), 25 pmol of each
primer, 250 µM deoxynucleotide triphosphate (dNTP) and 250 µM 10X Expand
HF buffer with 15 mM $MgCl_2$. Each reaction was overlaid with 25 µl mineral oil
(Sigma). Thermal cycling parameters were 95°C for 2 min, followed by 14 cycles
of 96°C for 35 sec, 53°C for 55 sec and 72°C for 35 sec, 11 cycles of 96°C for 35
sec, 53°C for 55 sec and 72°C for 2 min; 15 cycles of 96°C for 35 sec, 53°C for
55 sec and 72°C for 3 min, with a final extension of 72°C for 10 min (Simon *et al.*,

1992). A negative control, that did not contain spore DNA was included in every experiment. The PCR was performed with a Programmable Thermal Controller (MJ Research Inc., PTC-60 Thermal Cycler, Watertown, Mass.). DNA from each spore was amplified in five separate amplification reactions under the same conditions, to ensure reproducibility. Three Taq DNA polymerases were compared, Expand, Promega and Qiagen *Taq* DNA polymerases. The Expand system gave the highest quantity and quality of product and amplified most DNA samples. DNA fragments from PCR were separated by electrophoresis (1.2% agarose gel) for 2 h in Tris-acetate-EDTA buffer (100 mM Tris, 125 mM sodium acetate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide and visualised under UV light.

5.2.4 DNA purification, cloning and sequencing

PCR products were purified from agarose gels with the QIAquick Gel extraction Kit (QIAGEN, Santa Clarita, USA). Purified DNA was cloned into the pCR-Script TM Amp SK(+) cloning kit (Stratagene Cloning Systems, California, USA). Plasmid was introduced into Epicurian Coli XL1-Blue MRF'Kan supercompetent cells (Stratagene) and recombinant colonies were selected and checked for the presence of insert by restriction digests with *Sac*I and *Knp*I enzymes. For sequencing, recombinant plasmid DNA was purified with QIAprep Spin Miniprep Kit (QIAGEN). Plasmid templates were sequenced by an Applied Biosystem automatic sequencer by Dr. N. Shirley. The sequence data was analysed using DNASTAR software.

5.2.5 Sequences analysis

ITS sequences were aligned using the Pileup and Pretty programs (Wisconsin Sequence Analysis, Version 8, Genetic Computer Group - GCG). The pairwise number of synonymous substitutions per site was estimated using Diverge (GCG). The single scores in each grouping of sequences in each species were averaged to estimate the mean divergence between sequences. A similarity matrix was obtained using Homologies (GCG). The phenetic tree was constructed using Clustalw with weighted residue weight table (DNASTAR). BLAST (Altschul *et al.*, 1990) was used to compare the ITS sequences obtained in this study with DNA sequences in GenBank.

5.3 Results

5.3.1 ITS region amplification and sequencing

The ITS1, ITS2 and 5.8S regions of the rDNA from *G. mosseae* and *Gi. margarita* single spores from the pasture soil and *Gi. margarita* single and pooled spores from pot cultures were amplified by PCR using the ITS1/ITS4 universal fungal primers as described. The occurrence of *Gi. margarita* was less than one spore per g of soil as compared to *G. mosseae* spores which averaged three spores per g of soil. In *G. mosseae* over 100 PCR reactions of single spores were performed and only 20 gave enough DNA for cloning.

PCR was performed on 12 single field spores of *Gi. margarita* but amplification products were obtained from only one spore. The *Gi. margarita* ITS sequences from this single spore may not be representative of the field populations and therefore the field results were compared with those obtained from ITS sequences from pot culture material. A different picture might be obtained at a site where *Gi. margarita* sporulated more abundantly and where spores contained material that was more amenable to molecular analysis. Where the amplification was achieved from single spore extractions of AM fungal DNA from field soil, a single ITS product was visualised on the agarose gel. The ITS region from *G. mosseae* was 560-569 bp whereas the ITS from *Gi. margarita* was 470-480 bp (Fig. 5.2). DNA from one spore identified morphologically as *G. mosseae* gave an amplification product (Fig. 5.2, lane 4) that was slightly larger (571 bp) than other *G. mosseae* sequences obtained. The sequences (5) from this spore did not show any identity with those of AM fungi deposited in GenBank and were therefore excluded from the subsequent analysis.

Initial attempts at direct sequencing of PCR products were unsuccessful. Consequently all sequences were obtained from cloned DNA (see section 5.2.4). For both species the entire length of the ITS region was sequenced in both directions (Figs 5.3 and 5.4). For the three *G. mosseae* single spores collected from the field twelve, five and six amplification products respectively were cloned. The individual sequences (nine, three and seven) of *Gi. margarita* single spore from the field, and the single and pooled spores from pot culture, respectively were compared. With the, exception of the spore mentioned above the *G. mosseae* and

Gi. margarita ITS sequences are closely related to AM ITS fungal sequences in GenBank. The *G. mosseae* sequences showed as high as 97% identity with the corresponding published sequences from the same species (Lloyd-MacGilp *et al.*, 1996; Sanders *et al.*, 1995) (Table 5.1). The *Gi. margarita* ITS sequences are all clearly related to each other, and showed 99.7% identity with the published ITS sequences from *Gi. margarita* (Lanfranco *et al.*, 1999) (Table 5.2) and 82% with the *Scutellospora castanea* (Franken and Gianinazzi-Pearson, 1996), which belongs to the same family of Glomales, Gigasporaceae.

Figure 5.2. PCR amplification products using the ITS1 and ITS4 primer pair: lanes 1, 2, 3 and 4 each contain the PCR product from a reaction with a single spore of *Glomus mosseae* from field soil (Waite Campus, South Australia); lane 5 product from a single spore of *Gigaspora margarita* from field soil; lane 6 product from a single spore *Gi. margarita* from pot culture and lane 7, pool of 15 spores of *Gi. margarita* from pot culture. Lane 8, no DNA template; lanes a and b: molecular size marker, 1kb ladder (Gibco-BRL).

a 1 2 3 4 5 6 7 8 b

0.5kb >

Alignment of ITS *Gigaspora margarita* sequences

```
                    1                                                                                   100
Gi.margarita3A      ****************.*********.**g*****************  **c****caa***t*****t********........*************
Gi. margarita2B     ****************.*********.**g*****************  **c****caa***t*****t********........**************
Gi. margarita1A     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1E     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1F     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1B     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1D     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1H     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1I     ****************.**********************.********  **********.*********a**g***......****c*********
Gi. margarita1G     **************c.*****************************  *************.**************......*************
Gi. margarita1C     ****************.*********a*****************  **c***aaa*t*t****ttt*****caa......*************
Gi. margarita3C     ****************.********************.********  **********...*a***t*aa*****......t*************
Gi. margarita3B     ****************.********************.********  **c**.....*t*t***a*tt***aa*aa......**************
Gi. margarita3F     ****************.*******a*****************  **c**...******tatat*t********c*****************ac*
Gi. margarita3G     .........ccc***.*******a*****************  **c**...******tatat*t*******c*****************
Gi. margarita3E     ****************.*******a*****************  **c**...******tatat*t*******ca***************ct**
Gi. margarita2C     ****************.*******a*****************  **c**...******tatat*t*******.tc**************ct**
Gi. margarita2A     ****************.********************.********  *************tata**t*****.......***********ct**
Gi. margarita3D     ****************.*******a*****************  **c**...******tatat*t*******ca****************
Consensus           AAGGATCATTAAAAAACTGAGGTATTTTATACCTCTTGTATTTAAAACCC  AATTCTTTTTAAAAATATAAATTTTTTTT-AAAAAAAAAAAAAAAACTT
```

```
                    101                                                                                  200
Gi. margarita3A     c************************************************.*a*****  ***********************************t****.**
Gi. margarita2B     ****************************************g*******a*****  ***********************g*******************t****.**
Gi. margarita1A     ***********************************************  *************************************.**
Gi. margarita1E     ***********************************************  *************************************.**
Gi. margarita1F     ***********************************************  *************************************.**
Gi. margarita1B     ***********************************g*********  ***********************g*****************.**
Gi. margarita1D     ***********************************g*********  ***********************g*****************.**
Gi. margarita1H     ***************************c*******  ***********************g*****************.**
Gi. margarita1I     ***********************************g*********  ***********************g*****************a**
Gi. margarita1G     ***********************************g*********  ***********************g*****************.**
Gi. margarita1C     *****************a**a********c*a*****  *a***t*t*****************************t****.**
Gi. margarita3C     ***************************************  *******t**********************************.**
Gi. margarita3B     ***********************************a*****  *a***t*t*****************************t****.**
Gi. margarita3F     *****************a**a********c*******  *a***t*t*****************************t****.**
Gi. margarita3G     *****************************t**c*******  *******t******************************t****.**
Gi. margarita3E     c****************************g*********  ***********************g*******************t****.**
Gi. margarita2C     ca***************************g*********  ***********************g*******************t****.**
Gi. margarita2A     c****************************c*a*****  ***********************g*******************t****.**
Gi. margarita3D     *****************a**a********c*a*****  *a***t*******************************t*****.**
Consensus           TCAACAATGGATCTCTTGGCTCTCGCATCGATGAAAAACGCAGCGAAATG  CGATAAGTAATATGAATTGCAAAATTCCGTGAATCATCAAATCTTTG-AA
                                                                              5.8S
```

```
                    201                                                                                  300
Gi. margarita3A     ****************************************t****************  cag*......t******************t*************a****.*
Gi. margarita2B     *********************************.*g*****************  cag*......t******************t*************a****.*
Gi. margarita1A     ****************************************g.****************  *****......******************************
Gi. margarita1E     ***********************************.****************  *****......*******.*****************
Gi. margarita1F     ***********************************g.****************  *****......*****.*.*****************
Gi. margarita1B     ***********************************g.****************  ****......****************************.*
Gi. margarita1D     ***********************************g.****************  *****......******************************.*
Gi. margarita1H     ***********************************g.****************  *****......*****************************.*
Gi. margarita1I     ***********************************g.****************  *****aaaaa************************.*
Gi. margarita1G     ***********************************g.**t************  ****.....a***********************.........**g*
Gi. margarita1C     ***********************************g.****************  *****......*******a*c************************g****.*
Gi. margarita3C     *********************************t**t************  cag*......t***********g****c***************..*.*
Gi. margarita3B     *********************************gt************  cag*......t***********g****c***************..*.*
Gi. margarita3F     *********************************.*g************  **.*......****************g***********************a*.*
Gi. margarita3G     *********************************.*t************  **c*.....****************t***************a*.*
Gi. margarita3E     *********************************.*g************  **.*......************g***t************c***a*.*
Gi. margarita2C     *********************************.*g************  **.*......************g***t************c***a*.*
Gi. margarita2A     *********************************.*g************  ****.....a***********g***t************c***a*.*
Gi. margarita3D     *********************************t************  ca**.....*.g********************************.*
Consensus           CGCAAATTGCACTTCTTGGTATTCCGAGGAA-TACACATGCTTGAGGGTC  AGTTG----TAAAAAAAAAATCGTACATCATTGATGTTGCGGATCTGGGTT
                                5.8S
```

```
                                                                        400
Gi. margarita3A  ****c*a*******************************...********  **************g*g*********************catt**....
Gi. margarita2B  ****c*a*******************************...********  **************g*g*********************catt**....
Gi. margarita1A  *************************************************  *****************************************************
Gi. margarita1E  *************************************************  *****************************************************
Gi. margarita1F  *************************************************  ***********************t****************************
Gi. margarita1B  *************************************************  *****************************************************
Gi. margarita1D  *************************************************  *****************************************************
Gi. margarita1H  *************************************************  *****************************************************
Gi. margarita1I  *************************************************  *****************************************************
Gi. margarita1G  *************************************************  ***********************t****************************
Gi. margarita1C  *************************************************  *****************************************************
Gi. margarita3C  ****c*a*******************************************  *****************************************************
Gi. margarita3B  ****c*a*********************t*************a*******  ****************************t***********************
Gi. margarita3F  ****c*a*****t***c****t*******...***tta**********  **************...************************************
Gi. margarita3G  ****c*a*****t***c****t*******...***tta**********  **************...************************************
Gi. margarita3E  ****c*a*****t***c***********...***ata**********  **************...********a***************************
Gi. margarita2C  ****c*a*****t***c****t*******...***tta**********  **************...************************************
Gi. margarita2A  ****c*a*****t***c***********...***ata**********  **************...************************************
Gi. margarita3D  *************************************************  *****************************************************
Consensus  ATTCTGTTTTTATAAATTGGTTACCTAAAATTAATATGGTTATGTAATGT GAAGCGTACTAATTATATAGTCGCTAATCATTTATACTTATTACATTACA


           401                                                          480
Gi. margarita3A  ...*c*att**gaat**agt**t*****a**********...********  **************t***************
Gi. margarita2B  ...*c*att**gaat**agt**t*****a**********...********  **************t***************
Gi. margarita1A  ******.**t***********..*********************  *g***************************
Gi. margarita1E  ******.*cg***********..*********************  *g***************************
Gi. margarita1F  ******.*************..*********************  *g***************************
Gi. margarita1B  ******.*************..*********************  *g***************************
Gi. margarita1D  ******.*************..*********************  *g***************************
Gi. margarita1H  ******.*cg***********..*********************  *g***************************
Gi. margarita1I  ******.*************..*********************  *g***************************
Gi. margarita1G  ******.*************..*********************  *g***************************
Gi. margarita1C  ******.*************..*********************  *****************************
Gi. margarita3C  ******c**g**********..*********************  *****************************
Gi. margarita3B  ******.**g***********..***************a*******  *****************************
Gi. margarita3F  *......*t**gtat**ag***********************  *****************************
Gi. margarita3G  *......*t**gtat**ag***********************  *****************************
Gi. margarita3E  *taca*gtt**gtat**ag*********************t  *****************************g***
Gi. margarita2C  *......*t**gtat**ag***********************  *********g*................
Gi. margarita2A  *taca*ctt**gtat**ag***********************  *****************************
Gi. margarita3D  *......*t**gtat**ag***********************  *****************************
Consensus  ACTATA-CGAAAGTATTTAGTGGTGGTGTATAAATTATGAGAATGACCTC AAGTCATGTGAGAGTACCCGCTGAACTTAA
```
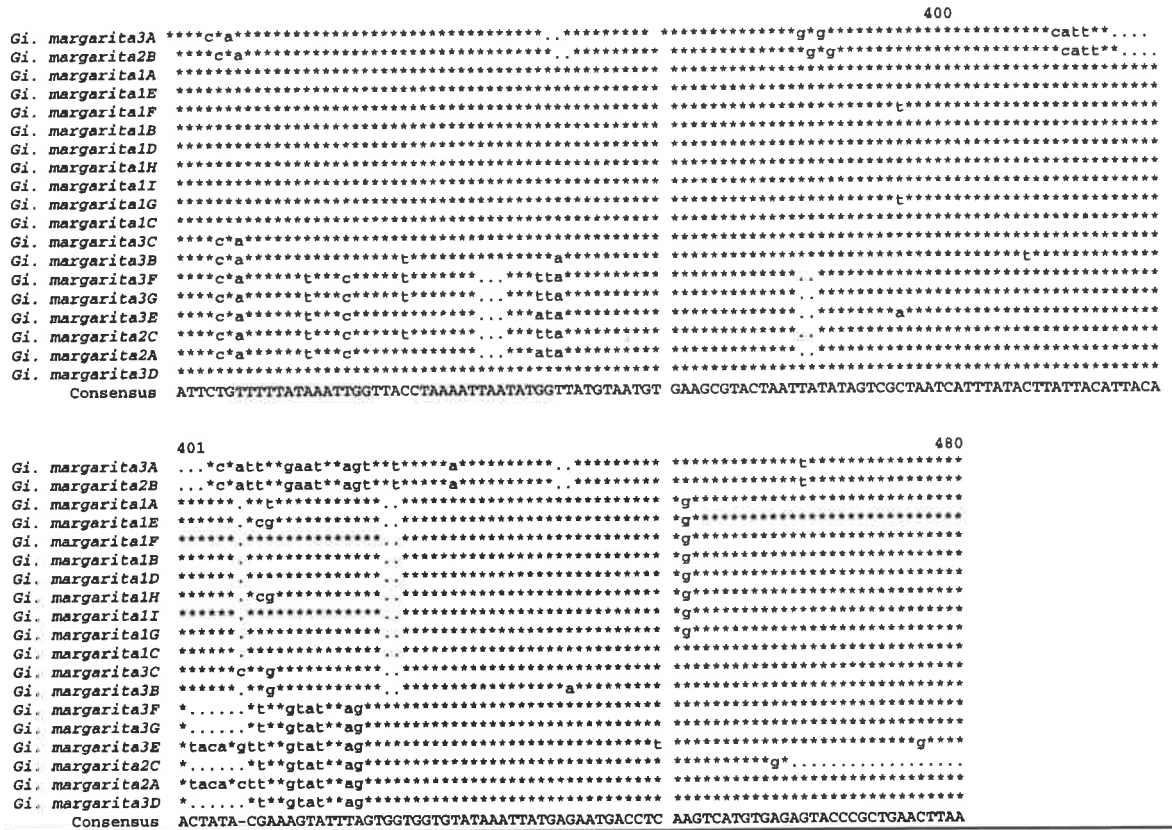
Figure 5.3. Multiple sequence alignments of the ITS region of *Gigaspora margarita* from a single field spore (1A-1I), pot culture single spore (2A-2C) and pool of spores from pot culture (3A-3G). Asterisk "*" indicates that the nucleotide is identical to the consensus sequence. Each "." denotes a gap resulting from insertion or deletion.

Alignment of ITS *Glomus mosseae* sequences

```
                 1                                                                                                  100
G. mosseae2E     *************t*a**a**gcg*gtcgag**tt***********c  *********************...***tgtaac*.ttttca***aa**
G. mosseae3F     *************t*a**a**gcg*gtcgag**tt***********c  **c*****************...***tgtaac*.tt*tca***aa**
G. mosseae3D     *************t*a**c**gcg*gtcgag**.t***********c  *********************...***tgtaac**tt*tta***aa**
G. mosseae3E     *************t*a**a**gcg*gtcgag**.************c  ******************tgtaac*tt*tt*a***aat*
G. mosseae3A     *************t*a**a**gcg*gtcgag**tt***********c  ****************.*a**c**tgtaac*.tt*ttat**aat*
G. mosseae3B     *************t*a**c**gcg*gtcgag**.t***********c  ***********t*****..**tgtaac**tt*tta***ggg*
G. mosseae3C     **************t****ac*gcg*gtc*at*ttt***ta*a*a*ggc  tgac***ata**t***a***c*a**c***.....g***ctttat**aa*a
G. mosseae2C     ***********c*************.***************  ***.****************c***a*****.*************
G. mosseae2D     ***********..a*************.*************  *****************.*****************a************
G. mosseae2A     ************.****************.*******  ***.********g*************************a*************
G. mosseae2B     ***********.***************.******c*******  ***.***************************a*************
G. mosseae1B     ***********.*.**************t****.******  ***********************************************
G. mosseae1J     ***********.*.************.*******  ***********************************************
G. mosseae1E     ***********.*.**************.*****  ***********************************************
G. mosseae1F     **************.*.*********.*****  ***********************************************
G. mosseae1D     ***********.****************gcgt****.****  ***.****************.*********g******
G. mosseae1G     ***********.*.**************gcg.******  ***********************************************
G. mosseae1H     ***********.****************a**********  *****************************a*ct.*************
G. mosseae1I     ***********.****************a*********  *****************************a*ct.*************
G. mosseae1A     ***********.****************a*********  ***********************************************
G. mosseae1L     ***********.*.**************a*******  ***********************************************
G. mosseae1C     ***********..a*****  ***********************************************
G. mosseae1M     ***********..a**********a***  ***********************.*****************
Consensus        AAGGATCATTAATGAATTTTTAAAGCGAGTCGACGCGTTAAGCGAGGCTT  GCGAAAATATTTAAAACCCCACTCTTTTTAACTTTTAAAAAATAAATCAT

                 101                                                                                                200
G. mosseae2E     t*a*tgaatt***t***********************.**********  **************c****.**********t****.**
G. mosseae3F     t*a*tgaatt***t******************.*********  ****************.**g*********.**********g****.**
G. mosseae3D     t*a*tgaatt***t****************g*.*********  *****t********.**g*********.**********g****.**
G. mosseae3E     a*atg*attt****************.*********  ***************.*****t****.**
G. mosseae3A     t*atttattt***t************.*********  *********.*******g**a**.***********t****.**
G. mosseae3B     ....tt*ttt***t********a************g*********  ********a***.*******g****g.gc**c*a*****a****.**
G. mosseae3C     *ggttgattt***t*********************.*********  *********c***.******c*tt*ttgtccc*c******g***c.**
G. mosseae2C     **c**********.*****************.*********  *************************************t****c**
G. mosseae2D     **c*********.**************.*********  *************g****.**********g****.**
G. mosseae2A     **c*********.**************.*********  ***************.**g*********.**********t****.**
G. mosseae2B     **c*********.**************.*********  *************c**.**********t*t****.**
G. mosseae1B     ***********************.*********  *************c**.**********t****.**
G. mosseae1J     *****t*t***********a****.*********  *************c**.**********t**t****.**
G. mosseae1E     *****t*******.************.*********  *************c**.**********t*t****.**
G. mosseae1F     ***********.************.*********  *********c**g**********.**********g****.**
G. mosseae1D     ***********.************.*********  ************.********.*****g******g******
G. mosseae1G     ***********.************.*********  ************.********c**.**********g****.***
G. mosseae1H     ***********.*******a***********.*********  *************a.********c**.**********t***.***
G. mosseae1I     ***********.*******a********.*********  *********a.********.******a*******t***.***
G. mosseae1A     ***********.*******.*********  **********.**g*****.**********g****.***
G. mosseae1L     ***********....****a***********.*********  ************.********c**.**********g****.***
G. mosseae1C     ***********....***************.*****  t***********.**c**g*****.**********t****.**
G. mosseae1M     ***********.**************.*********  *********.**g********.**********g****.**
Consensus        GATACATGAATTTAAAAAAAAAGATCACTTTCAACAAC-GGATCTCTTGG  CTCTCGCATCGATGAAAAAACGCAGCGAAAATGCGATAAGTA-TGTGGAA
                                                                                         5.8S

                 201                                                                                                300
G. mosseae2E     ***********.*************.*at*.******cg.**********  *t**g*tat*.**....***g*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae3F     ***********.*************.*at*.******cg.**********  *t**g*tat*.**.....*g*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae3D     ***********.************.*at*.******cg.**********  *t**g*tat*.**....***g*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae3E     ***********.************.*at*.******cg.**********  *t**g*tat*c**....***g*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae3A     **********.*************.*at*.******cg.**********  *t**g*tat*c********g*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae3B     **********.*************at*.******cgc*********  *t**g*tat*c*....***g*g*atgcc*gtt***a**gtcg*t*g*at
G. mosseae3C     ***********.********c****at*.******c*c**********  ***cg*t*****.c*****a*g*atgcc*g.t***a**gtcg*t*g*at
G. mosseae2C     ***********.*****************..**************c  ***c**t*ta*t*c****a*t*g*********...*********g****
G. mosseae2D     ***********.************.*c*.**********  ****g*********a*t*g*********...*********g***c
G. mosseae2A     ***********.************.****.***************  ***.*************a*t*g*********...*********g****
G. mosseae2B     ***********.************.****.***************  ***.****ta*t*c****a*t*g*********gg**********g****
G. mosseae1B     **********.************.****.**************  ***.************a*t*g*********...***.*******g****
G. mosseae1J     ***********.************.****.***************  ***.************aatt**g*********...***t******g****
G. mosseae1E     ***********.************.****.**************  ***.************a*t*g*********...***t******c****
G. mosseae1F     ***********.************.****.***************  ***.************a*t*g*********...***.******g****
G. mosseae1D     *g**********g*******t*g*at***g***********ac  t**.*******t*c****agta*g*ctg*tgga***tcgt*ag**.**
G. mosseae1G     *g**********g******t*g*at***g***********a*  t**********t*c****agta*g*ctg*tgga***tcgt*ag**.**
G. mosseae1H     **********.************..****.***************  ****.****.*t*c****aatta*g*ctg*tgag**ttcgt*ag***t*
G. mosseae1I     ***********.************.****.***************  ****.****.*t*c****agta*g*ctg*tgga***tcgt*agg**t*
G. mosseae1A     ***********.************t****tg***********ac  t***.****.*t*c****.agta*g*ctg*tg.a***tcgt*ag**..t
G. mosseae1L     ***********.***************.***************  ****g*****.*t*c****aagt*atgcc*g*ttga***tc*t*ag**..c
G. mosseae1C     **********.************...**tg*************  ***.************a*t**g*********...*********g*a*
G. mosseae1M     ***********.************...**tg*************  ***.************a*tt**g*********...***t******g*t*
Consensus        TTGCATAATTTTGGTGAATCATCGAATCCTTTTGAAACGCAAATTGCACT  CCCTTGGTATTCCGGGGGGG-ATTCCTGTTTGATTGGGGTCGTTAAAACA
                                                                5.8S
```

```
          301                                                                                          400
G. mosseae2E  ******************.*.******g*g******************  *****gtct**.**********c*****.************************
G. mosseae3F  ******************..*******g*g******************  *****gtct**.**********c*****.************************
G. mosseae3D  ******************..*******g*g*********a********  *****gtct**.**********c*****.************************
G. mosseae3E  ******************..*******g*g******************  *****gtct**.**********c*****.************************
G. mosseae3A  ******************..*******g*g******************  **c**gtct**c**********c*****.************************
G. mosseae3B  ******************..*******g*g**a*************c  ****gtct**.**********c*****.************************
G. mosseae3C  ******************..*******g*g**************  cga*****************c*****.********************.****
G. mosseae2C  ****tc.**************tt*******a***************  ***.*********************g***.a*******g*g***.****
G. mosseae2D  ******.cg*a*********tt*****.****************  ***.*********************g***.a*******g*g***.****
G. mosseae2A  ****tc.*************tt*****a*************  ***.*****************************.************.****
G. mosseae2B  ****tc.*************tt*****a***********  ***.*****************************.************.****
G. mosseae1B  ****tc.************c.***************  ***.*****************************.*********g***.****
G. mosseae1J  ****tcg*************.**************  **.*****************************.************.****
G. mosseae1E  ****tc.***c*********ct***********  ***.*****************************.*********g***.***c
G. mosseae1F  ****tc.**********t*c.*********g****.*****g***  ***.*****************************.*********g**g.****
G. mosseae1D  ****tcg************.*************  ***.*****************************.*********g***.****
G. mosseae1G  ****tcg************.************  ***.*****************************.************.****
G. mosseae1H  ********gcgtc*cac**.************  ***.*****************************.*********g***.****
G. mosseae1I  ****tcg***********.************  ***.*****************************.*********g***.****
G  mosseae1A  *****************.**************  ***.*****************************.*********g***.****
G. mosseae1L  ***********c****.****************c*a********  ***.*****************************.**c********g***.****
G. mosseae1C  ****tc.***********.*****************a*******  **ag*****************************.**c********g***.****
G. mosseae1M  *************.t*g*c********t*a**********c*****  **c**tc*t*cca*tg*t*a***c****t*****t**************
Consensus     AAAAATCGAAGCGTCGCTCT-CTTTTTTTAAGGGTGATCGCGTCGGAATT GAGCCCGTCTTTCAAATGTTAATTCATG-TCAAAGTGGCTTAAAATTTCA

          401                                                                                          500
G. mosseae2E  **ta***t*************************a*c*********  *********cgt*************gta*tacg**tgaccttt**t**.
G. mosseae3F  **ta**tt***********************a***********  *********cgt******t******gta*tacg**tgaccttt**t**.
G. mosseae3D  **ta***t**********************a**********  *********cgt******t*t***gta*tacg**tgaccttt**t**.
G. mosseae3E  **ta***t*********************a*c*********  *********cgt************gta*tacg**tgaccttt**t**.
G. mosseae3A  **ta***t*************ccc********a***********  *********cgt************gta*tacg**tgaccttt**t**.
G. mosseae3B  **ta***t************cc********a**********  *********cgt************gta*tacg**tgaccttt**t**.
G. mosseae3C  **ta***t******aa*****g*cccg*******a*c**********  *********cgt************gta*tacg**tgaccttt**t**.
G. mosseae2C  *.***********************t*.**********  ****************************.*********c**.g**a**
G. mosseae2D  *.*****************g***********t*.*********  ****************a*********.*********c**.g**a**
G. mosseae2A  *.*******************************t*.********  ************a******a*********.*********c**.g*****
G. mosseae2B  *.***************************t*.********  ***************************.*********c**.g*****
G. mosseae1B  *.*******************c***********.********  ****************************.****************
G. mosseae1J  *.*****************************.********  ****************************.****************
G. mosseae1E  *.*****************************.********  ****************************.****************
G. mosseae1F  *.*****************************.********  *********a*a****t********.************t**.***
G. mosseae1D  *.*****************************.********  ****************************.*********c****
G. mosseae1G  *.*****************************.********  ***************.*t***********c***.******a****g*******
G. mosseae1H  *.*****************************.********  ********.*t***********c***.******a****g*******
G. mosseae1I  *.*****************************.********  **********.************t*********.******a****g****
G. mosseae1A  *.*****************************.********  ***************.*t**********t*a******a****g******
G. mosseae1L  *g************c***************.******g*  ******g***cgtac*ataa*a*aaa*agt*******a*******g***
G. mosseae1C  *c***********************.*********g*  *******a***a.a*****t*********.*******a***g***t***
Consensus     TACCATCCGGTACGATTAAAGCGTATTTAAGATCAACTTTTGATTAAGAA CGCGCGATGAACGTACCATCTCATGTAGTGACGTTTGACTTGACGTCGTC

          501                                                           569
G. mosseae2E  *******.*************..**c*****************  *****************a****
G. mosseae3F  *******.*************..**c*****************  *****************a****
G. mosseae3D  *******.*************..**c*****************  *****************a****
G. mosseae3E  *******.*************..**c*****************  *********************
G. mosseae3A  ****a**.*************..**c*****************  *********************
G. mosseae3B  *******.*************..**c*****************  *********************
G. mosseae3C  *******.*************..**c****c************  *g******************
G. mosseae2C  ***t***.****************************  *g******************
G. mosseae2D  ***t***.****************************  *g******************
G. mosseae2A  ***t***.****************************  *g******************
G. mosseae2B  ***t***.****************************  *g******************
G. mosseae1B  *******.****************************  *********************
G. mosseae1J  *******.****************************  *********************
G. mosseae1E  *******.****************************  *******************
G. mosseae1F  *******.***********.***a*************  **********a***
G. mosseae1D  *******.****************************  *********************
G. mosseae1G  *******.****************************  *********************
G. mosseae1H  *******.****************************  *********************
G. mosseae1I  *******.****************************  *********************
G. mosseae1A  *************g***..****************  *********************
G. mosseae1L  ********ggtatat********..***************  ***********a*******
G. mosseae1C  **********g***gg**..*g***************  *g******g*********
G. mosseae1M  **********.****g**c****.***.*************  ***********a****
Consensus     AGGATCATTTCGTATACGATACTCTAAACTTTTGACCTCAAATCAGGTAA GAATACCCGCTGAACTTAA
```
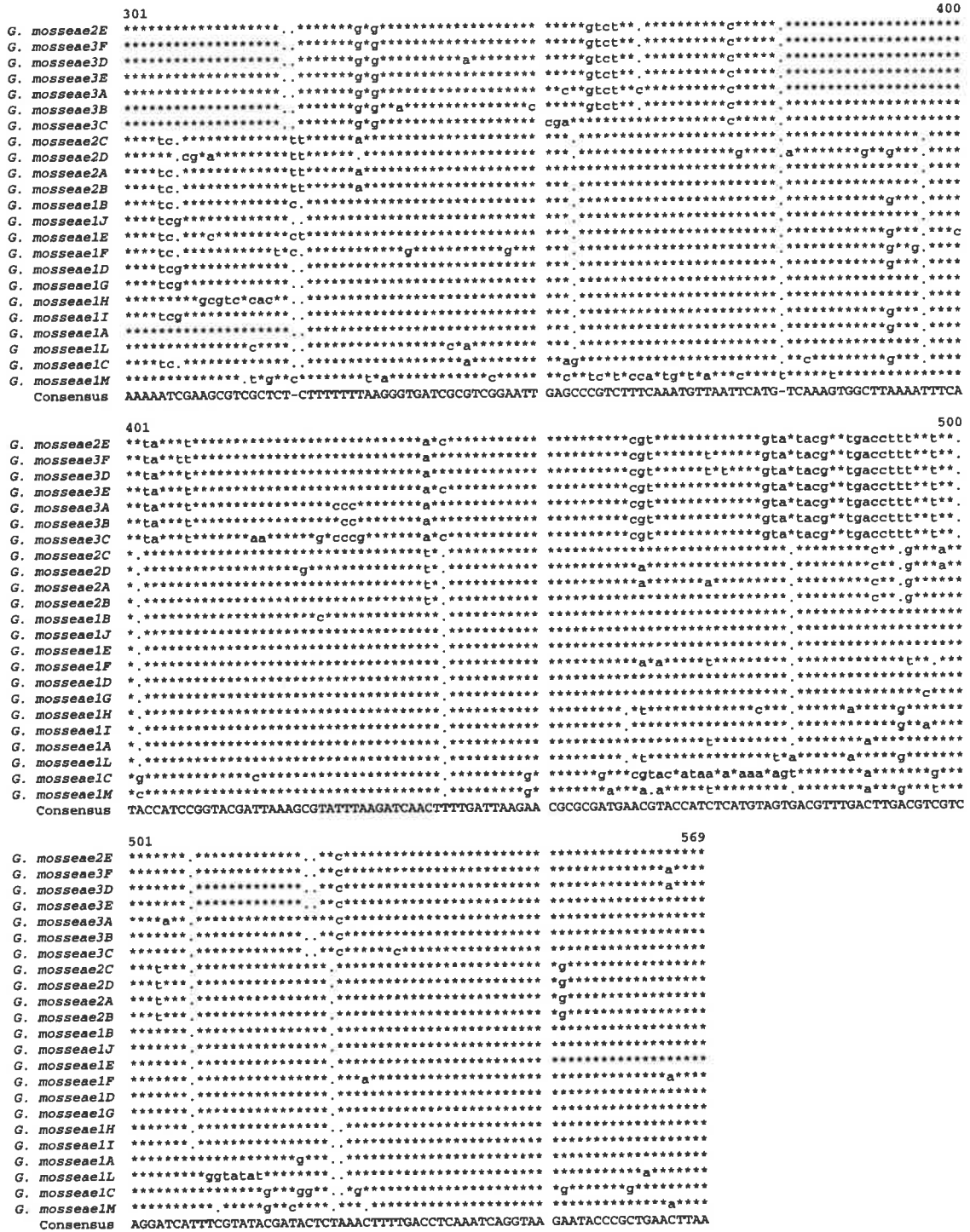
Figure 5.4. A) Multiple sequence alignments of the ITS region of *Glomus mosseae* from three field single spores (1A-1M, 2A-2E and 3A-3F). Asterisk "*" indicates that the nucleotide is identical to the consensus sequences. Each "." denotes a gap resulting from insertion or deletion.

Table 5.1.: Highest percent sequence identity of ITS sequences from *Glomus mosseae* field spores as compared to sequences in the GenBank.

Sequences from this study	Accession no.	Sequence from database (origin)	Accession no.	Identity (%) with the database sequences
*G. mosseae*1A	AF161043	*G. mosseae* (Finland)	X96834	96.3
*G. mossseae*1B	AF161044	*G. mosseae* (Indonesia)	X96832	96.5
*G. mosseae*1C	AF161045	*G. mosseae* (Finland)	X96834	92.3
*G. mosseae*1D	AF161046	*G. mosseae* (Indonesia)	X96832	93.9
*G. mosseae*1E	AF161047	*G. mosseae* (Indonesia)	X96832	95.1
*G. mosseae*1F	AF161048	*G. mosseae* (Indonesia)	X96830	94.7
*G. mosseae*1G	AF161049	*G. mosseae* (Indonesia)	X96830	85.8
*G. mosseae*1H	AF161050	*G. mosseae* (Indonesia)	X96832	95.1
*G. mosseae*1I	AF161051	*G. mosseae* (Indonesia)	X96832	95.8
*G. mosseae*1J	AF161052	*G. mosseae* (Indonesia)	X96832	95.4
*G. mosseae*1L	AF161053	*G. mosseae* (Finland)	X96834	94.1
*G. mosseae*1M	AF161054	*G. mosseae* (Venezuela)	X96837	92.7
*G. mosseae*2A	AF161055	*G. mosseae* (Indonesia)	X96830	96.2
*G. mosseae*2B	AF161056	*G. mosseae* (Indonesia)	X96832	96.5
*G. mosseae*2C	AF161057	*G. mosseae* (Finland)	X96834	94.5
*G. mosseae*2D	AF166276	*G. mosseae* (Finland)	X96833	95.2
*G. mosseae*2E	AF161058	*G. coronatum* (Italy)	X96834	91.6
*G. mosseae*3A	AF161059	*G. coronatum* (Italy)	X96846	89.6
*G. mosseae*3B	AF161060	*G. mosseae* (UK)	X96828	88.6
*G. mosseae*3C	AF161061	*G. mosseae* (UK)	X96828	85.0
*G. mosseae*3D	AF161062	*G. coronatum* (Italy)	X96844	91.4
*G. mosseae*3E	AF161063	*G. coronatum* (Italy)	X96846	92.2
*G. mosseae*3F	AF161064	*G. coronatum* (Italy)	X96844	91.6

Table 5.2: Highest percent sequence identity of ITS sequences from *Gigaspora margarita* field spore (1A-1I) and single (2A-2C) or pool (3A-3G) spores from pot culture as compared to sequences in the GenBank.

Sequences from this study	Accession no.	Sequence from database (*Gi. margarita* BEG 34)	Accession no.	Identity (%) with the database sequences
*Gi. margarita*1A	AF162453	*Gi. margarita* (single spore A)	AJ006840	98.9
*Gi. margarita*1B	AF162454	*Gi. margarita* (single spore A)	AJ006840	99.8
*Gi. margarita*1C	AF162455	*Gi. margarita* (single spore A)	AJ006840	94.6
*Gi. margarita*1D	AF162456	*Gi. margarita* (single spore A)	AJ006840	99.8
*Gi. margarita*1E	AF162457	*Gi. margarita* (single spore A)	AJ006840	99.4
*Gi. margarita*1F	AF162458	*Gi. margarita* (single spore A)	AJ006840	98.7
*Gi.margarita*1G	AF162459	*Gi. margarita* (single spore C)	AJ006846	96.6
*Gi. margarita*1H	AF162460	*Gi. margarita* (single spore A)	AJ006840	98.9
*Gi. margarita*1I	AF162461	*Gi. margarita* (single spore A)	AJ006840	98.2
*Gi. margarita*2A	AF162469	*Gi. margarita* (multispore)	AJ006850	95.7
*Gi.margarita*2B	AF162470	*Gi. margarita* (multispore)	AJ006848	91.9
*Gi.margarita*2C	AF162471	*Gi. margarita* (multispore)	AJ006849	99.1
*Gi. margarita*3A	AF162462	*Gi. margarita* (multispore)	AJ006848	90.5
*Gi. margarita*3B	AF162463	*Gi. margarita* (single spore A)	AJ006841	93.7
*Gi. margarita*3C	AF162464	*Gi. margarita* (single spore C)	AJ006844	97.8
*Gi. margarita*3D	AF162465	*Gi. margarita* (multispore)	AJ006849	93.1
*Gi. margarita*3E	AF162466	*Gi. margarita* (single spore A)	AJ006839	97.1
*Gi. margarita*3F	AF162467	*Gi. margarita* (multispore)	AJ006849	98.2
*Gi.margarita*3G	AF162468	*Gi. margarita* (multispore)	AJ006849	98.2

5.3.2 Alignment of ITS1/ITS2 and 5.8S region

All ITS sequences from *Gi. margarita* showed a high degree of similarity. A single spore of *Gi. margarita* from field soil, and spores from pot cultures contained ITS sequences that were grater than 80% (Table 5.3). Two sequences from the single field spore of *Gi. margarita* were 100% identical (Table 5.3). *Gi. margarita* showed differences in base substitution between 50 and 100 bp and 401 and 430 bp and single base substitutions were found in almost all sequences.

The multiple sequence alignments indicated that *G. mosseae* showed higher intraspecific variation in the ITS regions than *Gi. margarita* (Figs 5.3 and 5.4). The ITS regions from *G. mosseae* showed sequence similarity ranging from 66 to 98% (Table 5.4). There are numerous small differences in length which arise from insertions or deletions of a few base pairs. Among the sequences of *G. mosseae* most of the variation was found in the region between positions 220 and 350 bp. Apart from this region, the *G. mosseae* ITS 2E, 3F, 3D, 3E, 3A, 3B and 3C, showed differences in base substitution between position 16 and 115, 401 and 500. Some other isolated base substitutions occurred in the sequences (Fig. 5.4)

The average estimate of divergence among ITS sequences in single spores ranged from 2.4 to 5.7% for *G. mosseae* and from single or pooled spores of *Gi. margarita* ranged from 1.0 to 3.1%. The average divergence within the species was 14.44% for *G. mosseae* and 4.0% for *Gi. margarita* (Table 5.5).

Table 5.3. Similarity matrix(%) computed using Homologies (GCG) based on data obtained from ITS sequence of *Gigaspora margarita* from a single field spore (1A-1I), pot culture single spore (2A-2C) and pool of spores from pot culture (3A-3G).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1)	*Gi. margarita3A*	100.0	98.7	86.1	86.3	85.9	86.1	86.1	85.9	85.4	84.8	86.3	87.8	86.5	84.0	81.5	83.9	80.6	85.7	86.6
2)	*Gi. margarita2B*		100.0	85.5	85.7	85.3	86.3	86.3	85.7	85.6	85.0	85.7	86.9	85.6	84.0	81.0	84.3	81.0	85.6	85.7
3)	*Gi. margarita1A*			100.0	99.3	99.3	99.1	99.1	98.9	97.6	94.2	93.7	94.4	91.8	86.0	83.3	84.8	81.6	87.4	89.2
4)	*Gi. margarita1E*				100.0	98.9	98.7	98.7	99.1	97.2	93.8	93.3	94.6	91.6	86.3	83.5	85.0	81.8	87.7	89.4
5)	*Gi. margarita1F*					100.0	98.9	98.9	98.5	97.4	94.4	93.7	94.0	91.4	85.8	83.1	84.8	81.4	87.2	89.0
6)	*Gi. margarita1B*						100.0	100.0	99.1	98.5	94.8	93.7	94.2	91.6	86.0	83.3	85.6	82.4	87.8	89.2
7)	*Gi. margarita1D*							100.0	99.1	98.5	94.8	93.7	94.2	91.6	86.0	83.3	85.6	82.4	87.8	89.2
8)	*Gi. margarita1H*								100.0	97.6	94.0	93.7	94.2	91.6	86.2	83.5	85.0	81.8	88.1	89.4
9)	*Gi. margarita1I*									100.0	94.7	92.3	93.4	90.4	85.7	83.0	85.3	82.1	87.7	88.2
10)	*Gi. margarita1G*										100.0	90.1	91.9	88.4	83.7	81.0	84.0	80.5	86.4	86.5
11)	*Gi. margarita1C*											100.0	91.2	92.7	87.1	83.1	83.9	80.7	85.7	90.4
12)	*Gi. margarita3C*												100.0	92.5	86.0	83.5	84.6	81.2	87.6	88.8
13)	*Gi. margarita3B*													100.0	86.3	83.3	84.4	81.2	85.5	89.3
14)	*Gi. margarita3F*														100.0	95.4	94.4	92.8	92.5	94.4
15)	*Gi. margarita3G*															100.0	91.4	89.7	89.7	90.5
16)	*Gi. margarita3E*																100.0	92.2	95.3	90.4
17)	*Gi. margarita2C*																	100.0	89.7	87.7
18)	*Gi. margarita2A*																		100.0	88.6
19)	*Gi. margarita3D*																			100.0

Table 5.4. Similarity matrix (%) computed using Homologies (GCG) based on data obtained from ITS sequence of *G. mosseae*, from three field single spores (1A-1M, 2A-2E and 3A-3F).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1) *G. mosseae* 2E	100.0	98.2	97.2	97.1	94.9	93.1	84.9	74.3	73.5	74.4	74.7	75.3	75.2	74.6	73.5	73.9	74.1	73.7	74.4	74.1	73.0	71.0	71.5
2) *G. mosseae* 3F		100.0	98.0	95.6	94.2	92.6	83.8	73.4	73.4	74.4	73.5	74.3	74.2	73.5	74.1	73.4	73.6	72.4	73.5	74.3	72.1	70.7	72.2
3) *G. mosseae* 3D			100.0	95.4	93.7	93.1	83.4	73.4	73.4	74.4	73.5	74.0	74.0	73.4	74.1	73.4	73.6	72.4	73.5	74.3	72.5	71.2	72.0
4) *G. mosseae* 3E				100.0	94.8	92.8	84.3	75.2	74.4	75.3	75.3	76.0	75.9	75.0	74.4	74.6	75.0	74.2	75.4	75.2	73.5	71.9	72.3
5) *G. mosseae* 3A					100.0	92.5	85.7	73.8	73.0	73.8	73.7	74.4	74.7	73.9	73.0	73.4	73.4	72.6	73.7	73.4	71.9	70.5	71.5
6) *G. mosseae* 3B						100.0	83.8	72.0	71.5	72.3	72.1	72.6	73.3	72.2	71.5	71.9	72.0	71.6	72.8	72.2	71.1	68.8	69.4
7) *G. mosseae* 3C							100.0	69.5	68.7	70.1	69.8	70.5	70.4	69.6	69.1	69.8	69.3	68.6	69.5	69.6	68.3	66.9	66.4
8) *G. mosseae* 2C								100.0	94.2	96.0	96.7	93.3	93.0	92.9	92.0	87.8	87.5	86.9	88.7	87.5	86.0	87.4	85.5
9) *G. mosseae* 2D									100.0	95.1	93.3	92.4	91.7	92.2	92.2	85.5	85.9	85.8	86.9	87.1	85.8	87.7	86.8
10) *G. mosseae* 2A										100.0	97.1	94.9	94.5	94.2	94.7	88.1	88.0	87.3	88.6	88.2	86.5	89.6	88.4
11) *G. mosseae* 2B											100.0	94.9	94.5	94.5	93.1	88.6	88.5	88.6	89.7	88.0	87.4	87.9	86.5
12) *G. mosseae* 1B												100.0	98.0	97.4	96.5	90.3	90.5	89.5	91.3	90.1	88.3	90.0	88.8
13) *G. mosseae* 1J													100.0	97.6	95.4	90.4	91.0	90.2	91.8	90.1	89.0	89.5	89.2
14) *G. mosseae* 1E														100.0	95.4	89.8	89.9	89.3	90.6	89.4	87.7	89.1	89.0
15) *G. mosseae* 1F															100.0	88.7	88.8	87.7	89.4	89.2	86.8	90.0	89.5
16) *G. mosseae* 1D																100.0	98.2	89.9	93.3	93.9	88.3	83.2	82.9
17) *G. mosseae* 1G																	100.0	90.1	93.3	93.3	88.8	82.9	82.9
18) *G. mosseae* 1H																		100.0	95.1	92.5	91.4	83.0	83.8
19) *G. mosseae* 1I																			100.0	94.4	91.3	84.7	84.4
20) *G. mosseae* 1A																				100.0	91.1	85.6	85.7
21) *G. mosseae* 1L																					100.0	83.7	83.5
22) *G. mosseae* 1C																						100.0	86.8
23) *G. mosseae* 1M																							100.0

Table 5.5. Genetic diversity estimated from sequence divergence analysis within and among species

Taxon specimen	Origin (Number of sequences)	Mean of divergence*
G. mosseae 1	single spore field soil (12)	5.6
G. mosseae 2	single spore field soil (5)	2.4
G. mosseae 3	single spore field soil (6)	5.7
G. mosseae 1+2+3		14.44
Gi margarita 1	single spore field soil (9)	1.0
Gi. margarita 2	single spore pot culture (3)	3.1
Gi margarita 3	pool spores pot culture (7)	3.6
Gi margarita 1+2+3		4.0

* Divergence estimates by synonymous substitutions per 100 synonymous sites.

5.3.3 Phenetic analysis (or Dendrogram)

The sequences were edited and assembled using the program Phylogeny
(DNASTAR). The ITS regions were independently subjected to weighted pair
group analysis. The length of each pair of branches of the phenetic tree represents
the distance between sequence pairs calculated based on the sequence alignments.
When analysed together the sequences from *G. mosseae* and *Gi. margarita*
clustered into two groups which characterise each genus (Fig. 5.5). When the
sequences obtained in this study were compared with *G. mosseae* sequences from
Genbank, accession numbers X96830, X968832 and X96833, they form one cluster
with sequences from field spores *G. mosseae*1 and *G. mosseae*2. However, *G.
coronatum* sequences from GenBank, accession number X96846, and *G. mosseae*
accession number X96844 cluster more closely with sequences from *G. mosseae*3
spore from field (Fig. 5.5).

Figure 5.5. Dendrogram showing genetic distances between the *Glomus mosseae* and *Gigaspora margarita*. The tree was constructed from the sequence alignment data set shown in Fig. 5.3 and 5.4. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree represents distance between sequences. The units indicate the number of substitution events. The underlined sequences were obtained from GenBank under the accession number: *G. mosseae:* GMO30 = X96830, GMO32 = X96832, GMO33 = X96833, GMO34 = X96834, GMO44 = X96844; *G. coronatum:* GCOR6846 = X96846 and *Gi. margarita:* GIMAR44 = AJ006844, GIMARG40 = AJ006840, GIMARG48 = AJ006848, GIMARG41 = AJ006841 and GIMAR49 = AJ006849.
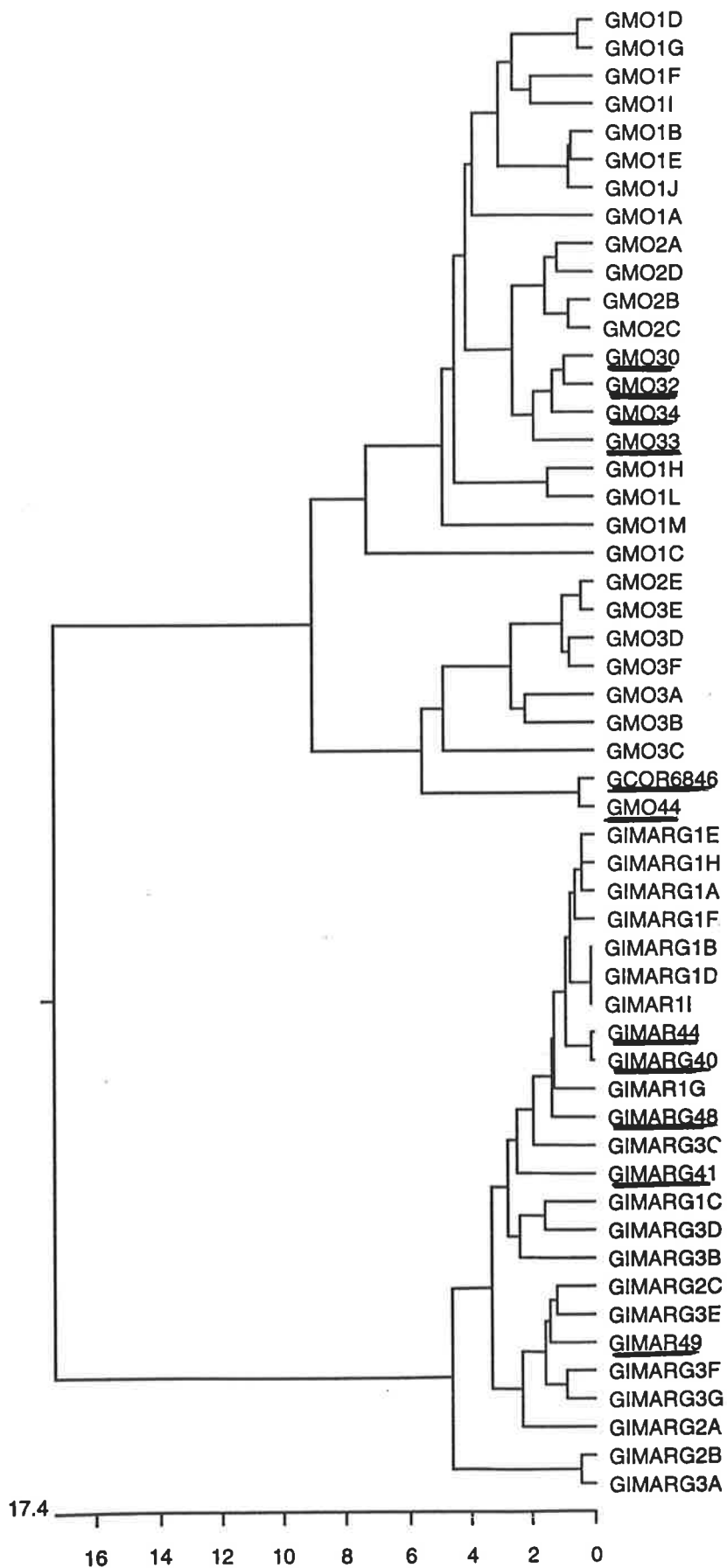
5.4 DISCUSSION

ITS regions in fungi have been found to evolve quickly and therefore contain sufficient nucleotide sequence variation to be used for studying phylogenetic relationships at the molecular level. Genetic diversity of AM fungi in natural and agroecosystems is poorly understood, so rDNA ITS sequences from *G. mosseae* from single field spores and *Gi. margarita* from single field spores and pot cultures were examined. All 42 sequences, except for five obtained from one spore were closely related to the mycorrhizal ITS sequences from species in the database. This is the first study from field collected spores that shows a large amount of heterogeneity in the ITS region within single spores of *G. mosseae*. This confirms the findings of Sanders *et al.*, (1995); Lloyd-Macgilp *et al.*, (1996) and Clapp *et al.*, (1995) whose analysis was completed on spores isolated from pot cultures. The heterogeneity was so large that none of the 23 sequences from three single spores of *G. mosseae* were identical. This result could not be attributed to PCR error based on the calculated error rate of the Expand™ system. Compared to other *Taq* polymerases, the Expand™ High Fidelity PCR System amplified large quantities of specific targeted DNA sequences (ITS). Based on the error rate of the Expand™ PCR system and the number of cycles used, we would expect at most 10% of the fragments to contain a single error. The two most similar sequences contain as many as 7 bp differences. Therefore, for *G. mosseae* the observed difference between sequences were not likely to be artefact of the PCR process. Based on the single field spores and spores from pot culture, *Gi. margarita* ITS sequences showed lower genetic diversity than *G. mosseae*. This result suggests that *G. mosseae* is a more genetically diverse species than *Gi. margarita* in natural populations. However conclusions on the genetic variation of *Gi. margarita* cannot be made from a single spore and it will be important to try to obtain additional sequences from field collected material. Future work on field populations with low spore numbers should be based on a higher number of field collected spores, supplemented by spores obtained from trap cultures initiated from single field-collected spores.

To quantify the degree of genetic diversity in *G. mosseae*, the GCG program Diverge was used. Estimation of the mean divergence was less (2.4 - 5.7%) when

sequences from single spores were analysed as compared from analysis of three spores together (14.4%) (Table 5.2). A similar high level of genetic diversity has been reported among *G. mosseae* from different origins (Lloyd-MacGilp *et al.*, 1996). Although the *G. mosseae* spores chosen were easy to distinguish morphologically there is a small possibility that spore number three belongs to a different species in the *G. mosseae* complex, such as *G. coronatum*, which possibly contributed to the much higher average estimate of divergence. This highlights an important problem in working with single spores of these unculturable fungi. Full identification (needed to distinguish *G. mosseae* and *G. coronatum* unequivocally) requires breaking the spores and mounting them in poly-vinyl-lactoglycerol (PVLG) and Melzer's reagent (Schenk, 1982). This procedure is not compatible with DNA extraction and molecular characterisation. In contrast, the mean sequence divergence from single spores of *Gi. margarita* (1.0 - 3.6%) was lower than in *G. mosseae* and more similar to the divergence estimate derived from the analysis of the pooled sequences (4.0). The divergence for the *Gi. margarita* spores from pot cultures was higher than from sequences amplified from the single field spore. In recent studies of genetic variation in individual spores of *Gi. margarita* using M13 minisatellite-primed PCR Zézé *et al.* (1997)reported fingerprints from 18 individual spores with several spores showing similar fingerprints. However, using the M13 core sequences Gadkar *et al.* (1997) did not find polymorphisms in the fingerprints of nine individual spores from *Gi. margarita*.

Spores from different locations, environmental conditions and small sample size are probably responsible for the different results between the field and pot cultures. These data confirm previous observations of Redecker *et al.* (1997) , where PCR and restriction fragment length polymorphism analysis were used to classify divergence which showed more variable restriction fragment patterns in the genus *Glomus* than in Gigasporaceae.

Some possible explanations have already been suggested to account for the variation in the ITS region. Variation can be the result of the presence of distinct genotypes in different nuclei in the same spore (heterokaryotic) or gene heterogeneity within single nuclei (Sanders *et al.*, 1996). This also, could occur by gene turnover (DNA sequences are maintained by a balance of sequence

amplification and deletion) (Hoelzel and Dover, 1991). Due to the fact that spores are almost certainly produced asexually and are highly multinucleate, another possibility is that (within an individual spore) there are multiple copies of ribosomal DNA (Clapp *et al.*, 1995; Tommerup, 1983). The high diversity in ribosomal genes in AM fungi can be used to understand genetic relationships and to develop a phylogenetic classification of the AM fungi in natural ecosystems.

The multiple sequences from *G. mosseae* and *Gi. margarita* were compared with those found in Genbank. From *G. mosseae* spore number 1, the 12 ITS sequences showed more than 86% identity with *G. mosseae* ITS sequences from spores found on three different continents (Lloyd-MacGilp *et al.*, 1996). In *G. mosseae* spore number 2, one out of five sequences showed more identity with *G. coronatum* than with other isolates of *G. mosseae* in the database. For *G. mosseae* spore number 3, four out of six sequences showed more identity with *G. coronatum* 5 (Table 5.1). The similarity of the some *G. mosseae* sequences to *G. coronatum* sequences present in the database, which highlights the difficulty in distinguishing between the two species. Because of the high degree of intraspecific variation in the ITS region and morphological similarities, it is difficult at the present time to differentiate between members of the *G. mosseae* complex (*G. mosseae, G. monosporum, G. dimorphicum, G. fragilistratum* and *G. fecundisporum*) (Dodd *et al.*, 1996). Studying the large subunit DNA sequences from many AM fungi Rosendahl (personal communication) found that two groups of *G. coronatum* sequences which one cluster with *G. mosseae* and another with *G. constrictum*. From *Gi. margarita* spore number 1, the nine sequences showed more than 95% identity with *Gi. margarita* (BEG 34) single spore ITS sequences. In *Gi. margarita* spore number 2, the three ITS sequences showed more than 91% identity with multispore ITS sequences of *Gi. margarita*. For *Gi. margarita* pool of spores ITS sequences showed more than 91% identity with ITS sequences of single or pooled spores of the published *Gi. margarita* (Lanfranco *et al.*, 1999)(Table 5.2). The ITS sequence variations between isolates indicate low genetic variability in *Gi. margarita* compare to *G. mosseae*.

Morphological characters (Blaszkowski, 1994; Dodd *et al.*, 1996; Meier and Charvat, 1992), PCR-RFLP of ribosomal DNA (Sanders *et al.*, 1995), RAPD-PCR (Lanfranco *et al.*, 1995), isoenzymes (Bago *et al.*, 1998; Rosendahl, 1989), total

soluble proteins (Dodd *et al.*, 1996), glycoproteins (Wright *et al.*, 1987), ITS sequences (Lloyd-MacGilp *et al.*, 1996) and fatty acids (Bentivenga and Morton, 1994; Jabaji-Hare, 1988) have been used to differentiate *G. mosseae* from *G. coronatum*. This study indicates that some ITS sequences within *G. mosseae* are closely related to those of *G. coronatum*. However, until more ITS sequences from field spores are provided, the phylogenetic relationship between these species will remain unclear. The level of variation in the rDNA ITS region suggests that either *G. mosseae* represents a group of species (the *G. mosseae* complex) (Dodd *et al.*, 1996) or a group of heterogeneous species in the genus *Glomus*. Studies of on SSU rDNA sequences and PCR-fingerprinting have also revealed variation in isolates of *G. mosseae* from eight different international collections (Vandenkoornhuyse and Leyval, 1998). Additional sequence data from several different parts of the genome may be required for accurate separation of members of the *G. mosseae* complex. The genetic divergence in ITS regions (Lloyd-MacGilp *et al.*, 1996) together with spore morphology need to be further examined in future studies on *G. mosseae* and *G. coronatum* to clarify the taxonomic relationship at the molecular level within this group.

The phenetic analysis of ITS sequences separated *G. mosseae* and *Gi. margarita* ITS sequences into two groups. These results support the morphological identification of spores from field soil based on characters described by Bentivenga & Morton (1994). Available sequence data from nuclear genes encoding ribosomal small subunit rRNA (SSU) from spore (Simon *et al.*, 1993) and from root DNA (Helgason *et al.*, 1998), have also been used to distinguish between different genera and species of Glomales.

5.5 Conclusion

The genetic variability in the rDNA ITS region from *G. mosseae* from field-collected spores showed higher heterogeneity within single spores than that found in spores of *Gi. margarita*. This finding that the *G. mosseae* ITS sequences are highly variable, is similar to those obtained earlier on spores collected from pot cultures. Future study is needed for a comparison between *G. mosseae* and *G. coronatum* ITS region. Until the publication of sequence information for the ITS region (Lanfranco *et al.*, 1999) in March 1999 during the writing of the thesis this was the

first report (Antoniolli *et al.*, 1998) of variation in *Gi. margarita* ITS sequences. The differences in the ITS sequences between the two species allowed the separation of these two groups in the genetic distance analysis.

The data from this study helps to refine the concept of genetic variation in the ITS region of natural populations of *G. mosseae* and *Gi. margarita* species. More ITS sequences will be needed from additional species of Glomales to clarify the level of inter- and intraspecific polymorphism within the rDNA ITS region of these important soil-borne fungi.

Based on these ITS sequences primers were designed for species-specific identification of *G. mosseae* and *Gi. margarita* from spores or colonised roots. These sequences were used as probes for quantification in colonised roots as described in Chapter 6.

Chapter 6

Species-specific PCR primers for detection and quantification of the mycorrhizal fungi *Glomus mosseae* and *Gigaspora margarita*

6.1 Introduction

Arbuscular mycorrhizal (AM) fungi form symbioses with the roots of plants and offer several advantages to the host, such as improved mineral nutrition and protection against water stress and pathogens (Harley and Harley, 1987; Smith and Read, 1997). To fully understand the relationship between fungus and plant, it will be important to determine which mycorrhizal species colonise roots and to quantify the relative extent of colonisation by different fungal species. This detailed identification cannot be achieved by conventional taxonomic methods that rely on morphological descriptions of spores. Molecular methods have considerable potential for providing a more detailed description which should enhance our understanding of the population dynamics of the fungi in ecosystems.

Identification of AM fungi from soil field spores is time consuming, requires considerable expertise and only provides information on those fungi that produce spores. The information can be used to evaluate the effects of environmental conditions, host and fungal genotype on spore production, but gives little information on the extent to which a particular species actively colonises the roots (Clapp *et al.*, 1995). Vegetative structures within colonised roots (arbuscules, vesicles and hyphae) have been used for identification (Abbott, 1982) but are of limited value because different fungal species are very similar and there may be host-species effects on fungal morphology (Smith and Smith, 1986). The morphological similarity between species (Rosendahl *et al.*, 1994), the difficulties of identifying spores from field material (Giovannetti and Gianinazzi-Pearson, 1994) and the lack of methods to culture AM fungi suggests that molecular tools for identification will have considerable advantages.

DNA-based techniques make it possible to identify species accurately, using specific PCR primers. The use of PCR primers to identify vegetative material of the fungi has several additional advantages of DNA-based identification tools over conventional methods. Primers based on selected ribosomal regions are of potential

value because the target DNA sequences are found in multiple copies per genome and are highly conserved. Primers can be developed from DNA extracted from individual spores, and then may be used for studying species diversity of AM fungi in host roots in natural communities (Clapp *et al.*, 1995; Dodd *et al.*, 1996; Sanders *et al.*, 1995). Such methods have the potential for rapid and sensitive identification of mycorrhizal species using DNA from spores or colonised roots collected from natural and agro-ecosystems. The methods adopted must have the appropriate level of specificity for use in mixed populations of AM fungi and distinguish these fungi from both roots and non-mycorrhizal root-infecting fungi. An ability to quantify the amounts of target DNA and relate this to biomass would also be an advantage. Species-specific synthetic oligonucleotides could also be used as probes for quantification of AM fungal DNA in soil or in roots and applied in studies of biodiversity.

This chapter reports the design and testing of PCR primers based on ITS sequences of *G. mosseae* and *Gi. margarita* (Chapter 5) and on the development of a method of quantification of fungal colonisation based on ITS sequences from AM fungi in colonised plant roots. PCR primers were developed from DNA isolated from spores of *G. mosseae* and *Gi. margarita* collected from field soil at the Waite Permanent Rotarion trial, South Australia (Chapter 3 and 4). The specificity of the primers was tested with DNA from 12 species of AM fungi and five other root-infecting fungi. The *G. mosseae* oligonucleotide primers were also used in a slot-blot hybridisation assay for the simultaneous detection and quantification of AM fungal DNA in colonised roots from trap cultures with field soil containing multiple fungal species and from pot cultures containing a single fungal species (Chapter 4).

The use of ITS sequences from *G. mosseae* and *Gi. margarita* is described in terms of:

1) designing species-specific PCR ITS primers,

2) testing the specificity and detection in spore or root DNA,

3) quantification of ITS DNA in colonised roots.

6.2 Materials and Methods

6.2.1 Fungi

The fungi used in this study are listed in Tables 6.1 and 6.2. Spores of AM fungi were obtained from several different sources. Some spores of *G. mosseae* (Waite, Mallala and Blanchetown) and *Gi. margarita* (Waite) were isolated directly from field soil and identification confirmed by Dr. C. Walker. Spores of *Entrophospora* sp. were provided by Dr P. A. McGee, and of *Glomus mosseae* WUM 9(6) and WUM 23 by Professor L.K. Abbott. The others were produced from pot cultures.

The ITS regions were cloned and sequenced from spores of *G. mosseae* and *Gi. margarita* collected from the Waite Permanent Rotarion trial and the sequences used (Chapter 5) to design primers. The remaining spores were used to check the specificity of these primers and to produce colonised root material to test the usefulness of the primers in the presence of plant tissue.

The non-Glomalean fungi used in this study were obtained as axenic cultures and were: binucleate *Rhizoctonia sp., Gaeumannomyces graminis* var *avenae* (Sacc.) Arx and D.L. Olivier and Walker, *G. graminis* var *tritici,* (Sacc.) Arx and D.L. Olivier and Walker, *Phythium echinulatum* V.D. Mathews and *Rhizoctonia solani* J.D Kuhn. They were chosen because they are amongst the most common soil-borne fungi in South Australia, are important root pathogens (Herdina *et al.* 1996, 1997) and are often found to be associated with mycorrhizal fungal spores.

6.2.2 Plant material

Two groups of plants were grown to provide spores and root material for testing the primers. Single species of AM fungi were grown in 'pot cultures' of *Allium porrum* or *Trifolium subterraneum.* Mixed populations of AM fungi originating from field soil were grown on plants of *Lolium perenne, Plantago lanceolata, Sorghum* sp. or *T. subterraneum.* These plants were grown in pots containing 1.2 kg of a mixture (1:1) of autoclaved sand and unsterilised field soil, pH 5.9, containing spores, mycelium and colonized plant roots. Non-mycorrhizal plants were grown in a sterilised soil/sand mix. All plants were grown under constant conditions (photoperiod 12 h, temperature 18-25°C, relative humidity 60 - 75%,

irradiance 240 $\mu E/m^2/s$ and received 15 ml of Long Ashton nutrient solution without phosphate weekly (Hewitt 1966).

6.2.3 DNA extraction from AM fungal spores

A pool of 10 spores of each species of AM fungus was used for extracting DNA. DNA was also extracted from single spores of *G. mosseae* and *Gi. margarita* isolated from field soil and single and pools of 15 spores of *Gi. margarita* from pot cultures. Groups of spores were cleaned three times by 20 second sonification, followed each time by a sterile deionised water rinse. Water was removed by transferring the spore suspension onto filter paper.

For the extraction of DNA, spores were then transferred to 1.5 ml Eppendorf tubes. Extraction buffer (50 μl) containing 100 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$ and 2% Triton(pH 8.0) was added and then crushed with a minipestle on ice. The samples were kept on ice and centrifuged at 2,900 g (g = acceleration due to gravity) for 2 min at room temperature. The supernatant was transferred into a fresh microcentrifuge tube. Samples were stored at -20°C.

6.2.4 DNA extraction from non-AM fungi in pure culture

Fungal cultures of non-mycorrhizal root-infecting fungi (Table 6.2) used in these experiments were grown on agar plates as described by Herdina *et al.* (1996).

Total DNA was extracted essentially as described by Raeder and Broda (1985) modified by Herdina *et al.*, (1997). The fungal material (0.1 g) was ground in liquid nitrogen, using a mortar and pestle and transferred to an Eppendorf tube containing five volumes of extraction buffer. The suspension was extracted with an equal volume phenol/chloroform (1:1, v/v) and centrifuged for 30 min at room temperature at 11,600 g. The resultant aqueous phase was incubated with RNAase (0.1 mg ml^{-1}) at 37°C for 20 min, extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) and centrifuged for 10 min at 11,600 g. DNA was precipitated from the aqueous phase with 0.54 volumes of cold isopropanol and collected by centrifugation. The DNA pellet was washed with 70% ethanol, dried *in vacuo*, resuspended in 100 μl TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0).

6.2.5 DNA extraction from roots

DNA from roots of plants grown in sterilised soil, in pot cultures infected with *G. mosseae* or *Gi. margarita* and in trap cultures grown in field soil extracted from approximately 1 g of fresh tissue, according to the method of Rogers and Bendich (1985). The root sample was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Ground tissue was suspended in 1 ml of 2x CTAB (100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 2% cetyltrimethylammonium bromide-CTAB; 1% polyvinyl polypyrrolidone) for 3 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and vortexed before centrifugation at 11,600 g for 2 min. The supernatant was transferred to a fresh 1.5 ml microfuge tube and 1/10 of 10% CTAB buffer (10% cetyltrimethylammonium bromide, 0.7 M NaCl) preheated at 65°C was added. An equal volume of chloroform/isoamyl alcohol (24:1) was added and vortexed before centrifugation at 11,600 g for 2 min. The supernatant was transferred to a fresh 1.5 ml microfuge tube, one volume of CTAB precipitation buffer (1% CTAB; 50 mM Tris/HCl, pH 8.0; 10 mM EDTA) added and centrifuged for 1 min. The pellet was resuspended in TEN buffer (10 mM Tris/HCl pH 7.0, 10 mM EDTA, 1 M NaCl) at 65°C for 10 min and 10 mg ml^{-1} RNAase A was added and the sample incubated further for 30 min at 37°C. The DNA was then precipitated by adding 2 volumes of cold ethanol 95% and 1 µl 2 M ammonium acetate (CH_3COONH_4) and centrifuged at 13,600 g for 20 min. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl of sterile deionised water. This DNA was used as template for PCR reactions using the PCR primers. The concentration of DNA in the samples was estimated by running an aliquot on a 1% agarose gel against a known amount of *Hind* III digested lamba DNA.

The percentage colonisation by AM fungi was determined on a sub-sample of all root material using the method described by Phillips and Hayman (1970), omitting phenol from the solutions.

6.2.6 Design of PCR primers

Multiple DNA sequence alignments from 42 sequences of the ITS regions (Chapter 5) were used to design species-specific primers for the identification of *G. mosseae* and *Gi. margarita,* using the "Oligo" software, Version 5 (National Biosciences Inc., Plymouth, MN, USA). The use of a large number of sequences from the ITS regions was important because of the high level of variability reported within and between individual *G. mosseae* spores (Lloyd-MacGilp *et al.,* 1996; Sanders *et al.,* 1995). Although *G. mosseae* ITS sequences showed a high degree of variability (Chapter 5), it was possible to find conserved stretches of bases from the ITS1 region between 142 to 160 bp (GOMSf) and from the ITS2 region between 402 to 430 bp (GOMSr). *Gi. margarita* sequences showed highly conserved stretches of bases in the ITS1 region between 109 to 126 bp (GIMARf) and in the ITS2 region between 340 to 357 bp (GIMARr). The primer sequences chosen were as follows:

for *G.mosseae*, GMOSf $^{5'}$(ACGGATCTCTTGGCTCTC$)^{3'}$ and GMOSr (CGTACCGGATGGATGAAT$)^{3'}$; and for *Gi. margarita* GIMARf $^{5'}$(GGATCTCTTGGCTCTCGC$)^{3'}$ and GIMARr $^{5'}$(CGCTTCACATTACATAAC$)^{3'}$.

6.2.7 PCR amplification with species-specific primers

The primers were used in amplification reactions to test their specificity with respect to taxa of AM fungi, plant roots and non-mycorrhizal root infecting fungi.

The amplification reactions were performed in a 25 µl volume containing 5 µl of spore, root or plant material DNA , 0.5 unit of Expand™ High Fidelity PCR System (Boehringer Mannheim, Germany), 25 pM GOMSf or GIMARf, 25 pM GOMSr or GIMARr, 250 µM dNTP and 250 µM 10x Expand HF buffer and 1.5 mM MgCl$_2$. Each reaction was overlaid with 25 µl mineral oil (Sigma). A negative control which did not contain AM fungal DNA was included in every experiment. PCR conditions were optimised (see Results) and the following procedure adopted for routine amplifications. For GOMSf/GMOSr primers, the reactions were performed as follows: initial denaturation step at 94°C for 4 min, 35 cycles at 94°C for 40 sec, 58°C for 50 sec, 72°C for 1 min, with a final extension of 5 min at 72°C. For GIMARf/GIMARr primers the reactions were performed as follows:

initial denaturation step at 94°C for 4 min, 35 cycles at 94°C for 40 sec, 56°C for 50 sec, 72°C for 1 min, with a final extension of 5 min at 72°C. The PCR was performed with a Programmable Thermal Controller (MJ Research Inc., PTC-60 Thermal Cycler, Watertown, Mass.). DNA from each spore was amplified in four separate amplification reactions under the same conditions to check for reproducibility. The amplification products were analysed using electrophoresis (1.2% agarose gel) in Tris-acetate-EDTA buffer (100 mM Tris, 125 mM sodium acetate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide and DNA was visualised under UV light.

6.2.8 Slot-blot assay

A Bio-Dot slot format micro filtration unit with a Zeta-Probe nylon membrane was assembled according to the manufacturer's recommendations (Bio-Rad). The DNA samples were denatured using NaOH and neutralised with CH_3COONH_4 as described by Herdina et al. (1997), before loading on the membrane.

6.2.9 DNA labelling and hybridisation

Oligonucleotide primers and the entire cloned ITS fragment from G. mosseae were both tested as DNA probes in the slot blot analysis. They were labelled with $[\alpha^{32}P]dCTP$ using a 3'-end labelling kit (Amersham Life Science). The oligonucleotide primers (GOMf/GOMr) were concentrated to 10×10^{-12} moles in 50 µl for labelling and hybridisation. The cloned ITS fragment was amplified by a PCR reaction performed with the same conditions described above. The product was purified from the agarose gel with QIAquick Gel Extraction Kit (QIAGEN, Santa Clarita, USA), and concentrated to 10×10^{-12} moles in 50 µl for labelling. The oligonucleotide primers and ITS fragment were labelled to specific activity of about 1.7×10^8 cpm µg^{-1} and separated from unincorporated nucleotides through a Centri-Spin^{TM}-10 Column (Princeton Separations).

Hybridisation of the membranes was performed at 42°C overnight in fresh hybridisation buffer containing 5.55 ml deionised sterile water, 2.5 ml 5x SSC (3 M NaCl, 0.3 M trisodium citrate), 0.2 ml 20 mM NaH_2PO_4 (pH 8.0), 1.25 ml 2.5% SDS, 0.5 ml 5x modified Denhardts solution (25 polyvinylpyrrolidone, 2% Ficoll

400, 2% polyethylene glycol 8000), 0.1 ml denatured salmon sperm DNA (5 µg ml^{-1}). After hybridisation, the membranes were washed at 42°C using sequential washes of 3x SSC and 2.5% SDS, 0.5x SSC and 2.5% SDS and 0.2x SSC and 1% SDS for 15 min each (relatively low stringency). Higher stringency conditions were also tested, as follows: hybridised at 60°C for 16 h and washed twice with solution 1 at 60°C for 15 min, solution 2 at 60°C for 15 min and solution 3 at 60°C for 15 min. The washed membranes were either exposed to Cronex X-ray film with intensifying screens (Dupont) at -80°C for 24 - 48h for those probed with the ITS fragment from *G. mosseae* or exposed to a phosphor screen overnight and quantified using a Storm Phosphor Image software (Molecular Dynamics) for those probed with the oligonucleotide primers.

6.3 Results

6.3.1 Primer specificity

The primers used in this study have the characteristics of efficient primers as outlined by Van Tuinen (1998b). They were designed from multiple ITS sequences and were 18 bp in length, with annealing temperatures above 55°C, GC content between 39-65% and with all four bases randomly distributed. The calculated melting temperatures (Tm) were: GOMSf (65.2°C), GOMSr (63.8°C); GIMARf (56.2°C) and GIMARr (55.4°C). PCR conditions were optimised by testing different annealing temperatures. Precise annealing temperatures were identified using spore DNA from either *G. mosseae* or *Gi. margarita* as template.

The GOMSf/GOMSr primers when used at 58°C gave amplification products with most of the *G. mosseae* isolates listed in Table 6.1 and Fig.6.1 DNA from isolates LPA5 from France and WUM BR4-1 from New South Wales, Australia were not amplified under the conditions tested (Fig. 6.1, lanes m and n). The *G. mosseae* primers were also tested against DNA from spores of 11 other species of AM fungi and did not produce amplification (Table 6.1 and Fig. 6.2). These results indicate that the primer pair GMOSf/GMOSr is highly specific for *G. mosseae* and does not detect other AM fungi.

The GIMARf/GIMARr primers used at 56°C were tested against DNA from two isolates of *Gi. margarita* and 11 other species of AM fungi. The primers amplified a fragment from *Gi. margarita* samples only. No amplification product was detected from DNA of the other species (Table 6.1 and Fig. 6.3). Control reactions where no DNA template was added gave no amplification product with either primer pair.

Differences in fragment length amplified by the two primer pairs were noted, with the *G. mosseae*-specific primers amplifying a 239 bp product and *Gi. margarita* primers a 261 bp product (Figs. 6.1 and 6.3). These species-specific differences in size of amplification products enhances their use as diagnostic tools.

Table 6.1 Fungi used to assess of the specificity of GOMSf/GOMSr and GIMARf/GIMARr primers for amplification of part of the internal transcribed region.

Source of DNA	Origin (name of isolate)	GOMSf/GOMSr	GIMARf/GIMARr*
AM fungi spores			
Acaulospora laevis	France (†BEG 13)	-	-
Entrophospora sp.	New South Wales	-	-
Glomus caledonium	France (BEG 20)	-	-
G. "City Beach"	Western Australia (†WUM-16)	-	-
G. etunicatum	USA (†MD 107-1)	-	-
G. fasciculatum	France (BEG 53)	-	-
G. mosseae	France (†LPA 5)	+	-
G. mosseae (field)**	South Australia (Waite Campus)	+	-
G. mosseae	France (BEG 5)	+	-
G. mosseae	Queensland (WUM 9 (6))	+	-
G. mosseae	Queensland (WUM 23)	+	-
G. mosseae	New South Wales (WUM Br4-1)	-	-
G. mosseae (field)	Mallala - South Australia	+	-
G. mosseae (field)	Blanchetown - South Australia	+	-
G. macrocarpum	New South Wales (†KCSC 54)	-	-
Gigaspora rosea	France (BEG 9)	-	-
Gi. margarita	France (before BEG collection)	-	+
Gi. margarita (field)**	South Australia- Waite Campus	-	+
Scutellospora calospora	New South Wales (WUM 12 (2))	-	-

* The PCR amplification showed at least two bands, ~ 700bp and ~ 260bp. "+" indicates the expected amplification for the primer pairs, "-" indicates no amplification products for the primer pairs. ** *G. mosseae* and *Gi. margarita* used for the primer design.

† Origins of fungal material as follows: BEG, La Banque European des Glomales; WUM, Western Australian Culture Collection; MD, INVAM collection, West Virginia; LPA, Labroatoire de Phytoparasitologie, Dijon, France; KCSc, University of Sydney Culture Collection.

Figure 6.1: *Glomus mosseae*-specific primers (GMOSf/GMOSr) recognise most isolates of *G. mosseae*. Lanes a) and k), 1 kb ladder (Gibco-BRL); lanes j) no DNA template; remaining lanes, spore DNA from b) *G mosseae* (Waite- SA), c) *G. mosseae* (Mallala - SA), d) *G. mosseae* (Blanchetown - SA), e) *G. mosseae* (BEG 5), f) *G. mosseae* (WUM 9(6)), g) *G. mosseae* (WUM 23), h) *G. mosseae* (LPA 5), i) *G. mosseae* (WUM Br4-1).

bp   a   b c d e   f   g h i j k

239 →
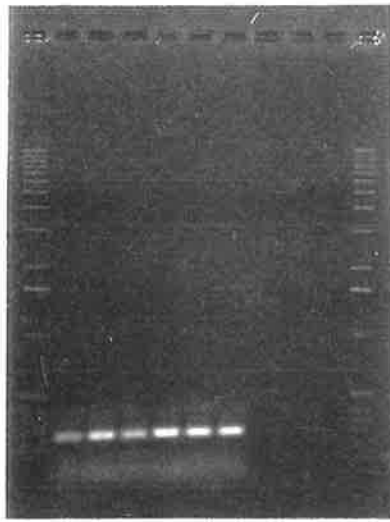
Figure 6.2: PCR amplification with *Glomus mosseae*-specific primers (GMOSf/GMOSr) shows that the primers are specific for *G. mosseae* and do not recognise other mycorrhizal fungal species. Lanes a and p), 1 kb ladder (Gibco-BRL); lane o) no DNA template; remaining lanes, spore DNA from b) *Acaulospora laevis*, c) *Entrophospora* sp., d) *G. macrocarpum*, e) *G. fasciculatum*, f) *G. etunicatum*, g) *G. caledonium*, h) *Scutellospora calospora*, i) *G. mosseae* (field spores), j) *Gigaspora rosea*, l) *Gi. margarita*, m) *G. 'City beach'*, n) *G. mosseae* (cloned ITS DNA template).
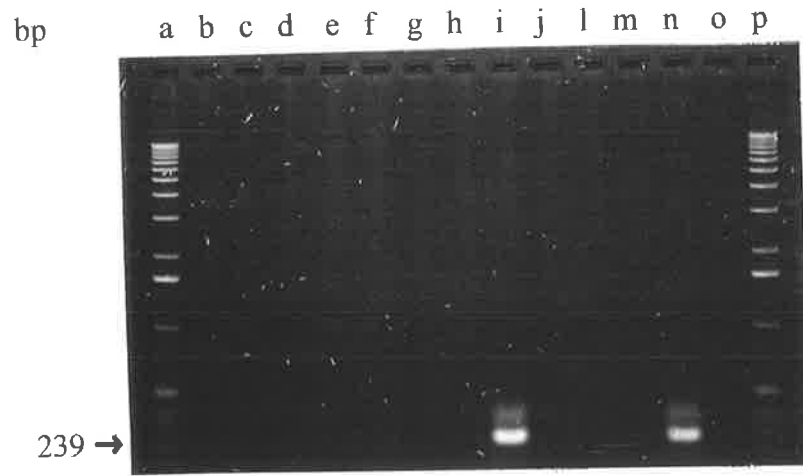
Figure 6.3: PCR amplification with *Gigaspora margarita*-specific primers (GIMARf/GIMARr) shows that primers are specific for *Gi. margarita* and do not recognise spores of other mycorrhizal fungal species. Lanes a and p) 1 kb ladder (Gibco-BRL); lane o) no DNA template; remaining lanes, spore DNA from b) *Acaulospora laevis*, c) *Entrophospora* sp., d) *Glomus caledonium*, e) *Scutellospora calospora*, f) *G. etunicatum*, g) *G. mosseae* (BEG 5), h) *G. mosseae* (Waite Campus), i) *G. fasciculatum*, j) *G. macrocarpum*, l) *Gi. rosea*, m) *Gi. margarita* , n) *Gi. margarita* (cloned ITS DNA template).

bp   a b c d e f g h i j l m n o p
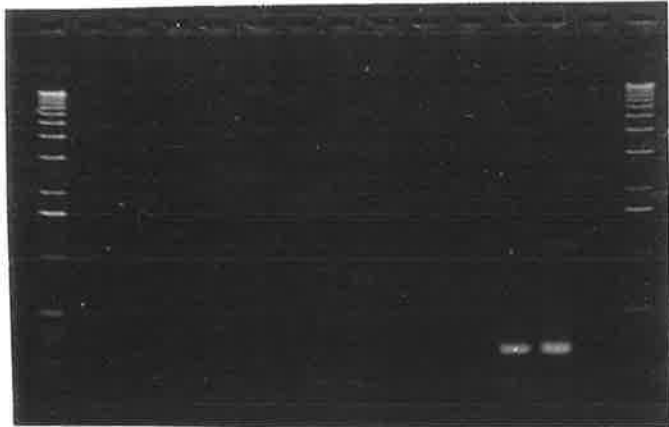
261 →

6.3.2 Detection of mycorrhizal fungal colonisation

The primers were tested to determine whether they were sufficiently specific and sensitive to detect the presence of mycorrhizal fungi in plant roots. The DNA was isolated from 'trap cultures' grown in field soil and from 'pot cultures' of individual fungi grown with various plant species (Table 6.2). DNA was also extracted from uncolonised roots of *A. porrum* and *T. subterraneum*, for use as controls. Specific products with the appropriate primers were obtained from DNA extracted from roots colonised by *G. mosseae* (Fig. 6.4, lanes b, c, d and Table 6.2) or *Gi. margarita* (Fig. 6.5, lane f and Table 6.2), whereas no product was obtained from DNA extracted from uncolonised roots (Fig. 6.4, lanes e, f, g, h and Fig. 6.5, lanes b, c, d, e, g, h) or roots colonised by *G. intraradices* (Fig. 6.4, lane e). The identity of the host plant had no effect on the amplification of the fungal sequences.

Table 6.2 Plant roots material and other fungi used to assess the specificity of GOMSf/GOMSr and GIMARf/GIMARr primers for the amplification of part of the internal transcribed region.

Source of DNA	Origin (name of isolate)	GOMSf/GOMSr	GIMARf/GIMARr*
Plant roots			
Trifolium subterraneum	Trap plant - field soil or pot culture	+	+
Sorghum sp	Trap plant - field soil	+	+
Lolium perenne	Trap plant -field soil	+	-
Allium porrum	Pot culture - *G. intraradices*	-	-
Allium porrum	Pot culture - *Gi. margarita*	-	+
Other fungi			
Binucleate *Rhizoctonia sp.*	South Australia- soil (R78)	-	+*
Gaeumannomyces graminis var avenae	New South Wales- *Agrostis sp.* (137T)	-	-
G. graminis var tritici	South Australia - *Triticum aestivum* (800)	-	-
Phytium echinulatum	South Australia -soil (BH3)	-	-
Rhizoctonia solani AG 2-1	South Australia -soil (1344)	-	+*

* The PCR amplification showed at least two bands, ~ 700bp and ~ 260bp. "+" indicates the expected amplification for the primer pairs, "-" indicates no amplification products for the primer pairs.

Non-mycorrhizal root infecting fungi are from the collection in CSIRO Soil and Water, Adelaide.

Figure 6.4: PCR amplification with *Glomus mosseae*-specific primer (GMOSf/GMOSr) shows that the *G. mosseae* primers recognise *G. mosseae* DNA from trap culture roots. Lanes a) and l) 1 kb ladder (Gibco-BRL); lane j) no DNA template; lanes b-d), DNA from colonised (trap culture) roots of b) *Trifolium subterraneum*, c) *Sorghum* sp. d) *Plantago lanceolata*; lane e) *Allium porrum* colonised with *G. intraradices*; lane f) *A. porrum* colonised with *Gigaspora margarita*; lane g) *A. porrum* uncolonised; lane h) *T. subterraneum* uncolonised; lane i) Cloned ITS DNA from *G. mosseae*.

Figure 6.5: PCR amplification with *Gigaspora margarita*-specific primers (GIMARf/GIMARr) shows that the primers recognise *Gi. margarita* DNA from trap culture roots. Lanes a) and l) 1 kb ladder (Gibco-BRL); lane j) no DNA template; lanes b-d trap cultures of b) *Trifolium subterraneum*, c) *Sorghum* sp., d) *Plantago lanceolata*; lane e) *Allium porrum* colonised with *G. intraradices*; lane f) *A. porrum* colonised with *Gi. margarita*; lane g) *A. porrum* uncolonised; lane h) *T. subterraneum* uncolonised; lane i), Cloned ITS DNA from *Gi margarita*.
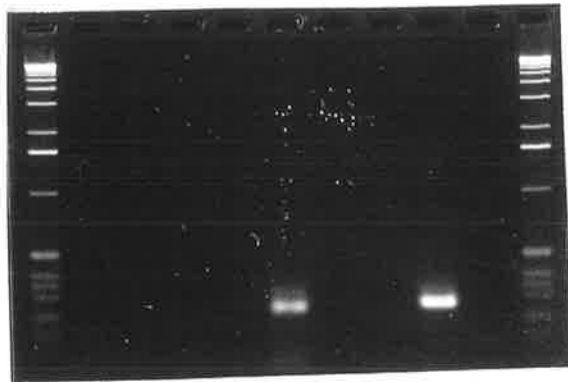
6.3.3 Detection of associated fungal species

To determine whether other root-infecting fungi interfered with the use of GOMSr/GOMSf and GIMARr/GIMARf, PCR was performed using the same conditions as above, with DNA extracted from cultures of *R. solani* AG2-1 1344, *G. graminis* var *avenae*, *P. echinulatum* and binucleate *Rhizoctonia sp.* with and without the addition of DNA from spores of *Gi. margarita*. *G. mosseae* primers (GOMSf/GOMSr) did not yield products with any of these fungal species (results not shown). However, *Gi. margarita* primers (GIMARf/GIMARr) amplified at least two bands, ~750 bp and ~261 bp, from DNA from *R. solani* AG2-1 and binucleate *Rhizoctonia* sp. R78. (Fig. 6.6, lanes b, e, f and Table 6.2). No amplification products were obtained from *G. graminis* var *avenae* or *P. echinulatum*. The *G. mosseae* primers therefore appear highly suitable for the detection of most isolates of this species and the presence of other root-infecting fungi in root material would not interfere with their usefulness. The *Gi. margarita* primers could also be used in most situations except where spores or roots are contaminated with *Rhizoctonia* AG2-1 and binucleate *Rhizoctonia* sp. R78.

Figure 6.6: *Gigaspora margarita* primers tested against DNA from other fungi show recognition of sequences in *Rhizoctonia* but not other common root-infecting fungi. Lanes a) 1kb ladder (Gibco-BRL); lane h) no DNA template; remaining lanes, b) *Rhizoctonia solani* AG2-1, c) *Gaeumannomyces graminis* var *avenae*; d) *Pythium echinulatum*; e) Binucleate *Rhizoctonia* sp.; f) Binucleate *Rhizoctonia* sp. plus *Gi. margarita*; g) Cloned *Gi. margarita* ITS DNA
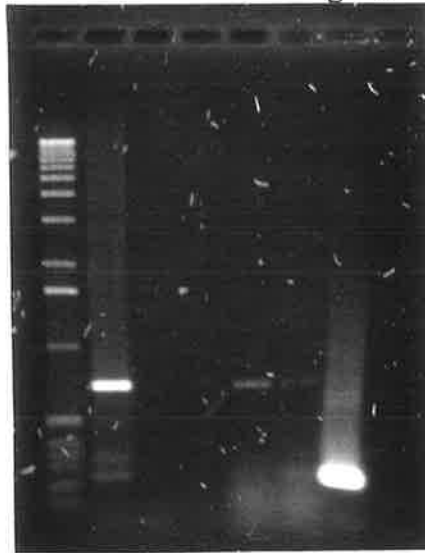
6.3.4 Detection and quantification of *G. mosseae* using oligonucleotide probes

Slot blot analysis was performed using the entire cloned ITS region from *G. mosseae* as a labelled probe. This probe hybridised to all DNA tested, including DNA from colonised roots of *T. subterraneum*, *L. perenne*, uncolonised roots of *T. subterraneum* and DNA from mycelium of *P. echinulatum* and *R. solani* AG2-1 1344 (Fig. 6.7a). When the oligonucleotides GOMSr/GOMSf were used as probes, hybridisation was detected only when *G. mosseae* DNA was present (Fig. 6.7b). No hybridisation was detected with DNA from binucleate *Rhizoctonia* sp. R78 or *G. graminis* var. *avenae* 137T (data not shown). Neither the cloned ITS fragment of *G. mosseae* nor the GOMSr/GOMSf primers hybridised to any DNA when high stringency conditions were used. This experiment was repeated twice and similar results were obtained. Therefore, the cloned fragment was not sufficiently specific for diagnostic purposes, but 18 bp oligonucleotides from *G. mosseae* can be used to detect and estimate the amount of DNA from this species.

To quantify the amount of fungal DNA in plant roots, we used known amounts of the cloned ITS fragment as a standard. Known quantities of DNA from the cloned fragments of *G. mosseae*, *Gi. margarita* or a mixture of the two were hybridised to the labelled oligonucleotide probes GOMSr/GOMSf. A hybridisation signal was successfully obtained with the *G. mosseae* DNA or a mixture of DNA from *G. mosseae* and *Gi. margarita* DNA, but not with *Gi. margarita* DNA alone (Fig. 6.8). These primers detected as little as 10 ng of cloned DNA from *G. mosseae* (Fig. 6.8).

To confirm that these oligonucleotide probes could be used to detect and quantify *G. mosseae* present in roots a serial dilution of DNA extracted from roots of *T. subterraneum* (trap culture; 68% colonised, see Table 6.3) was loaded onto the membrane. The DNA was probed with the labelled GOMSr/GOMSf oligonucleotides primers. A signal was detected in slots containing 100 ng of total DNA from colonised roots (Fig. 6.9, slot 2b). Similar results were obtained when a different DNA extraction method (Raeder and Broda, 1985) was used (Fig. 6.9: slots 3a-3d). Tests with other mycorrhizal plant species (data not shown) also gave positive results. Signal from colonised roots of *L. perenne* (trap culture) was obtained only when 1 µg of plant

DNA was used, probably because of the low fungal colonisation (19%) in these roots (Fig. 6.9, slots 3e-3h and Table 6.3).

Hybridisation with DNA from *T. subterraneum* roots colonised by *G. mosseae* BEG-5 (95% colonisation) was slightly stronger than from roots of the trap cultures. No signal was detected when DNA from *A. porrum* roots colonised by *G. intraradices* or *Gi. margarita* or from leaf DNA of *T. subterraneum* was used (Fig. 6.9).

The amount of ITS DNA from *G. mosseae* present in colonised roots was estimated by slot-blot analysis, using the oligonucleotide species-specific primer GOMSr/GOMSf. The labelled membrane was exposed to a phosphor screen overnight and quantified using Storm Phosphor Image Software (Molecular Dynamics). This result showed that 100 ng of total DNA extracted from roots of *L. perenne* in trap cultures (19% colonised) contained 0.013 ng of ITS AM fungal (*G. mosseae*) DNA and that 100 ng roots of *T. subterraneum* (95% colonised) contained 0.78 ng of ITS AM fungal *(G. mosseae)* DNA (Table 6.3).

Figure 6.7a: Slot-blot hybridisation using the entire 569 bp ITS fragment from *G. mosseae* with DNA from different sources. Lanes 1a-1d) DNA from the cloned fragment of *G. mosseae* amplified with ITS1/ITS4 primers using PCR (0, 100, 200, 400 ng); 2a-2d) DNA from uncolonised roots of *Trifolium subterraneum* (100, 200, 400 and 600 ng); 3a-3d) DNA from colonised roots of *T. subterraneum* (100, 200, 400 and 600 ng); 4a) *Pythium echinulatum* (20ng); 4b) *Rhizoctonia solani* AG2-1 265 (20ng); 4c-4d) DNA from colonised roots *Lolium perenne* (1000 and 500 ng).

b: Slot-blot hybridisation using the 18 bp oligonucleotides from *G. mosseae* -GOMSr/GOMSf with DNA from different sources. The membrane from a) was stripped and re-hybridised. Slots are same as in Figure 7a.

a)

	1	2	3	4
a				
b				
c				
d				

b)

	1	2	3	4
a				
b				
c				
d				

Figure 6 8: Slot-blot hybridisation of GOMSr/GOMSf oligonucleotide primers to cloned DNA from *G. mosseae* and *Gi. margarita*. Slots 1a-1e, *G. mosseae* DNA, 0, 10, 20, 30, 40 ng. Slots 2a-2e, *Gi. margarita* DNA, 0, 10, 20, 30, 40 ng; slots 3a-3e, *G. mosseae* DNA, 0, 5,10, 20, 30 ng, plus *Gi. margarita* DNA 0, 5, 10, 20, 30 ng.

Figure 6.9: Slot-blot hybridisation using GOMSr/GOMSf oligonucleotide primers to DNA extracted from different sources. Slot 1a-1h DNA from the cloned fragment of *G. mosseae* amplified with ITS1/ITS4 primers using PCR 0, 100, 200, 400, 600, 800, 1200, 1600ng). Slot 2a; uncolonised roots of *T. subterraneum* (200 ng); slots 2b-2g, colonised roots from *T. subterraneum* trap culture (100, 200, 400, 800, 1600, 3200 ng); slots 3a-3d, colonised roots from *T. subterraneum* trap culture (100, 200, 400, 800 ng); slots 3e-3h, colonised roots of *L. perenne* trap culture (100, 200, 400, 1000 ng), slot 4a, uncolonised roots of *T. subterraneum* (200 ng); slots 4b-4e, *T. subterraneum* roots colonised by *G. mosseae* BEG5 (100, 200, 400, 700 ng); slot 4f, *Allium porrum* roots colonised by *G. intraradices*; slot 4g, *A. porrum* roots colonised by *Gi. margarita*; slot 4h, *T. subterraneum* leaf DNA.
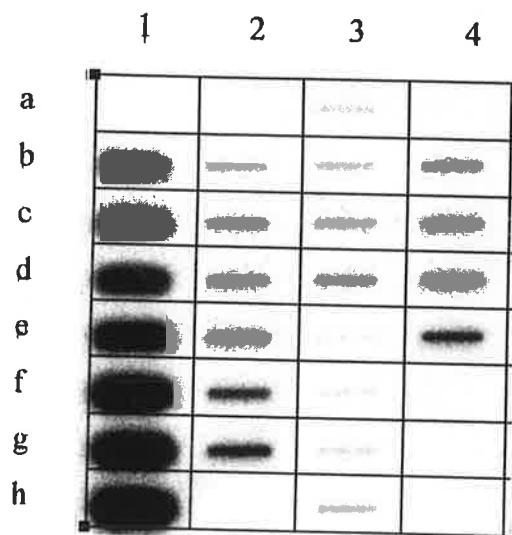
Table 6.3 The percentage of colonisation and the estimated amount of *G. mosseae* ITS DNA in colonised roots using primers GOMSf/GOMSr.

Host	AM fungi	Colonisation (%)	*G. mosseae* ITS DNA (ng/100ng of total root DNA)
Trifolium subterraneum	uncolonised	0	0
T. subterraneum	multiple colonisation	68.0	0.19
T. subterraneum	*G. mosseae* BEG5	95.0	0.78
Allium porrum	*G. intraradices*	75.0	0
A. porrum	*Gi. margarita*	20.0	0
Lolium perenne	multiple colonisation	19.0	0.013

6.4 Discussion

The ITS region of the ribosomal DNA genes has the potential to be an excellent region for design of primers for species-specific identification because of the large variability in this region of the genome. However, it has also been suggested that hypervariability of this region in Glomalean fungi might actually be a disadvantage for its use in taxonomic studies. The ITS sequences were successfully isolated from DNA from field spores to design species-specific primers for identification of *G. mosseae* and *Gi. margarita* and to quantify *G. mosseae* in colonised roots from single-species pot cultures and mixed-species trap cultures. This is the first report where molecular tools have been used directly to estimate the amount of ITS DNA from *G. mosseae* in roots colonised by multiple fungal species.

It had been suggested that the ITS sequence is too variable to be used as a diagnostic tool (Van Tuinen *et al.* 1998a), but this study shows that GOMSf/GOMSr and GIMARf/GIMARr primers used are robust, species-specific, and can be used to identify *G. mosseae* and *Gi. margarita* in DNA isolated from spores and colonised roots. The failure of primers to identify two isolates of *G. mosseae* will not detract from their usefulness at sites where 'recognisable' isolates are present, such as the permanent rotation trial at the Waite Campus and other sites in Australia. A large number of sequences were compared (19 from *Gi. margarita* and 23 from *G. mosseae)* in order to identify highly conserved sequences in the ITS region and to attempt to overcome potential difficulties relating to the high variability of the region (Chapter 5). The GMOSf/GMOSr primers detected *G. mosseae* DNA from six out of the eight isolates of this fungus that were tested. The inability of these primers to recognise DNA from two isolates (LPA 5 and WUM Br-4) may be due to the high degree of variation in the ITS region of this species (Clapp *et al.* 1995, Sanders *et al.* 1995, Redecker *et al.* 1997). Cloning and sequencing the regions from these isolates will be needed to confirm this suggestion and to determine whether the morphological identifications (as *G. mosseae*) are correct. It is also possible that there are polymorphisms within the units of the rDNA repeat, as already reported for ectomycorrhizal fungi (Gardes *et al.* 1991). Again, this requires further investigation. Using RAPD-PCR, Lanfranco *et al.*

(1995) also found that the primers used failed to identify one of the nine isolates of *G. mosseae* tested. Insufficient data is available for other fungi, so it is not possible to determine at this stage whether the problem is confined to the highly variable *G. mosseae* or whether it is more general.

The utility of ITS sequences is confirmed by the recent work of Millner *et al.* (1998) who also designed species-specific primers for *G. mosseae*. Only two ITS sequences were used for the design of their primers (compared with the 23 in this study) and identified 12 out of 12 *G. mosseae* isolates from pot cultures. However, full identification at the required specificity needed a two stage reaction; PCR amplification using the primers GMOS1 and GMOS2, followed by probing with GMOS5. No attempts to quantify colonisation were made by Millner *et al.* (1998). No interference from root DNA of the plant species tested was observed either by Millner *et al.* (1998) or this study. It is not yet known whether the primers designed by other groups will be useful in the detection of AM fungal species in field cultures containing fungal populations of mixed species.

The ITS sequences of *Gi. margarita* have also been used by Lanfranco *et al.* (1999) to design species-specific primers. The reverse primer is the same in Lanfranco *et al.* (1999) and this thesis. However, the forward primer is different. In this study the primer is located between 106 and 124 bp, that of Lanfranco *et al.* (1999) it is located between 13 to 33 bp. The specificity of these primers has been demonstrated by Lanfranco *et al.* (1999) and this work. Lanfranco *et al.* (1999) did not test their primer pair against non-glomales fungi. This will be important because this study shows that the primers GIMARf and GIMARr can detect the presence of *Rhizoctonia* sp. in AM fungi material. Furthermore, in this study the primers tested in a large range of plant species, which again confirms their specificity. *Gi. margarita* seems to have low variation the ITS region between different isolates which should allow the use of these primers for a larger number of isolates.

Contamination of root material in the field with non-mycorrhizal, root-infecting fungi represents a potential problem in the use of the ITS region for PCR because the ITS sequences are found in all organisms. The *G. mosseae* primers did not amplify

products from any of the non-mycorrhizal fungi tested, increasing the potential value of the primers for field studies. However, the *Gi. margarita* primers produced amplification products from the soil-borne *Rhizoctonia spp.*, although not from the other pathogenic fungi tested. The difference in size of the amplified products from *Rhizoctonia* compared to *Gi. margarita* indicates that it will be possible to differentiate between *Gi. margarita* and the contaminant by determining fragment length. If no contamination is found, the primers can then be used for specific detection and quantification of *Gi. margarita*.

The experiments performed in this study indicate that fungal DNA can be identified and potentially quantified in the total DNA extracted from mycorrhizal roots. However, to be able to convert the amount of fungal DNA into a percentage of root colonization, a titration curve is needed. Further development of this technique will be important in order to construct this relationship. In this study, the root systems used were several months old so that parts of the fungus in the root may have been dead, explaining the lack of a close positive correlation. A more detailed analysis of the relationship between amount of DNA and % colonisation using vital stains, needs to be undertaken before the method can be confidently applied in ecological studies. Successful quantification of the contributions of individual species in mixed populations of fungi (*G. intraradices, G. mosseae, Gi. rosea* and *S. castanea*) in roots has previously only been achieved by carrying out nested PCR reactions on very large numbers of separate, small root samples in order to determine the frequency of occurrence of the different fungi using taxon-discriminating probes from 25S rDNA. This approach was successful for analysing multiple colonisation by these fungi in a microcosm community (Van Tuinen *et al.* 1998a) but its utility in field studies remains to be tested. Because the quantification was based on the detection frequency in root fragments it may not be possible to use those techniques to estimate the absolute amount of fungal material in the roots.

The quantification of DNA using a slot-blot hybridisation assay on colonised roots has the potential to be more accurate and reliable than the conventional methods of evaluating the extent of colonisation. The *G. mosseae* oligonucleotide probe was

observed to be more specific than the entire cloned ITS region, which suggests that longer probes may be more degenerate and therefore less reliable (Manak 1993) or they may contain more regions that are similar to other ITS sequences. Another advantage of synthetic oligonucleotides is that they can be easily synthesised and used as probes. The methodology described in this chapter is similar to that already reported for the quantification of *G. graminis* var. *tritici and R. solani* AG 8 (Herdina *et al.* 1996, 1997). In those studies slot-blot assays were used to quantify the amount of *G. graminis* var. *tritici* in roots and in field soils (Herdina *et al.* 1997). Similar methods might be used to determine the level of mycorrhizal inoculum in soil and colonisation in roots, both of which will enhance the study of ecology of AM fungi. The oligonucleotide probes have not yet been tested on fungal DNA extracted from soil (Claasen *et al.* 1996), but the results with plant roots suggest they will also be useful for this purpose, as long as DNA extraction can be optimised. The *G. mosseae* oligonucleotide probe is sensitive and has the appropriate level of specificity to detect several different *G. mosseae* isolates and therefore could be used for the quantification of this species. Overall, the results show that the use of species-specific oligonucleotides provide new possibilities for analysing colonised roots to determine the contributions of different fungi to the symbiosis. Such information is crucial for interpreting the outcomes of studies of the effects of different AM fungi on plant growth in natural communities (Van der Heijden *et al.* 1998), competition and selection of the best species for inoculation programs. In summary, the primer pairs described in this study have the potential to provide robust and sensitive tools for the identification and quantification of mycorrhizal species based on spores found in the field and roots colonised with different fungi.

6.5 Conclusion

The PCR primers described in this study are based on ITS sequences (Chapter 5) and they provide useful tools for studying *G. mosseae* and *Gi. margarita*. The *G. mosseae* primers are more specific than *Gi. margarita* primers, in relation to non-mycorrhizal

fungi. The *Gi. margarita* primers can generate different size bands, due to the presence of *Rhizoctonia.*

The quantification of DNA using slot-blot hybridisation assay in colonised roots has the potential to be an accurate, specific and sensitive method for routine evaluation of colonisation. The technique is relatively simple to perform and thus can be of practical value.

The specificity and sensitivity of these primers have the potential to permit the reliable detection, quantification and identification of *G. mosseae* and *Gi. margarita* species of AM fungi in the soil and therefore will be potent tools for understanding the efficacy of the symbiotic relationship with host plants.

Chapter 7

General discussion, summary of findings and future research

7.1 General discussion

7.1.1 Introduction

The general aim of this project was to fully describe an AM fungal community based on spore populations at a field site using traditional and molecular classification methods. The molecular identification of the mycorrhizas from natural communities has received more attention in recent years for a number of reasons. First, species and strains differ in their ability to promote plant growth. Second, the presence of mycorrhizas seems to be essential for the establishment of some plant species in the field (Smith *et al.*, 1998). Third, the traditional identification of AM fungi is based on the morphology of spores from pot cultures and precise identification of spores from the field is difficult, because variation can occur in the spore morphological characteristics. Because of the limitations associated with morphological identification, molecular identification methods potentially provide an invaluable tool for studying AM fungi diversity in the field.

7.1.2 Site selection and comparison of fungal populations

A survey of AM fungal communities in different agroecosystems (Chapter 3) was initiated because these systems had not been characterised with respect to spore populations of AM fungi. The survey showed that the permanent pasture phase of the Waite Permanent Trial had the highest AM fungal diversity as compared to other sites (Chapter 3). Spore identification of this AM fungal community was assessed by traditional methods. Two fungal species, *G. mosseae* and *Gi. margarita*, were selected for the development of a molecular detection and identification method (Chapter 6), because they represented the most abundant species in the families Glomaceae and Gigasporaceae, respectively. The Waite trial was used because plant growth and nutrition of the pasture has been documented for more than 25 years (Grace *et al.*, 1995). Field samples were taken over two years and trap cultures were established

with *Lolium perenne, Plantago lanceolata, Trifolium subterraneum* and *Sorghum* sp. in order to fully describe the AM fungal spore populations. Single spore isolation was also attempted with a number of fungal species in order to provide material for future physiological and competition studies. This was successful with *G. mosseae, G. fasciculatum, Glomus* sp. and *Gi. margarita* (Chapter 4).

In addition to studying fungal populations at the Waite Permanent pasture, other sites were also studied because different cultural practices may influence fungal populations. This work provided a baseline for future studies on field populations of AM fungi and allowed for comparisons to be made with the Waite site. The survey undertaken showed that the number of spores and diversity of genera varied between sites. The information obtained indicated that the permanent pasture contained high AM spore populations compared to the other systems (Chapter 3). However, firm conclusions cannot be made because, at the sampling time (May 1996) most of the other sites had few host plants present (Chapter 3). The sites also differed with respect to soil disturbance which could be important in determining spore numbers (Smith, 1978). The spore populations of these sites may not be fully representative of the AM fungi present in the soil, because some fungal species may be not have sporulated at the sampling times and may exist only as hyphae in the soil or in roots.

The initial studies of the permanent pasture (Waite) indicated that a large number of fungal species were present and therefore this site provided a very good starting point for initial descriptions of a fungal spore population, and for the development of molecular tools to investigate fungal populations in roots and soil. Some studies have reported the detection of different AM fungi in plant roots using techniques such as analysis of isoenzymes and antibodies (Rosendahl *et al.*, 1989); Hepper *et al.*, 1988a). However, the number of different AM fungi that can be identified simultaneously is limited. PCR opens the possibility of simultaneous identification of multiple AM fungal species in plants roots because small amounts of AM fungal DNA can be amplified, when plant and fungal DNAs are mixed. Many PCR approaches have been used to detect AM fungal populations in roots such as: PCR-RFLP (Bonito *et al.*, 1995), PCR primers developed from RAPD fragments (Lanfranco

et al., 1995), from small ribosomal subunit (Simon *et al.*, 1993; Clapp *et al.*, 1995), from genomic clones (Zézé *et al.*, 1996) and from large ribosomal subunit rDNA (Van Tuinen *et al.*, 1998a). These techniques have only been tested in single AM fungal species and with plants grown under controlled conditions.

Arbuscular mycorrhizal fungal spore populations at the Waite permanent pasture, based on four field collections, gave a partial picture of the AM fungal species present in soil. The field sampling did not recover all species, as several additional species were recovered from trap cultures. Trap-cultures also provided an incomplete inventory of sporulating fungi (Chapter 4). Therefore, these studies have shown that field or trap cultures alone were not a good indicator of AM fungi present. The sampling method used in this study involved the disturbance of the hyphal network, so that trap cultures would favour species with robust propagules in soil (spores and root fragments) which later formed mycorrhizas and sporulated. Another methodology which may give a more realistic composition of species in the field is the use of intact cores. Studies (Braunberger *et al.*, 1994; Brundrett and Abbott, 1995) on intact soil cores and *T. subterraneum* to assess the morphology of the mycorrhizas at a generic level in the roots, have been carried out, but did not provide an analysis of the AM fungal spore populations. The spore populations provide identification at species level, but colonised roots give identification at a genus or higher taxon level (Merryweather and Fitter, 1998a; Merryweather and Fitter, 1998b).

The identification of the Glomalean fungi by root colonisation patterns using intact soil cores and host specific plants (Abbott, 1982; Brundrett *et al.*, 1996) could be influenced by host plant root structure (Brundrett and Kendrick, 1990), making identification difficult. In addition, it is important for morphological identification to become familiar with the distinguishing features of the AM fungi present in soil and the root plant structure (Brundrett *et al.*, 1996; Brundrett and Kendrick, 1990). It is also important to know which fungi are involved in a mycorrhiza so that their individual contributions to the system may be assessed (Merryweather and Fitter, 1998b). Another consideration for future studies of AM fungal communities is to increase the number of samples of field soil for spore recovery. Bioassays with trap cultures using

intact cores would provide another method for gaining a more realistic picture of the mycorrhizal community present in the field soil.

The choice of host plant species is essential for studying AM fungal spore populations because of the different levels of sporulation between hosts. *P. lanceolata, T. subterraneum* and *Sorghum sp.* are used for the maintenance of pot cultures and as hosts in trap cultures in many laboratories (eg. Abbott and Robson, 1984a; Jasper *et al.*, 1993; Smith and Read, 1997; Stutz and Morton, 1996; Sanders, 1993; Simpson and Daft, 1990b). In this thesis results showed that recovery of AM fungi from trap cultures of field-collected soil differed according to host plant species used. This confirms the problem of this methodology. *Lolium perenne* was a poor host for trap cultures. It indicated the lowest richness and diversity of AM fungi from field soil collected in December 1996 (Table 4.4). Another consideration for recovery of maximum fungal diversity in pastures is the composition of the plant species in the field. For example, members of the Gramineae in the field were associated with low AM fungal diversity and with *Glomus* sp. as the main genus recovered (Chapter 3, Table 3.5). Future work should consider the type of pasture and use a methodology which reveals the maximum richness and diversity of these fungi from field soils. The effects of plant, soil and interactions between AM fungal species need to be considered (Johnson *et al.*, 1992b; Smith and Read, 1997; Stutz and Morton, 1996). Further investigations are needed for an understanding of the factors that regulate populations of these fungi.

7.1.3 Molecular characterisation of *Glomus mosseae* and *Gigaspora margarita*

Molecular studies of AM fungi have developed rapidly since this study started in 1996 (eg. Bago *et al.*, 1998; Lanfranco *et al.*, 1999; Millner *et al.*, 1998; Redecker *et al.*, 1997; Rosendahl and Taylor, 1997; Zézé *et al.*, 1997). These studies used AM fungal spores from pot cultures for DNA extraction. In this thesis field-collected spores were used for designing primers from ITS regions. Genetic variation in the ITS regions was detected both for *G. mosseae* single spores (2.4 - 5.7%) and *Gi. margarita* single spores (1.0 - 3.0%) and pool of spores (3.6%) (Chapter 5). These findings support

previous studies that showed considerable ITS sequence heterogeneity in AM fungi (Sanders *et al.*, 1995; Lloyd-MacGilp *et al.*, 1996). Lanfranco *et al.* (1999) recently reported intersporal and intrasporal genetic variability ranging from 0.2 to 9% sequence divergence in *Gi. margarita* BEG 34. These levels of sequence divergence cannot be directly compared to what was reported in this thesis because in this study was used field collected spore. *Gi. margarita* sequences from this study (Table 5.2) are almost identical to those reported by Lanfranco *et al.* (1999). *Scutellospora castanea* that belongs to the same family *Gi. margarita* also show different sequences of ribosomal DNA even between nuclei from the same fungal individual (Hijri *et al.*, 1999). The divergence in rDNA sequences could be due to a lack of recombination on different nuclei and the occurrence of compatible anastomosis and exchange of nuclei (Bécard and Pfeffer, 1993; Rosendahl and Taylor, 1997; Sanders, 1999; Tommerup, 1987).

7.1.4 Methods for molecular detection of *Glomus mosseae* and *Gigaspora margarita*

Initially, the plan was to use traditional and molecular methods to identify AM fungal spore field populations during the project. However, problems with designing sufficiently specific primers delayed the application of this methodology to field grown plants. The direct sequencing of ITS regions from single field collected spores was not possible in spite of many attempts. An investigation published during the course of the work (Lloyd-MacGilp *et al.*, 1996) also showed that it was not possible to use direct sequencing of AM fungal DNA from single spores. In order to establish a protocol that could be used on DNA isolated field spores of AM fungi, DNA was cloned and sequenced from a pool of *Gi. margarita* spores from pot culture (Chapter 6). The same protocol was used successfully for three single spores of *G. mosseae* and a single *Gi. margarita* from field material.

The identification of polymorphisms in an individual species depends on the methodology used and the level of genetic variation. For example, the intersporal variability of *Glomus* based on amplified fragment length polymorphisms (AFLP) (Rosendahl and Taylor, 1997) and of *Gigaspora margarita* with the M13 minisatellite-

primed PCR (Zézé, 1997) showed a high level of genetic variability, which may be related to asexual reproduction. However, a particular sequence may not be constant in all members of one taxonomic group, as demonstrated in *G. mosseae* (Lloyd-MacGilp *et al.*, 1996; Millner *et al.*, 1998; Sanders *et al.*, 1995) and in this study (Chapter 5). The *G. mosseae* and *Gi. margarita* primers designed in this study were constructed by comparing large numbers of ITS sequences and were tested against 11 different AM fungal species, five host plants and five different species of non-AM fungi. *G. mosseae* primers did not amplify ITS sequences from two out of ten isolates of *G. mosseae* tested, and *Gi. margarita* primers amplified two products from *Rhizoctonia* (Chapter 6). The high specificity and sensitivity of these primers (with the exceptions stated above) suggests that they will be a good tool for detection, identification and quantification of these two species of AM fungi. Primers with a high level of specificity would be important for the detection of all AM fungi at group (Glomalean), family or genus level and could be used to quantify the fungal DNA involved in the mycorrhizas, and used in conjunction with the species specific probes to determine the relative importance of these species to the community as a whole.

The quantification of AM fungi using slot-blot hybridisation (Chapter 6) will have many advantages over conventional bioassays. These include rapid analysis, and the ability to process a large number of samples simultaneously. Furthermore, DNA hybridisation assays are potentially more accurate and reliable than traditional quantification methodologies. For a DNA hybridisation assay it is not necessary either to culture the fungus for a long time with the host and or stain the roots followed by microscopy. Moreover, the DNA slot-blot hybridisation assay is potentially specific and sensitive, and can be used for routine quantification of AM fungi by scientists who are not expert in identification of AM fungi. Once additional specific oligonucleotides for identification of different AM fungal species become available, this method will be more useful for studying AM fungi communities in roots from field material.

The molecular work in this thesis demonstrated that species-specific oligonucleotides for *G. mosseae* were sufficiently sensitive to identify and quantify this species in spores and colonised roots under greenhouse conditions with field soil. The

next step will be to use these oligonucleotides in colonised roots grown under field conditions. While morphological techniques will continue to be important in the taxonomy of the Glomales, less ambiguous and easier methods would be helpful for non-experts to identify these fungi. Therefore, the determination of AM fungal populations in soil using methods that rely only on spores should be avoided because they are: a) time consuming; b) unreliable (the presence of a single spore species in the soil does not necessary mean that this particular species is forming a mycorrhizal association) and c) non-specific (Gerdemann, 1955) or poorly specific (Giovannetti and Hepper, 1985; Smith *et al.*, 1992) for AM fungi.

7.2 Summary of findings

The main outcomes were:

- the permanent pasture trial at the Waite showed high numbers of spores as well as a high degree of AM fungi diversity;

- *G. mosseae* was the dominant AM fungal species in both years of survey;

- field-collected soil in conjunction with trap cultures with *P. lanceolata* or *Sorghum* sp. or *T. subterraneum* were an effective tool to study populations and diversity of indigenous AM fungi;

- *P. lanceolata, Sorghum* sp. and *T. subterraneum* as hosts for trap culture showed no differences in richness and diversity of AM fungi, however *L. perenne* gave the lowest richness and diversity, particularly in December 1996,

- genetic variation in the ITS region of natural populations of *G. mosseae* was higher than *Gi. margarita*,

- *G. mosseae* and *Gi. margarita* primers were shown to be robust tools for detection, quantification and identification of these species of AM fungi.

7.3 Future research

This study of AM fungal spore populations recovered from field soil and trap cultures showed that studying the communities of these fungi by only using spores from field-collected soil does not provide a complete picture of the fungal community. Some

mycorrhizal fungi sporulate easily in the field or in trap cultures and have the advantage of speeding up the process of colonisation, sporulation and potentially protecting roots from plant pathogens (Newsham *et al.*, 1995b). However, other species of AM fungi show low sporulation and the use of trap cultures could be of great value for increasing the number of spores, facilitating morphological identification and as inoculum for physiological studies.

In future work it will be important:

1) to have baseline information on AM fungal spore communities from other ecosystems,

2) to isolate single spore cultures from field-collected spores from more species,

3) to study the efficiency in nutrient uptake and effectiveness by the fungal species isolated during this work (Chapter 4),

4) to develop a more practical method for recovery of a maximum number of species from the field soil, using successive trap cultures (Stutz and Morton, 1996) and/or intact cores (Brundrett and Abbott, 1995) with more than one host in each pot culture, because in this thesis the low number of field soil samples and single host trap cultures showed a different composition in the AM fungal species,

5) to establish an efficient mycorrhizal partnership in pasture systems that could be used for inoculation programs.

Further research to characterise AM communities in the field soil should not only take the spore populations into account, but include associated information on the AM fungal populations in the plant roots (Merryweather and Fitter, 1998a). An important future aim will be to combine the conventional identification of AM fungi with molecular identification using species-specific PCR primers to measure the activity of mycorrhizas under field conditions.

The suggested approach for future molecular work will be to identify the AM fungal species that form mycorrhizas in field conditions using species-specific PCR primers described in this thesis, in plant roots under different soil and crop management and to study the effect host plants have on the plant-fungal interaction. The species

which colonise roots can be quantified with species-specific primers and compared with the diversity of AM fungi in soil.

Appendix 1

Authorities of AM fungi

Acaulospora melleae Spain & Schenck

Acaulospora morrowiae Spain & Schenk

Acaulospora myriocarpa Spain, Sieverding & Schenck

Acaulospora. laevis Gerdemann & Trappe

Entrophospora colombiana Spain & Schenck

Gigaspora decipiens Hall & Abbott

Gigaspora gigantea (Nicolson & Gerdemann) Gerdemann & Trappe

Gigaspora margarita Beck & Hall

Gigaspora roseae Nilcoson & Schenck

Glomus aggregatum Schenk & Smith emend. Koske

Glomus ambisporum Smith & Schenck

Glomus caledonium (Nicolson & Gerdemann) Trappe & Gerdemann

Glomus clarum Nicolson & Schenk

Glomus constrictum Trappe

Glomus coronatum Giovannetti

Glomus etunicatum Becker & Gerdemann

Glomus fecundisporum Schenk & Smith

Glomus fragilistratum Skou &Jakobsen

Glomus globiferum Koske & Walker

Glomus intraradices Schenck & Smith

Glomus invermaium Hall

Glomus macrocarpum Tulasne & Tulasne

Glomus microaggregatum Koske, Gemma & Olexia

Glomus monosporum Gerdemann & Trappe

Glomus mosseae (Nicolson & Gerdemann) Gerdemann & Trappe

Glomus occultum Walker

Scutellospora castaneae Walker

Scutellospora calospora (Nicolson & Gerdemann) Walker & Sanders

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