A POLYMERASE CHAIN REACTION AND DENATURING GRADIENT GEL ELECTROPHORESIS PROCEDURE FOR ANALYSIS OF ARBUSCULAR MYCORRHIZAL FUNGI IN SOIL

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are important components of agro-ecosystems and are especially significant for productive low-input agriculture. Traditional spore morphology-based identification of AMF in biodiversity studies is subjective and requires expertise and time. Researchers have used molecular techniques to investigate community composition of AMF in uncultivated, disturbed, or contaminated soils, but this approach to community analysis of AMF in agricultural soils has not been reported. In this study, a polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) procedure for the detection of fungal 18S ribosomal RNA gene was developed with reference cultures. Five AMF species were procured from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM). These reference cultures were chosen because isolates of their species were putatively identified in a previous survey of farm field soils in Saskatchewan, Canada. A reference PCR-DGGE profile was generated using DNA extracted and amplified from the spores of these INVAM cultures. The method's technical limitations were investigated. The optimized procedure's effectiveness was tested by its application to soil samples from 38 farms. Bands from the PCR-DGGE profiles of these samples were excised for sequence analysis. The total number of species recovered was low in comparison to other AMF community surveys of temperate climate locations. The majority of the sequences recovered were *Glomus* species. *Scutellospora calospora*, a previously undetected AM fungus in Saskatchewan was found. A trend in AMF distribution in Saskatchewan was observed and it was relatable to their

phylogenetic taxonomy. Though not without its drawbacks, this approach to community composition analysis of AMF was faster than conventional trap cultivation methods.

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LIST OF ABBREVIATIONS

AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic Local Alignment Search Tool
CTAB	Hexadecyltrimethyl ammonium bromide
ddH_2O	Distilled and deionized water
D_e	Effective diffusion coefficient
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
FAME	Fatty acid methyl ester
GC	Guanine and cytosine
INVAM	International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi
ITS	Internal transcribed spacer
MPN	Most probable number
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
PVPP	Polyvinyl polypropylene
RAPD	Randomly amplified polymorphic DNA

RDP	Ribosomal Database Project
RNA	Ribose nucleic acid
SDS	Sodium dodecyl sulphide
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
TAE	Tris-acetic acid-EDTA
T-RFLP	Terminal restriction fragment length polymorphism
TE	Tris-HCl EDTA

1 INTRODUCTION

Mycorrhizal fungi are symbionts in roots of the majority of terrestrial plants. The arbuscular mycorrhizal fungi (AMF) are the largest sub-group of these fungi, and members of this group are obligate symbionts. These fungi are so named because the fungal symbiont produces characteristic finely branched hyphal structures, termed arbuscules, inside the cortical cells of plant roots (Douds and Millner, 1999). AMF are probably the most ubiquitous fungi in agricultural soils, accounting for 5 to 36% of the total biomass in soil and 9 to 55% of the biomass of soil microorganisms (Olsson et al., 1999). The earliest fossil record of this association dates back more than 400 million years ago, and researchers have postulated this association was required for the successful colonization of land by early plants (Remy et al., 1994). Indeed, it is the non-mycorrhizal plant that is the exception in nature (Douds and Millner, 1999).

Arbuscular mycorrhizal fungi are a critical component in agricultural systems because these organisms can increase plant growth (Smith and Read, 1997), plant reproductive capacity (Lu and Koide, 1994), plant water stress tolerance (Gupta and Kumar, 2000), and plant health through antagonistic and competitive effects on pests and pathogens (Gange and West, 1994). The main benefit to the host plant in the mycorrhizae symbiosis is the enhanced uptake of immobile soil nutrients, in particular phosphorus (Jakobsen, 1999). Arbuscular mycorrhizal associations increase nitrogen accumulation in plant tissues as a result of the hyphae out competing for mineralized organic soil nitrogen (Ibijbijen et al., 1996). Arbuscular mycorrhizal fungi also interact

with other soil organisms involved in important nutrient cycles. For example, biological nitrogen fixation by *Rhizobium* in legume hosts can be enhanced through co-infection with AMF (Xavier and Germida, 2002). Such ecological roles are of special importance in low-input farm management systems because these systems rely on natural nutrient cycles to provide the nutrients required for plant production.

Research on AMF in Saskatchewan has focused on the mycorrhizal effect on crop production rather than AMF diversity and function. The first study of AMF in Saskatchewan dates back nearly 50 years (Bakerspigel, 1956). Bakerspigel (1956) identified spores of *Glomus fasciculatum* in 35 soils from across Saskatchewan and Manitoba. Later, Pang and Paul (1980) isolated *Glomus mosseae* from soils cropped with fababeans. Kucey and Paul (1983) reported that AMF numbers exist in significant numbers to affect crop production. Talukdar (1993) was the first to assess the occurrence of AMF across Saskatchewan through monospecific propagation, and he linked this work to indigenous AMF inoculum development to promote growth in wheat and lentil (Talukdar and Germida, 1994). The inoculum work was used to examine the ability of native AMF to promote lentil and wheat growth with changes in phosphorus levels (Xavier and Germida, 1997) and interactions with other soil microbes such as *Rhizobium* (Xavier and Germida, 2002) and *Pseudomonas* (Walley and Germida, 1997). Xavier et al. (2000) were the first to attempt identifying AMF by a method other than spore morphology (e.g., protein profile). Helgason (2002) studied the response of three wheat cultivars to indigenous AMF colonization. With the exception of Talukdar (1993), limited attention has been placed on identifying the components of the field AMF community.

Evidence of the ecological importance of AMF is abundant, but an understanding of the distinct roles of individual fungal species is limited. Spore morphology and spore enumeration are the traditional methods for taxonomic identification and AMF diversity studies. Field recovered spores are often unidentifiable and require trap cultivation on host plants to produce identifiable spores (Bever et al., 2001). However, fungal diversity information gathered by this approach is incomplete because sporulation is dependent on the species, host, seasonality, growth conditions, and other environmental factors (Oehl et al., 2003). Molecular techniques that assess the AMF diversity directly present in soil avoid many of the challenges associated with spore production and cultivation.

The objective of this study was to develop a polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) assay to detect AMF in soils of Saskatchewan, Canada. The method developed involves an adapted extraction procedure to consistently produce amplifiable fungal template and a nested PCR strategy where the first reaction enriches a sample's general fungal DNA content. A second (nested) PCR reaction amplified AMF templates in particular. The subsequent nested PCR product was subjected to DGGE, and the resultant bands were excised and sequenced to identify the AMF represented by the band. To investigate its efficacy, the technique was applied to soil samples from a survey of 38 farm field soils across Saskatchewan, Canada. The achievement of the objective will test the hypothesis that the PCR-DGGE technique (this study) is at least as accurate as morphological techniques (Talukdar, 1993) in the determination of the number of AMF species in Saskatchewan field soils.

2 LITERATURE REVIEW

2.1 Functions of Arbuscular Mycorrhizal Fungi

The arbuscular mycorrhizal (AM) symbiosis is typically mutualistic where both members of the symbiosis derive benefits from the association. The main benefit to the host plant in the mycorrhizal symbiosis is the enhanced uptake of insoluble soil nutrients, in particular phosphorus (P) (Jakobsen, 1999). In addition, AM associations increase nitrogen (N) accumulation in plant tissues as a result of the hyphae out competing other organisms for mineralized organic soil N (Ibijbijen et al., 1996). Arbuscular mycorrhizal fungi also interact with other soil organisms involved in important nutrient cycles. For example, biological N fixation by *Rhizobium* in legume hosts can be enhanced through co-infection with AMF (Xavier and Germida, 2002). In return, the host plant allocates a portion of the fixed carbon (C) compounds it produces to the obligate fungal symbiont (Johnson et al., 2002). However, plant growth response to AMF colonization may range from mutualism, to indifference, to antagonism depending on the host/symbiont species involved (Talukdar and Germida, 1994), nutrient availability (Xavier and Germida, 1997), interactions with other soil organisms (Wilson, 1984; Walley and Germida, 1997), and other environmental factors (Leyval et al., 1997; Gupta and Kumar, 2000; Johnson et al., 2002; Karasawa et al., 2002).

Besides the impact on the plant host, AMF affect the subterranean environment. Soil aggregation is an important aspect of soil structure, which determines characteristics

such as water inflow rate, pore space, and erosion resistance (Douds and Millner, 1999). Arbuscular mycorrhizal fungi have been linked to soil aggregation and aggregate stability (Miller and Jastrow, 1990; Wright and Upadhyaya, 1998). These fungi play an important role in the production and maintenance of water-stable aggregates through extra-radical hyphae entanglement of soil particles and particle adhesion by a hyphae secreted glycoprotein (glomalin). Moreover, colonization of plant roots by AMF alters the amount and types of exudates released by the host. Root exudates are important regulators of microbial community composition and activity, and these compounds are a source of reduced C and amino acids for microbial consumption. Certain exudates (e.g., flavenoids) are signal molecules that induce/attract or repress/repel other microbes important for nutrient cycling (e.g., *Rhizobium* in N fixation).

2.1.1 Nutrient Transport

In the majority of mycorrhizal types, carbohydrates produced by photosynthesis moves from the autotroph (host plant) to the heterotroph (fungal symbiont), while nutrients acquired from the soil solution pass in the opposite direction (Smith and Read, 1997; Jakobsen, 1999). The contribution of AMF to plant nutrient uptake is mainly through the acquisition of nutrients (especially P) from soil by the extra-radical fungal hyphae, especially from root-distant soil not depleted of nutrients by the root (George, 2000). The effects of AMF on nutrient uptake and mobilization processes have different importance for different nutrients. Mechanisms of nutrient translocation to the host and the C drain of the fungus on the host have significant effects on plant growth (Schellenbaum et al., 1998).

Fungal hyphae are functionally analogous to fine root hair as both are nutrient uptake organs. Diameters of fine root hair, 5 to 20 μm (Wulfsohn and Nyengaard, 1999), and hyphae, 3 to 7 μm (Bago, 2000; Dodd et al., 2000), are comparable, but hyphal length densities of AMF in soil of chamber and field experiments range from ten to hundred-fold greater than root length densities in the corresponding studies (Miller et al., 1995; Ravnskov et al., 1999; Schweiger et al., 1999; Dodd et al., 2000). Fungal hyphae extend the plant's effective absorption surfaces beyond the nutrient depleted zone that develops around the root caused by direct root uptake processes. However, greater hyphal density is not of equal significance for uptake of all ions in soil (Jakobsen, 1999; George, 2000). It is of importance for ions with small effective diffusion coefficients (D_e) in soil, such as $H_2PO_4^-$ (10^{-8} to 10^{-11} cm^2 s^{-1}) (Barber, 1984). Mycorrhizal plants will deplete distal available P faster than non-mycorrhizal plants because the diffusion distance of $H_2PO_4^-$ to the nearest hypha will probably be less than to the nearest root (Li et al., 1991). In contrast, non-mycorrhizal roots are as adept at depleting ions with larger D_e such as K^+ (10^{-7} to 10^{-8} cm^2 s^{-1}) and NO_3^- (10^{-6} to 10^{-7} cm^2 s^{-1}) (Barber, 1984; George, 2000). For these ions, only in situations where diffusion is low (e.g., dry soil) or root growth is decreased (e.g., compacted soil) will the contribution of fungal hyphae to plant nutrient absorption be predictable (Li et al., 1997).

The sources of available nutrients to the fungi for uptake are less certain. The soil solution, in dynamic equilibrium with the labile inorganic fraction, is the primary source (Smith and Read, 1997). Colonized and non-colonized roots absorb P in solution at similar rates (Joner and Jakobsen, 1995). However, hyphae are better suited than roots to utilize patches of nutrient separated in space and time (Cui and Caldwell, 1996) or small

pores not accessible by roots (O'Keefe and Sylvia, 1992). Fungal membrane bound high affinity active transporters facilitate rapid removal of low concentration nutrients such as P and potassium (K) from solution at sites of release thereby driving the equilibrium from adsorption towards desorption. No evidence supports the hypothesis that AMF lower the threshold concentration for uptake (Jakobsen, 1999).

Evidence suggests AMF produce extracellular phosphatase that mineralize organic P for uptake (Joner et al., 2000; Koide and Kabir, 2000), but the activity of AMF phosphatase is relatively small in comparison to the activity of other soil microbes and autolysis (Joner et al., 2000). Although evidence indicates mycorrhizal plants grow better with organic amendments than non-mycorrhizal plants, the likely benefit of AMF in these situations is competition of extra-radical hyphae with other microbes for solubilized organic P (Ibijbijen et al., 1996; Koide and Kabir, 2000).

Localized changes in rhizosphere pH can alter the availability of non-labile P from inorganic P (P_i) sources such as rock phosphate. Li et al. (1991) observed similar pH changes caused by AMF hyphae. This is accomplished as a response to uptake of NH_4^+ (acidification) or NO_3^- (alkalization). The production of chelating compounds by plants and soil microbes (e.g., citrate) can increase solubility of aluminium (Al) and iron (Fe) phosphates thereby increasing the amount of P entering solution, but AMF chelates have not been demonstrated.

Fungal colonization and activity is negatively correlated to soil nutrient (e.g., P) level increase (Amijee et al., 1989; Thomson et al., 1991; Jakobsen, 1999), but this correlation varies with host dependency on mycorrhizae for nutrient acquisition (Table 2. 1). An increase in host dependency requires higher levels of P to depress AMF

Table 2.1. Correlation of AMF colonization suppression with increasing soil phosphorus levels is a host-dependent response.

Plant	P added	% root colonized	Reference
	mg P kg^{-1} soil		
Barley			(Baon et al., 1993)[†]
(*Hordeum vulgare* L.)			
cv. Shannon	0	28	
	10	16	
	20	8	
cv. Galleon	0	20	
	10	21	
	20	9	
	mg P kg^{-1} soil		
Leek	0 (0.23)	16	(Amijee et al., 1989)[‡]
(*Allium porrum)*	150 (0.31)	22	
	300 (0.44)	18	
	450 (0.71)	8	
	600 (1.69)	6	
	750 (2.26)	4	
	mg P l^{-1} solution		
Sunflower	0	5	(Koide and Li, 1990)[§]
(*Helianthus annuus* L.)	0.31	5	
	0.94	15	
	2.8	6	

[†] Genotypic variation in AMF-dependency of barley cultivars Shannon and Galleon resulted in Galleon requiring higher P levels to generate a suppression response.

[‡] Leek is highly dependent on the AM symbiosis for nutrient acquisition. Hence, very high levels of P are required for colonization suppression. The growth medium was a 2:1 soil/sand mix with a basal $NaHCO_3$-extractable P concentration of 0.23 mg P l^{-1} soil solution 28 days after P application. Values in parentheses ( ) are $NaHCO_3$-extractable P concentrations in mg P l^{-1} soil solution 28 days after P application.

[§] Colonization is limited under any condition in which infection cannot promote growth. When available P levels are too low, the extra absorptive surface area provided by fungal hyphae cannot increase P uptake. When available P levels are too high, host root system does not require the symbiosis for P uptake. The growth medium was sand with a basal $NaHCO_3$-extractable P concentration of 0.03 mg P kg^{-1} sand.

colonization (Amijee et al., 1989; Koide and Li, 1990; Baon et al., 1993). The mechanism of colonization suppression is host controlled. In split root experiments, P addition to one half of the root system suppressed AMF colonization in the unfertilized half (Koide and Li, 1990; Thomson et al., 1991). Regulation of mycorrhizae development is related to the availability of host C supplied to the fungus. Continuous uptake and translocation of nutrients to the host when host nutrition levels are above optimal (i.e., beyond the capability of the host to utilize) is a C drain on the host because the fungus requires energy for active uptake and transport and hyphal growth and maintenance (Koide and Li, 1990; Jakobsen, 1999).

2.1.2 Water Stress

Many studies have reported enhanced survival of mycorrhizal plants over non-mycorrhizal plants under water stressed situations (Busse and Ellis, 1985; Hetrick et al., 1987). One may hypothesize a mechanism of direct uptake and translocation of water via the hyphal network similar to the manner of hyphae mediated nutrient uptake. Experimental result does not support this hypothesis (Kothari et al., 1990; George et al., 1992). The decrease of the host plant's hydraulic conductance has been suggested because of observations of increased transpiration rates in mycorrhizal plants (Hardie, 1985; Koide, 1985). However, transpiration rates are related to photosynthesis and respiration. Both processes are related to plant size and nutrition (Kothari et al., 1990).

The likely effect of mycorrhizal colonization on plant drought tolerance is related to nutrient acquisition (Smith and Read, 1997). As the soil dries, nutrients become less available because the tortuosity of the diffusion path increases (Barber, 1984). As mentioned previously, the higher hyphal length density of mycorrhizae decreases the

diffusion distance for nutrients to reach an absorptive surface. Under drought conditions, the contribution of hyphae to nutrient uptake is advantageous to mycorrhizal plants.

2.1.3 Soil Structure

Soil structure determines characteristics such as water inflow rate, biogeochemical cycling processes, erosion resistance, and C storage (Wright and Upadhyaya, 1998; Rillig and Steinberg, 2002). Soil organic matter plays a major role in aggregation, and organic matter accumulation is a function of biotic activity (Oades, 1993; Jastrow, 1996). Mechanistically, the role of fungal hyphae and plant roots in soil aggregation can be viewed as a "sticky-string bag" (Miller and Jastrow, 2000). The hyphae of AMF entangle and enmesh soil particles to form aggregates in a hierarchal fashion with the smaller aggregates held together by stronger forces than the larger aggregates (Miller and Jastrow, 1990; Oades and Waters, 1991).

The glycoprotein glomalin is secreted onto hyphal surfaces in copious amounts (Rillig et al., 2001). In terms of fungal physiology, glomalin is a recalcitrant hydrophobic molecule that enables aerial growth beyond the gas-water interface (Miller and Jastrow, 2000). Its concentration in soil has a strong correlation to water-stability of aggregates (Wright and Upadhyaya, 1998). The hydrophobicity of this molecule may reduce macroaggregate disruption during wetting and drying cycles by retarding water movement into the pores, thereby allowing the non-disruptive escape of displaced gases from the pores (Miller and Jastrow, 2000). Rillig and Steinberg (2002) hypothesized glomalin production is a mechanism of habitat modification by AMF to generate more favourable growth space. They found in chamber systems where the growth medium texture is finer (e.g., microaggregate sized) hyphal growth was reduced while extractable

glomalin increased. The converse was discovered in coarser (e.g., macroaggregate sized) growth medium.

2.1.4 Pest Control

The effects of mycorrhizal colonization on disease incidence and severity caused by plant pathogens depend on variables such as plant nutrition, inoculum level of pathogen versus AMF, and time of AMF colonization, i.e., before or after pathogen challenge (Graham, 1988; Fitter and Garbaye, 1994). Incidence and severity of disease associated with root-infecting fungi and nematodes may be reduced by mycorrhizal colonization. However, mycorrhizae have no effect or have negative effect on shoot diseases (Smith and Read, 1997).

A number of mechanisms to explain suppressed plant yield losses to diseases have been suggested. Increase plant resistance to infection as a function of improved nutrition (Declerck et al., 2002), competition for colonization sites (Fitter and Garbaye, 1994), anti-microbial compounds produced by fungal hyphae (Benhamou et al., 1994), and priming of plant immune system by fungal colonization (Pozo et al., 2002) are the most accepted mechanisms.

2.1.5 Effects of Intensive Agriculture on AMF Communities

2.1.5.1 Tillage

Extra-radical hyphae function as the nutrient absorption and translocation organ of the mycorrhizal association and as inoculum for colonization of new roots. Soil disturbance such as tillage disrupts the physical hyphal network (Jasper et al., 1991; Kabir et al., 1998). A series of growth chamber experiments showed that maize plants

grown in disturbed soil were less colonized by AMF and had lower shoot P and zinc (Zn) concentrations than plants grown in undisturbed, field collected soil (Evans and Miller, 1988; Fairchild and Miller, 1988; Evans and Miller, 1990; Fairchild and Miller, 1990). No effect of soil disturbance was observed for spinach (*Spinacea oleracea*) and canola (*Brassica napus*), two species which are not colonized by AMF (Evans and Miller, 1988). In addition, application of the fungicide benomyl, which disrupts the cytoskeleton of fungal hyphae, to disturbed and undisturbed soils decreased the effect of disturbance by decreasing P translocation by mycorrhizae in undisturbed soils. Fairchild and Miller (1990) also observed no effect of soil disturbance on the P concentration of plants when the availability of P in the soil was high enough to preclude any benefit from mycorrhizae, even though mycorrhizae formation, measured as percentage of root colonized, was greater on plants in undisturbed soil. These experiments suggested that previously non-infected plants or seedlings can benefit from an established AMF hyphal network by tapping into the hyphae for locally limited or unavailable nutrients from distal sources.

2.1.5.2 Crop Rotation

Crop rotations with periods of bare fallow and non-mycorrhizal plants have been known to cause stunting and P and Zn deficiencies in subsequent planting with species highly dependent on mycorrhizae for mineral nutrition such as maize (*Zea mays*), sorghum (*Sorghum bicolour*), sunflower (*Helianthus annuus*), chickpea (*Cicer arietium*), and flax (*Linum usitatissimim*) (Thompson, 1987; Thompson, 1994). These symptoms are related to a decline in mycorrhizal propagules in the soil and the consequent decrease in colonization and nutrient uptake (Thompson, 1994).

The principle of certain hosts being less conducive to the reproduction of particular AMF was demonstrated in a unique situation where growth suppression of tobacco (*Nicotiana tabacum*) was caused by an AMF. The AM fungus *Glomus macrocarpum* causes tobacco stunt disease (An et al., 1993). Tall fescue (*Festuca arundinacea*) in rotation decreased populations of *G. macrocarpum* below detrimental levels to tobacco (An et al., 1993), whereas sorghum-sudangrass increased populations of *G. macrocarpum* to disease levels (Hendrix et al., 1995).

2.1.5.3 Fertilizers

Changes in AMF communities in response to different management practices raises the question of whether those AMF abundant in high nutrient, well-fertilized soils are plant growth promoters or just more aggressive than other AMF at acquiring host C for their own reproduction (Douds and Millner, 1999). Johnson (1993) observed a decline in four fungal species, including *Gigaspora gigantea*, and an increase in dominance by *Glomus intraradices* in soils with a long-term fertilization history. This supported other work that showed *Gi. gigantea* (the genus *Gigaspora* in general) associated more with natural or low-input systems than in conventional agriculture (Douds et al., 1993) and that *G. intraradices* (the genus *Glomus* in general) has a positive response to high nutrient situations (Sylvia and Schenck, 1983). In chamber studies, plants inoculated with the unfertilized AMF community were larger than those inoculated with the fertilized AMF community (Johnson, 1993). Microscopic observations of the mycorrhizae suggested AMF from the fertilized community may have been a greater carbon drain on their host than those from the unfertilized community. The former produced the same proportion of root length with vesicles as those from the unfertilized

community, but a lower proportion of root length with arbuscules, the site of nutrient transfer to the host.

These examples show crop production in the field is often dependent on the indigenous AMF population. However, production practices are not always synergistic with the mycorrhizal symbiosis whether a high-input/conventional or low-input/organic system is practiced. In fact, one could argue that AMF are generally not crucial for nutrition, growth or health of plants in many production agricultural systems because methods that negate the effect of mycorrhizae are practiced (Ryan and Graham, 2002).

Management of inherent biological and ecological cycles to preserve soil resources and maintain economic productivity is the central tenant of organic farming (Atkinson et al., 2002). However, non-standardized organic practices may result in the use of some modern agricultural methods such as continuous monoculture, fallow and non-host crop in rotation, and tillage that have adverse effects on the diversity and activity of AMF. Therefore, describing the community of AMF at a site becomes an important first step in determining the effects of agricultural treatments upon AMF and the eventual development of management regimes for these fungi.

2.2 Current Methods used to Study AMF Communities

A complete description of an AMF community would entail the identification and quantification of all fungi present as spores, extra-radical hyphae, and intra-radical hyphae (Douds and Millner, 1999). One may appreciate this to be an exhaustive proposition. Classical techniques for AMF identification are based on spore morphology. This approach is time consuming and subjective, but the systematics are well understood and easy to teach – though difficult to master. Recent importation of molecular

techniques such as the polymerase chain reaction has begun to expedite our ability to characterize field AMF communities. However, molecular techniques have limitations.

2.2.1 Cultivation and Morphological/Phenotypic Methods

Spore characteristics, especially spore wall structure and morphology, and ontological changes are the most important criteria for AMF identification (Franke and Morton, 1994; Morton and Bentivenga, 1994). Talukdar (1993) presented a concise treatise on the topic. An illustrated hard copy (Schenck and Perez, 1990) and online electronic taxonomic guides/keys (http://invam.caf.wvu.edu/) explain and apply the systematics. Approximately 150 species are described to date. Undoubtedly the majority of AMF species remains undescribed (Bever et al., 2001). For example, The International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) maintains approximately 40 isolates that do not belong to any of the currently described species.

The procedure for AMF spore extraction from field soils for identification involves several steps: *i*) collection of representative soil samples – majority of AMF species inhabit the top 20 cm of a soil profile but some species have been shown to be more abundant deeper in the soil profile (An et al., 1990; Douds et al., 1995); *ii*) separation of spores from soil by wet sieving and decanting followed by sucrose density gradient centrifugation (Clapp et al., 1996); *iii*) separation of extracted spore mixture into similar looking groups by observation under a stereomicroscope; *iv*) confirmation of spore group identity by detailed observation of permanently mounted spore samples under a light microscope.

In field samples, low spore number, parasitization of spores, and age and environmental alteration of spores (e.g., discolouration) will hinder accurate identification (Bever et al., 2001). Hence, trap cultivation in a greenhouse, i.e., propagation of field AMF on a host plant in a controlled environment, is often practiced to increase spore numbers. In this approach, spores of some species detected in the original inoculum may not be detected or some species undetected in the original inoculum may be detected because of unknown stimulatory or inhibitory cultivation conditions (Talukdar, 1993; Bever et al., 2001). To understand the difficulties associated with this methodology, the factors affecting AMF sporulation must be discussed.

2.2.1.1 Host Specificity, Seasonality, and Abiotic Affects on Sporulation

Arbuscular mycorrhizal fungi are considered to have low specificities of association with host species, but this conclusion is based mostly on experiments in which individual isolates of fungal species are grown separately, apart from competitive interactions (Bever et al., 2001). When fungi are examined as a community, evidence suggests fungal growth rates are highly host specific. In an experiment in which AMF were trapped on different plant hosts, isolates of different fungal species sporulated differentially, with the relative dominance of fungal species being reversed, depending on the plant species with which they were associated (Bever et al., 1996). As this pattern of host specificity of growth rates in this "non-specific" association has been observed in other systems, including tallgrass prairie (Johnson et al., 1992), California grasslands (Nelson and Allen, 1993), chalk grasslands (Sanders and Fitter, 1992), and agricultural fields (Douds and Millner, 1999), this appears to be a general property of this interaction.

This specificity of fungal response could contribute to the maintenance of diversity within the AMF community (Bever et al., 2001).

Schultz et al. (1999) found evidence that fungal spore density differs seasonally, with some fungi sporulating in late spring and others sporulating at the end of summer. As the spores represent the dormant state of the fungus, the physiologically active state is most likely the mirror image of the seasonal spore counts. For example, *Gi. gigantea*, which sporulates most abundantly in the fall and appears to overwinter as spores, is likely to be physiologically active during the warm season. Similar patterns have been seen for *Gi. gigantea* in a sand dune on the coast of Rhode Island (Gemma et al., 1989; Lee and Koske, 1994). Alternatively, *Acaulospora colossica*, which sporulates most profusely at the beginning of summer and oversummers as spores, is physiologically active with the cool season plant community (e.g., wild garlic [*Allium vineale*]).

Arbuscular mycorrhizal fungi activity is correlated to soil mineral nutrient levels, especially with soil P levels. The same correlation was observed for sporulation (Johnson et al., 1992). Because nutrient levels are spatially and temporally heterogeneous in any given field, sampling strategies must account for this in order to be representative. Other soil factors such as pH have a marked effect on AMF distribution and abundance. Some AMF are restricted to either acid or alkaline soils, while others are found in both (Porter et al., 1987).

2.2.1.2 Difficulties Associated with Trap Cultivation of AMF Spores

How does one elucidate an accurate description of a field AMF community with a cultivation and morphology-based approach given the factors influencing sporulation variability? The exhaustive work of Bever and Morton's group (Bever et al., 1996; Bever

et al., 2001) in a 1 ha field at Duke University is the best example of the effort needed. Their initial assessment of field samples found 11 AMF species. By trap cultivation, over the course of the next five years, they found a total of 37 species (although 31 were found after two years). For them, it seemed with each variant on the sampling methodology, whether it was greenhouse conditions of the trap cultures, species of plant host used in the traps, treatment of soil prior to trapping, or season of sampling field soil, additional fungal species were revealed (Bever et al., 1996). The distinct conditions favourable to successful growth and sporulation of each species reflect differences in fungal ecologies. From this brief description of the process, the time and effort required to fully describe a field AMF community can be appreciated. However, there are undoubtedly additional fungal species present at the site for which they have not yet adequately met growth and sporulation requirements (Bever et al., 2001).

2.2.1.3 Other Phenotypic Methods

Obviously spore morphology based AMF community analysis has limitations because not all AMF will have sporulated during the time of direct assessment or under trap cultivation. Because spores represent the dormant stage of the fungal life cycle, how is the active AMF population, i.e. those species colonizing host roots in functional mycorrhizae, assessed? The methods of most probable number (MPN) and percent of root colonized (e.g., grid-line intersect method) have been used to quantify fungal population and activity, respectively. However, MPN depends on cultivation and percentage of root colonized is weakly correlated to fungal activity (Kabir et al., 1998). Automated methods examining a sample's fatty acid content (especially for the fungi-specific fatty acid ergosterol) such as phospholipid fatty acid (PLFA) profiling and fatty

acid methyl ester (FAME) profiling provide information on functional diversity and avoid the need for cultivation. These biochemical methods do not, however, enable fine-scale analysis of diversity that is required to determine the importance of species diversity, and of a particular species, on ecosystem function (Prosser, 2002).

2.2.2 Molecular/Genotypic Methods

An alternative approach that avoids the limitations of cultivation and morphology is the direct analysis of fungal nucleotide sequences within the soil. For taxonomic purposes, most applications have analyzed the genes encoding ribosomal RNA (rRNA) (van Tuinen et al., 1998a; Kowalchuk et al., 2002). Ribosomal RNA genes are ideal for this application because regions of conservation and variability facilitate sequence alignment and comparison, respectively. Available models for base substitution rates in rRNA genes allow for phylogenetic analysis of individuals and groups within and between populations. Regions of conservation are useful for primer or probe design to amplify copies of the desired gene for analysis (e.g., polymerase chain reaction) (Schussler et al, 2001a) or for *in situ* detection (e.g., fluorescence *in situ* hybridization) (Kuhn et al., 2001).

2.2.2.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) enables researchers to amplify small quantities of the targeted nucleic acid from crude extracts (van Tuinen et al., 1998b; Schwarzott and Schussler, 2001). Thus, there is potential for this technique to be used in biodiversity assessments of soil. However, there are two technical challenges that need to be addressed in the development of PCR based techniques to assess AMF diversity.

Firstly, amplification from soil, spore and rhizosphere samples is hampered because of inhibitory substances co-extracted from soils, spores and rhizosphere (van Tuinen et al., 1998b). Sample purification by polyvinyl polypropylene (PVPP) (Berthelet et al., 1996), hexadecyltrimethyl ammonium bromide (CTAB) (Zeze et al., 1998), or Glass Milk and dilution of samples (Schwarzott and Schussler, 2001) are techniques to decrease the level of inhibitors in a sample. Secondly, during amplification using PCR, one template may be preferentially annealed to, and amplified, by PCR primers because of that template's favourable secondary structure (Innis and Gelfand, 1999). The use of BSA (bovine serum albumin) or gelatin helps to prevent excessive secondary structure formation during the annealing phase of the PCR.

Different PCR strategies are used to gather information on various community parameters (e.g., composition and activity). A nested PCR strategy, where the first reaction enriches a sample's general fungal DNA content followed by a second (nested) reaction to target AMF templates in particular, is useful in situations where the target DNA concentration is relatively low within the general population (van Tuinen et al., 1998a). The gene targeted for analysis can provide different levels of taxonomic resolution. The genes of the rRNA operon can provide consistent distinction at the genus level (18S) and species level (ITS or 25S) (Simon et al., 1992; Sanders et al., 1995; van Tuinen et al., 1998a), and short sequence repeats (microsatellites) and randomly amplified polymorphic DNA (RAPD) can resolve intra-species variations (Wyss and Bonfante, 1993; Vandenkoonhuyse et al., 2001).

Researchers use different primer designs to achieve different goals. When investigating highly conserved genes for community and phylogenetic analysis, specific

primers are used (Morton and Redecker, 2001; Schussler et al., 2001a). Degenerate primers may be designed to amplify functionally related but genetically non-conserved genes for functional diversity analysis. Short, non-specific primers, such as those used in RAPD analysis, are useful for looking for markers or length polymorphic sequences for identifying strains and groups within a community (Wyss and Bonfante, 1993).

2.2.2.2 Analysis of PCR Amplified Product

Once amplified, there exists a variety of complimentary techniques to separate out the mixture of amplified sequences based on denaturing characteristics of sequences' nucleotide composition, fragment length polymorphism analysis, and cloning approaches (Sanders et al., 1995; Speksnijder et al., 2001; Kowalchuk et al., 2002). Cloning of PCR amplified rRNA gene fragments and sequencing the clones from a library is the most often used method. Sequences obtained can be compared and submitted to an ever-growing online database for taxonomic determination of individual cloned fragment. The sequence data can then be used for phylogenetic analysis to determine evolutionary relationships using freeware computer programs such as PHYLIP (Felsenstein, 1989; Felsenstein, 1997). In addition, sequences may be used to refine primers design to target a range of taxonomic groups – from genus level (for community studies) to individual isolates (for monitoring inoculum released). Cloning and sequencing is limited to studies of individual spores or monocultures because of its relative high cost and the potential large number of clones to screen for soil samples. With this approach, Schwartzott, Schussler, and Walker's group (Schussler et al., 2001a; Schussler et al., 2001b; Schwarzott and Schussler, 2001; Schwarzott et al., 2001) investigated and developed the current phylogenetic taxonomy for AMF that allowed the reclassification of AMF from

its traditional placement within the non-monophyletic phylum, Zygomycota, and into their own monophyletic phylum, Glomeromycota.

Analysis by denaturing characteristics of nucleotide composition and fragment length polymorphism are known as 'fingerprinting' techniques because of the characteristic banding patterns generated from electrophoresis of gene fragments. Denaturing gradient gel electrophoresis (DGGE) was first used for studying environmental bacterial communities in 1993 (Muyzer et al., 1993) and fungal communities in 1997 (Kowalchuk et al., 1997). This technique separates PCR products of same size but different sequences by chemical denaturation. Temperature gradient gel electrophoresis (TGGE) is a similar technique but it is based on temperature denaturation characteristics of the DNA fragment. Theoretically, each band within a DGGE/TGGE profile is representative of a related group of organisms, and if primer specificity is high enough, each band may represent a species or even an isolate (Kowalchuk et al., 2002).

A number of fingerprinting techniques involve restriction analysis of PCR products. These include terminal restriction fragment length polymorphism (T-RFLP) (Sanders et al., 1995) and amplified ribosomal DNA restriction analysis (ARDRA) (Helgason et al., 1998). These approaches are premised on the theory that individuals or unrelated groups of organisms will have a different number and location of restriction sites. Whole community scale analysis is achievable because computer programs such as Bionumerics® can analyse banding patterns like bar codes to monitor changes in a community after different treatments or compare communities at different sites (Terefework et al., 2001). For these fingerprinting techniques, higher resolution, such as

identification of individuals and species, is possible by excising bands from the gels for sequencing (Helgason et al., 1998).

Two inherent problems confound the use of PCR for quantitative analysis: *i*) the plateau of product accumulation and *ii*) variable reaction efficiency in different samples (Williams and Tucker, 1999). Competitive PCR and real-time/kinetic PCR are two strategies developed to overcome these problems (Edwards et al., 2002; Landeweert et al., 2003). In competitive PCR, a normalizing spike is added into the reaction with the sample. The spike and the target gene must compete for amplification substrates equally. Thus, a known amount of the spike in the reaction serves to normalize the quantity of the target gene. The theory behind real-time PCR is described in detail elsewhere (Gibson, 1996; Heid, 1996).

2.3 Current State of AMF Taxonomy

The taxonomy of the kingdom Fungi is in a state of flux. Traditionally, major groups of fungi and the relationship between taxonomic groups have been based on comparative morphology, biochemical analysis and the developmental patterns of the sexual reproductive structures (Deacon, 1997). Today, these relationships are being reassessed by nucleic acid sequence analysis, with special emphasis on the nuclear DNA encoding the 18S rRNA gene. Information is patchy because certain groups have received more attention than others. The two best characterized phyla, Ascomycota and Basidiomycota, share a common ancestor, i.e., these phyla are monophyletic (van de Peer et al., 2000). Other major phylogenetic groupings, including the Zygomycota, are not yet clearly defined.

The phylum Zygomycota is polyphyletic; therefore, it is not accurate to describe it in a phylogenetic classification (Tehler et al., 2000; O'Donnell et al., 2001). Even its existence from a morphological point of view is questionable because the majority of the organisms assigned to it are not known to have a sexual stage, i.e., members of this phylum lack fusion of gametangia followed by the subsequent formation of zygosporangia (Benny, 1995; Deacon, 1997). The inclusion of superficially similar morphological entities in the zygomycetes results in an unsustainable artificial taxonomic group.

Morphologically, the nearest similar group of fungi to the AMF with known sexuality belongs to the genus *Endogone*, and by analogy the AMF were placed with the *Endogone* in a single family, the Endogonaceae. This was a tenuous grouping because other members of *Endogone* all produce zygosporangia, whereas the AMF species do not. A comprehensive review of the group was carried out (Gerdemann and Trappe, 1974) during which two new genera (*Acaulospora* and *Gigaspora*) were erected within the Endogonaceae. Later, a morphological cladistic analysis produced a species tree with a new order, Glomerales, containing two suborders and three families (Morton and Benny, 1990). However, some of the conclusions of this work have been questioned. For example, it was suggested that the largest genus, *Glomus*, is nonmonophyletic and probably reflects several genera or even families (Simon et al., 1993), and recently the monophyly of the AMF has itself been brought into doubt (Morton, 2000).

Based on comprehensive 18S rRNA gene analysis, Schwartzott, Schussler, and Walker's group (Schussler et al., 2001a; Schussler et al., 2001b; Schwarzott and Schussler, 2001; Schwarzott et al., 2001) demonstrated that the AMF are a monophyletic

clade. This clade – group of organisms with a common ancestor – is not related to any zygomycetous group but probably shares common ancestry with the Ascomycota-Basidiomycota clade (Schussler et al., 2001b). Based on this genetic evidence, the AMF was placed into its own phylum – the Glomeromycota (Figure 2.1). The Glomeromycota divides into four main orders: Glomerales (family Glomeraceae), Diversisporales (ined.) (families Gigasporaceae, Acaulosporaceae, and Diversisporaceae [ined.]), Paraglomerales (family Paraglomaceae), and Archaeosporales (families Archaeosporaceae and Geosiphonaceae). Paraglomerales and Archaeosporales are ancestral lineages (Morton and Redecker, 2001; Schussler et al., 2001b; Schwarzott et al., 2001), and, indeed, Paraglomerales is so ancient, it does not cluster with the rest of the Glomeromycota (Figure 2.1). The Glomerales are polyphyletic, as it is understood from Morton and Benny (1990). Particularly, the sub-clade represented by *Glomus versiforme* needs removal from the Glomerales given the molecular evidence (Schwarzott et al., 2001). Though sequence data supports the inclusion of the *G. versiforme* clade in the Diversisporales (ined.), its disparate morphological characteristics make its inclusion in Diversisporales tenuous without further evidence (Schussler et al., 2001a; Schussler et al., 2001b; Schwarzott et al., 2001).

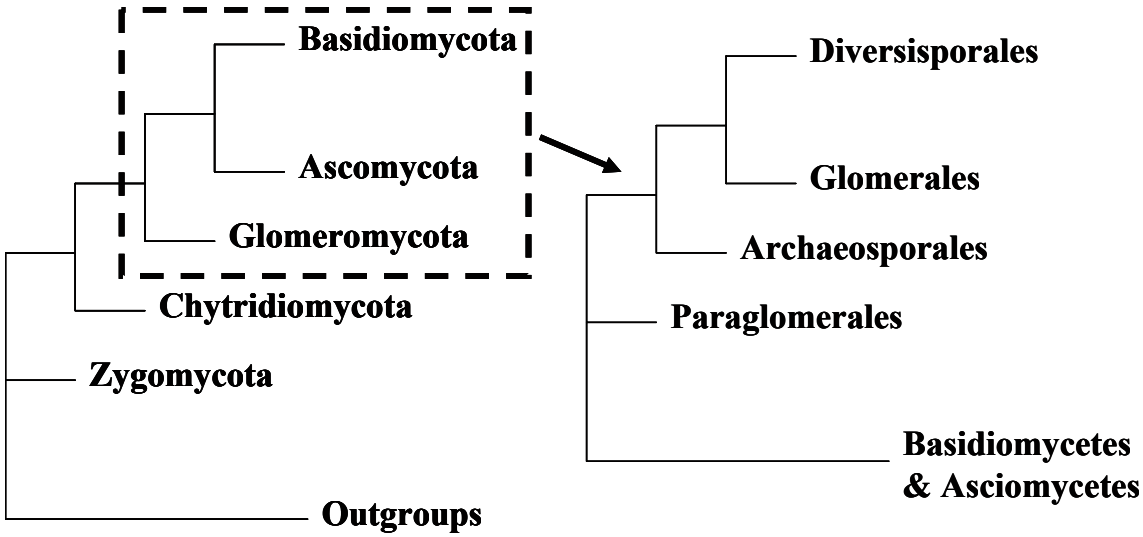

Figure 2.1. Current phylogenetic tree of the Fungi (left) and the phylum Glomeromycota (right) based on sequence analysis of 18S rRNA gene (adapted from Schussler et al. 2001b).

2.4 Ecological Significance of AMF Diversity: Inter- and Intra-Species Variations

The number of AMF species in a field community provides only a cursory look at the true level of ecological diversity present at a site. Within populations of a fungal species, or a single spore of the species, there is evidence of abundant genetic variation, in spite of the asexual nature of these organisms (Sanders et al., 1995; Clapp et al., 1999; Jansa et al., 2002b). Sanders et al. (1995) found a different ITS sequence in each of 10 morphologically identical *Glomus mosseae* spores. Clapp et al. (1999) and Jansa et al. (2002a) found several distinct 18S and ITS sequences within a single spore of a *Scutellospora* sp. and *Glomus intraradices*, respectively. It is difficult to draw direct links to the genetics of AMF from the study of ribosomal genes because of their multicopy nature and possibly different evolutionary processes from other genes (Jansa et al., 2002b). However, it became clear that the genetic heterogeneity of nuclei within AMF spores exists also for single copy locus such as the gene encoding the BiP protein (Kuhn et al., 2001). It is not clear whether intra-individual genetic diversity of nuclei in the AMF plays a role in their physiology and ecological tolerance. The observed diversity could be evidence of an adaptive mechanism that allows symbiosis with different plants in a whole range of environments (van der Heijden et al., 1998; Kuhn et al., 2001; Jansa et al., 2002a; Jansa et al., 2002b).

3 MATERIALS AND METHODS

3.1 Reference AMF Isolates

Seven reference AMF isolates were used for the development of the assay (Table 3.1). Species represented by these isolates were putatively identified in a previous survey of farm field soils in Saskatchewan (Talukdar and Germida, 1993). Five of the isolates were procured from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, http://invam.caf.wvu.edu/). Approximately 150 g of each culture (containing soil, infected roots, and spores) was received and kept refrigerated at 4˚C.

Two other isolates were from regenerated pot cultures from the University of Saskatchewan's Soil Microbiology Laboratory. Briefly, 1.5 kg of each culture, consisted of soil and roots, from 1998 (stored at 4˚C) was planted with corn (*Zea mays* var. Golden Bantam) that was surface sterilized for 3 min in 10% (v/v) Javex® bleach and rinsed in autoclave sterilized distilled water five times (Jarstfer and Sylvia, 2002). Corn was planted in 2 kg pots on June 10, 2002 and harvested three months later. After harvest, the potting soil was placed in plastic bags and stored at 4˚C. All reference cultures were separated into soil (containing soil, infected roots, and spores), root, and extracted spore samples prior to use in DNA extraction. Spore samples were collected by wet sieving and sucrose density centrifugation of 5.0 g soil aliquots (Clapp et al., 1996). Root samples were collected during the wet sieving step of spore collection.

Table 3.1. Reference arbuscular mycorrhizal fungi species used as controls.

INVAM accession no.	Species	Source
AU102	*Gigaspora decipiens*	INVAM
UT316	*Glomus etunicatum*	INVAM
SA101[†]	*Glomus luteum*	INVAM
WY110	*Glomus mosseae*	INVAM
IT104	*Glomus versiforme*	INVAM
NT4[†‡]	*Glomus luteum*	University of Saskatchewan, Soil Microbiology Lab Culture Collection
NT7[‡]	*Glomus versiforme*	University of Saskatchewan, Soil Microbiology Lab Culture Collection

[†] *Glomus luteum* SA101 and *Glomus luteum* NT4 are the same isolate. *G. luteum* NT4 was the voucher specimen submitted by Talukdar and Germida (1993) to INVAM for classification and archive. It was given the INVAM accession no. SA101 and has been maintained in successive cultures for research use since submission. It was originally classified as *Glomus clarum* based on spore morphology (Talukdar and Germida, 1993) but subsequently reclassified as *Glomus luteum* (Kennedy and Morton, 1999).

[‡] The designations NT4 and NT7 are not INVAM accession numbers. These are the University of Saskatchewan Soil Microbiology Lab Culture Collection's isolate numbers.

3.2 DNA Extraction: Optimized Procedure

In order to obtain amplifiable genomic DNA, a DNA extraction method was developed based on the methods of Griffiths et al. (2000), Kowalchuk et al. (2002), and Schwarzott and Schussler (2002) (Figure 3.1). Selected spores (10 to 40 per isolate) were vortexed at maximum speed two times for 30 s each in 100 µL 1% SDS (w/v), and washed with 100 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediamine tetra-acetic acid [EDTA])between vortex steps. Excess TE buffer was removed after the final wash. Three cycles of crush/freeze/thaw were performed on the spores using a flame sterilized glass micropestle and liquid N_2. Raw lysates were suspended in 60 µL TE buffer. An equal volume of phenol:chloroform:isoamylalcohol (24:24:1) was added to each lysate and vortexed twice for 30 s at maximum speed. The tubes were centrifuged for 3 min at 3000X *g*. Aqueous layers (containing the extracted DNA) were removed, placed in new tubes, and kept on ice. Another equal volume of TE was added to the raw mixture of lysate/phenol:chloroform:isoamylalcohol, and the extraction procedure repeated to increase yield. To remove phenol in the collected aqueous phase, an equal volume of chloroform:isoamylalcohol (24:1) was added to the collected aqueous phase and the tube was inverted gently for 10 s. The aqueous phase was placed in a new tube with two volumes of precipitating solution with 30% (w/v) polyethylene glycol 4000 and 1.6 M NaCl and incubated at room temperature for two hours to precipitate the DNA. Precipitated DNA was spun at 14 000X *g* for 10 min to pellet. The supernatant was removed and the pellet washed with 100 µL -20˚C 70% (v/v) ethanol. Ethanol was.

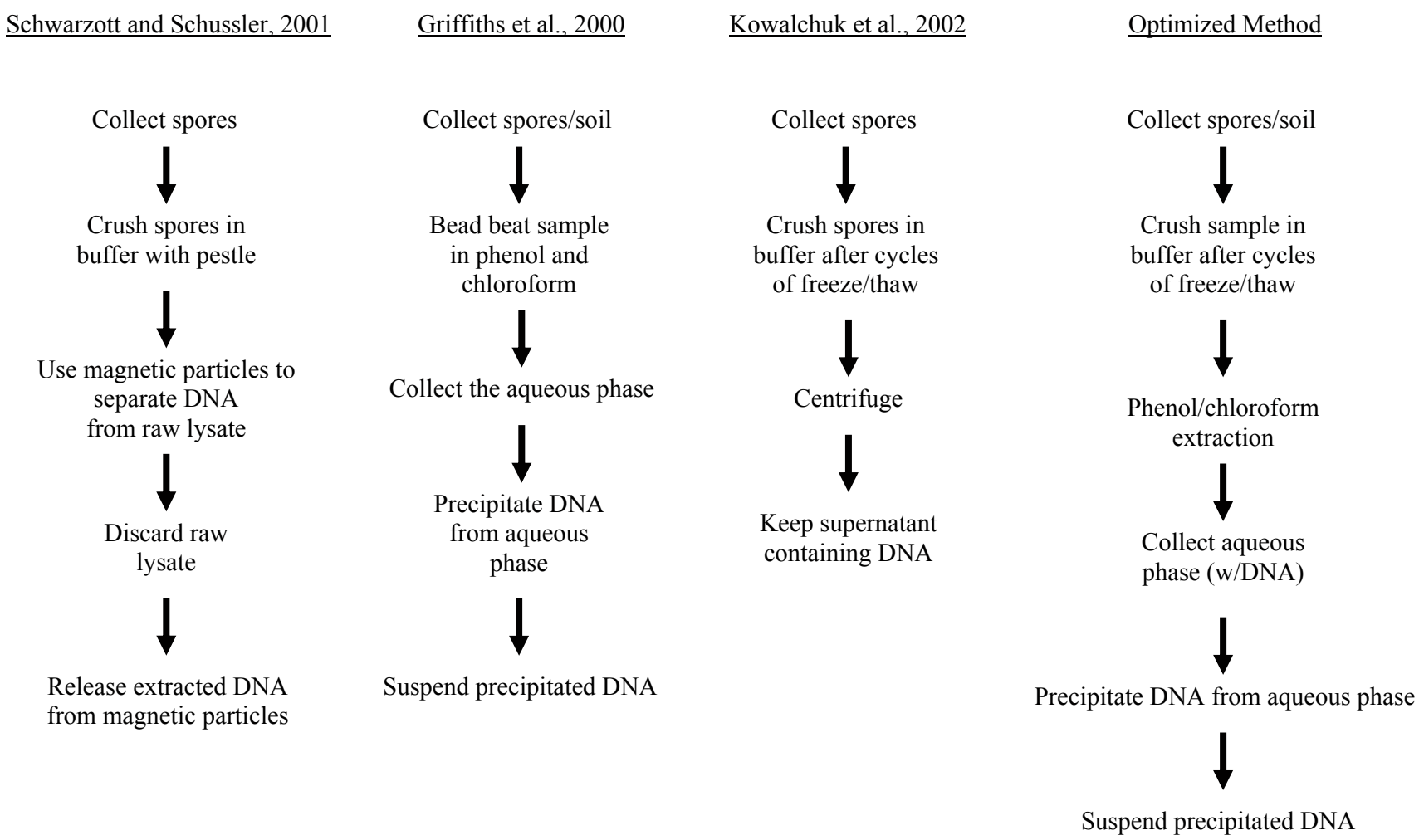

Figure 3.1. Flow charts of DNA extraction procedures attempted and used develop the optimized extraction procedure to extract amplifiable AMF DNA from spores and soil.

drained and the pellet allowed to air dry for 10 min. Finally, the pellet was suspended in 30 µL autoclaved distilled and deionized water (ddH_2O). DNA extraction from soil followed these steps except a 0.5 g sample was crushed in 750µL TE (in three 250 µL aliquots) using a flame sterilized mortar and pestle during the preparation of the raw lysate.

3.3 Nested PCR Strategy and Conditions

The extracted DNA was subjected to a first PCR using primers (0.5 µM each) GeoA2 (5'CCAGTAGTCATATGCTTGTCTC3') and Geo11 (5'ACCTTGTTACGACTTTTACTTCC3') to amplify an approximately 1.8 kb fragment of the 18S rRNA gene (Schwarzott and Schussler, 2001). The first PCR primers are universal fungal primers. All primers were made by Sigma/Genosys (Oakville, Canada). PCR was done in 20 µL volume with 2.0 µL template DNA (~10 ng μL^{-1}) using the *Taq* PCR Master Mix system (Qiagen; Hilden, Germany) with the manufacture's recommended buffer, enzyme, and nucleotide conditions (1X Qiagen PCR buffer contains 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase, and 200 µM of each dNTP). Product was amplified on a Robocycler Gradient 96 (Stratagene; California, USA) using the following conditions: 94°C for 2 min; 30 x (94°C, 30 s; 59°C, 60 s; 72°C, 2.5 min.); 72°C, 10 min. PCR product was analyzed by agarose gel electrophoresis (1.0% (w/v) agarose; 100V, 20 to 30 min.) and ethidium bromide staining (Kowalchuk et al., 2002).

First stage PCR product with a visible band was diluted 1:100 (PCR product without a visible band was undiluted) and used as template in subsequent nested PCR using the same reaction mixture described above except for primers. The second stage primers (AM1 (5'GTTTCCCGTAAGGCGCCGAA3') (Helgason et al., 1998) and NS31-

GC (which corresponds to NS31 (5'TTGGAGGGCAAGTCTGGTGCC3') described by Simon et al. (1992) plus a 5' GC clamp (5'CGCCCGGGGCGCGCCCCGGGCGGGGC-GGGGGCACGGGGGTTGGAGGGCAAGTCTGGTGCC3') sequence described by Kowalchuk et al. (1997)) produce an approximately 550 bp fragment. The nested PCR primers are AMF specific. Thermocycling used the following condition: 94°C for 2 min; 30 x (94°C, 30 s; 67°C, 60 s; 72°C, 60 s); 72°C, 10 min. Nested PCR product was analyzed same as described for first PCR.

3.4 DGGE Analysis

Ten micro-litres of PCR product were used for DGGE analysis. Gels contained 4% (w/v) polyacrylamide (37:1 acrylamide/bis-acrylamide) 1x Tris/acetic acid/EDTA (TAE) buffer, and were 1.5 mm thick (20 x 20 cm). The linear gradient used was from 32% to 50% denaturant, where 100% denaturing acrylamide was defined as containing 7 M urea and 40% (v/v) formamide. A 10 mL stacking gel containing no denaturants was added before polymerization was complete (~2 h). All DGGE analysis was run in DCode system (Bio-Rad Laboratories, Hercules, CA, USA) at a constant temperature of 60°C. Electrophoresis was for 10 min at 75V, after which the voltage was lowered to 45 V for an additional 16 h. Gels were stained in 1x TAE containing 4 μL Sybr Green (Sigma/Genosys, Oakville, Canada) per 20 μL TAE and visualized by UV illumination. Gel images were digitally captured by a Nikon CoolPix 4500 digital camera with a Sybr Green filter from the manufacturer.

3.5 Sequence Analysis of Partial 18S rDNA

Prominent DGGE bands were excised from the UV illuminated acrylamide gels, and the DNA was eluded from the excised gel by incubation in 30 µL ddH_2O at 28˚C overnight. The gel-eluted DNA was used as template for PCR amplification (same conditions as described for the nested PCR). The product of this PCR was again analysed by DGGE using a narrower gradient (34% to 46% denaturant) to ensure a single DGGE band was produced. PCR products that produced a single band in this second DGGE gel were purified for sequence analysis using the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) with a final elution volume of 30 µL. The National Research Council – Plant Biotechnology Institute DNA Sequencing Lab (Saskatoon, Canada) performed the sequencing reactions using the primer NS31 (without GC clamp). Similarity comparison of the partial 18S rDNA sequences were performed using the National Centre for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/). Possible chimeric sequences (Speksnijder et al., 2001; Wang and Wang, 1997).were screened for using the Ribosomal Database Project (RDP) online Chimera Check program (http://rdp.cme.msu.edu/html/analyses.html).

3.6 Detection Limit of the Optimized Procedures

3.6.1 Sensitivity

The detection limit of the optimized procedures for INVAM culture *Gigaspora decipiens* AU102 was determined. Zero, two, four, six and eight spores were spiked into each of five 0.5 g soil samples and subjected to the optimized extraction and PCR-DGGE

procedures previously described. The detection limit is expressed as the number of spores required for detection by DGGE per gram of soil sample (sp g^{-1}).

3.6.2 Primer Bias

Equal concentrations (100 ng μl^{-1}) of nested PCR product from *G. luteum* SA101, *G. versiforme* IT104, *Gi. decipiens* AU102, and *G. mosseae* WY110 were used as template for the primer bias test for the primer pair NS31-GC/AM1. A matrix was set up for each of the four species at four different template compositions (1%, 5%, 10%, and 25% for the species tested with the remainder template composition divided equally amongst the other three species) (Table 3.2). The PCR condition was same as the condition of the nested PCR, and PCR product was visualized by DGGE under the conditions described previously.

Table 3.2. A sample matrix used for primer bias determination in *G. luteum* SA101.

Reference species	Reaction 1	Reaction 2	Reaction 3	Reaction 4
	-------- Percent of DNA template in PCR reaction -------			
G. luteum SA101	25	1	5	10
G. versiforme IT104	25	33	31.7	30
Gi. decipiens AU102	25	33	31.7	30
G. mosseae WY110	25	33	31.7	30

3.7 Organic Farm Soil Sampling

Thirty-eight soil samples from organic farm fields were collected across Saskatchewan, Canada, during a weed survey conducted in May, 2002. A 5.0 cm diameter hydraulic soil probe was used to collect the samples. In each field, 16 soil cores were taken to a 45 cm depth. The soil cores were divided into 15 cm depths and bulked

together to form three composite samples of each field. Where the field appeared level and uniform, the sampling was done following a W-pattern (Thomas, 1985). Irregular fields were sampled avoiding irregularities such as depression and knoll regions, roads or paths and ditches, power lines, oil wells, saline areas, etc. Collecting a representative sample was considered to be more important than following a rigid collection regime. The 0 to 15 cm sample from each site was used for DNA extraction. The properties of these samples are listed in Table 3.3.

3.8 Phylogenetic Analysis of Isolated AMF 18S rDNA Sequences

Sequences were aligned with ClustalX (version 1.81) and visualized and edited with GeneDoc (version 2.6). A phylogenetic tree was created using the programs DNADIST (Jukes-Canter model), NEIGHBOR (neighbour-joining method; out-group: *Schizosaccharomyces pombe*), and SEQBOOT available in the PHYLIP (version 3.5c) computer programs package (Felsenstein, 1989; Felsenstein, 1997). The program TreeExplorer was used to visualize the resultant phylogenetic tree. Default settings were used for all analysis, and these programs were freeware obtained from links at http://evolution.genetics.washington.edu/phylip/software.html.

Table 3.3. Some physio-chemical properties of the field soil samples.

Site	Soil Zone	Soil Texture	pH	P (0 to15 cm)	N (0 to15 cm)
				----------kg ha^{-1}----------	
1-1	Dark Brown	loam	7.02	17.72	29.77
1-2	Dark Brown	loam	6.95	23.43	65.48
2-1	Black	sandy clay loam	6.42	10.76	7.93
4-1	Dark Grey	sandy loam	6.55	8.82	0.05
4-2	Dark Grey	sandy loam	6.56	9.93	18.85
6-1	Dark Grey	sandy loam	7.65	7.01	0.39
12-1	Dark Brown	loam	7.68	10.68	15.24
13-1	Black	loam/sandy clay loam	6.91	8.87	36.60
13-2	----------not available----------				
14-1	Dark Grey	loam/sandy clay loam	6.59	10.86	9.85
15-2	Black	silty clay loam	7.31	18.16	3.09
16-1	Black	loam	7.05	7.57	19.39
16-2	Black	loam	6.65	9.28	13.59
19-1	Dark Grey	clay	7.03	14.93	10.07
19-2	Dark Grey	clay	6.84	12.45	5.56
20-2	Dark Grey	clay	7.21	15.68	17.78
21-1	Dark Grey	clay loam	7.66	13.96	66.86
21-2	Dark Grey	clay loam	6.57	10.71	40.32
28-1	Black	loam	7.17	21.63	53.17
32-1	Dark Brown	loam	7.40	24.30	5.17
33-1	Dark Brown	loam	6.55	21.93	6.37
34-1	Grey	loam	6.77	8.23	7.97
34-2	Grey	loam	6.42	6.89	6.62
36-1	Dark Grey	sandy loam	7.72	9.78	4.85
39-1	Dark Brown	sandy loam	6.44	14.36	9.84
39-2	Dark Brown	sandy loam	6.95	8.80	3.14
41-1	Dark Grey	sandy loam	7.70	5.65	5.60
46-2	Black	loam	7.68	9.06	10.18
47-2	Dark Grey	loam	6.53	16.55	29.53
51-2	Brown	loam	7.33	14.96	15.47
55-1	Brown	loam	7.06	19.00	40.94
55-2	Brown	sandy loam	6.27	19.22	39.35
59-1	Dark Brown	loam	7.20	17.53	44.16
60-1	Brown	loam	5.88	16.77	24.26
68-1	Black	sandy loam	7.29	12.06	27.16
70-2	Black	loam	7.27	20.37	50.15
74-1	Grey	loam	7.24	10.14	37.45
74-2	Grey	loam	7.39	7.52	21.13

4 RESULTS

4.1 DNA Extraction from Reference Samples

A number of methods for DNA extraction from spores were tried (see Figure 3.1). However, no consistent PCR amplifiable template was produced by any these procedures. The optimized method developed required the use of a mortar and pestle to consistently extract amplifiable DNA from reference fungal spores. Upon sequential amplification with the GeoA2/Geo11 and NS31-GC/AM1 primer pairs, spore PCR products of the expected size (~1.8 Kbp and ~550 bp, respectively) were observed for all the reference isolates tested (Figure 4.1 and 4.2, respectively).

The DNA extraction method of Griffiths et al. (2000) was able to extract amplifiable template from reference root and soil samples, but the desired AMF 18S rDNA fragments were not produced consistently (Figure 4.3). Except for Lane 2, no corresponding AMF DGGE signature was observed (reference AMF mobility signatures were represented by Lanes 1, 4, and 7 which corresponded to *G. luteum* SA101, *G. versiforme* IT104, and *Gi. decipiens* AU102, respectively). To overcome the inconsistent DNA extraction from soil, a scaled-up version of the 'mortar and pestle' method adapted from the reference spore DNA extraction was used to generate the soil PCR-DGGE profile of Figure 4.4. Except for Lane 11 (reference soil of *Gi. decipiens* AU102 extracted with optimized method without extra spores spike), the optimized DNA methodology produced detectable AMF signatures in all reference soil samples.

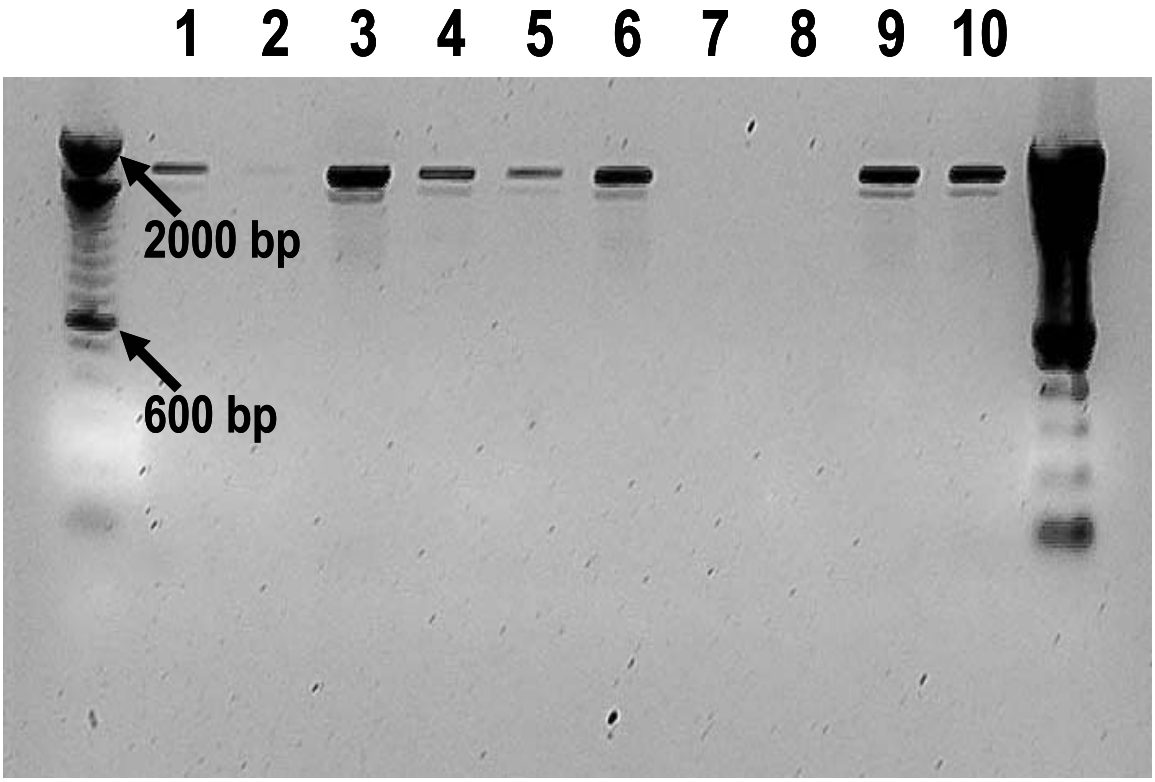

Figure 4.1. Agarose gel showing 18S rRNA gene fragment (~1.8 kbp) amplified from extracted genomic DNA of reference AMF spores using primers GeoA2 and Geo11. Lane 1 – *Glomus luteum* SA101, Lane 2 – *Glomus versiforme* IT104 (a faint band is present but did not reproduce properly on paper), Lane 3 – *Gigaspora decipiens* AU102, Lane 4 – *Glomus mosseae* WY110, Lane 5 - *Glomus etunicatum* UT316, Lane 6 – mixed spores (*G. mosseae* WY110 and *Gi. decipiens* AU102), Lane 7 – extraction blank, Lane 8 – water blank, Lane 9 – *Glomus versiforme* NT7, Lane 10 – *Glomus luteum* NT4. Fragment size determined by 100bp fragment ladder from Invitrogen (Burlington, Canada).

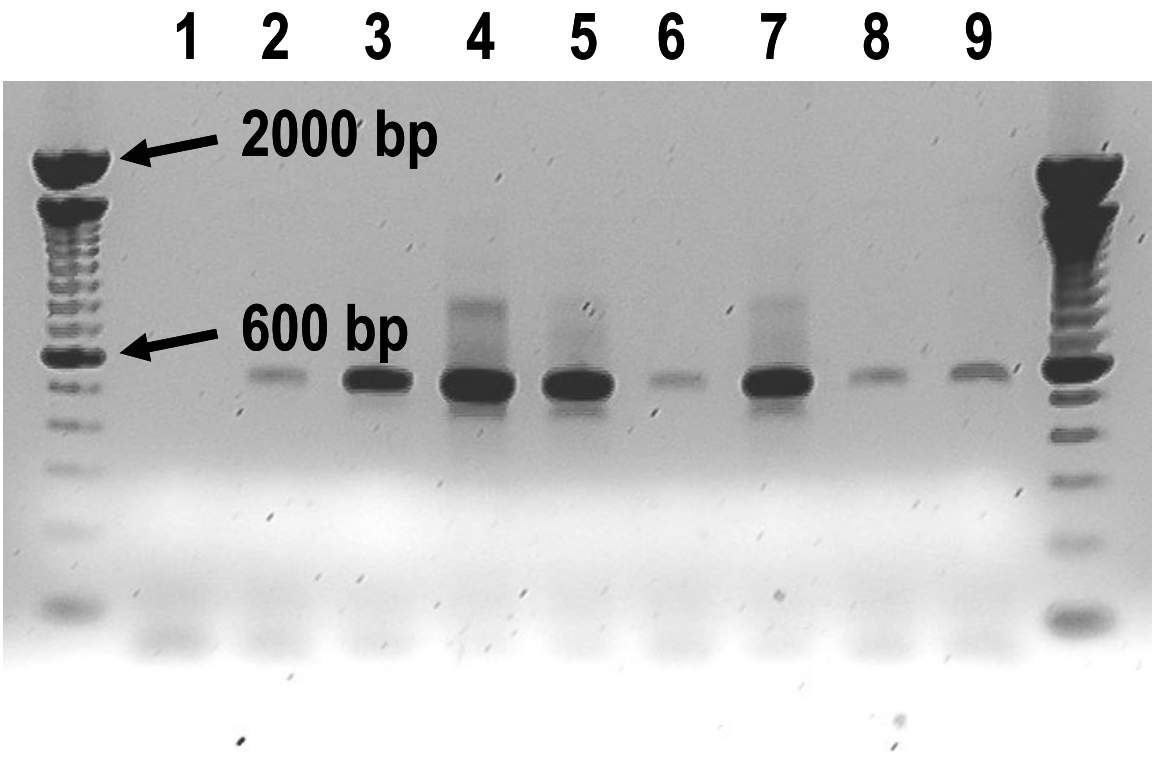

Figure 4.2. Agarose gel showing the 18S rRNA gene fragment (~550 bp) amplified from the first PCR product (Figure 4.1) of reference AMF spores using primers NS31-GC and AM1. Lane 1 – water blank, Lane 2 – *Glomus luteum* SA101, Lane 3 – *Glomus versiforme* IT104, Lane 4 – *Gigaspora decipiens* AU102, Lane 5 – *Glomus mosseae* WY110, Lane 6 – *Glomus etunicatum* UT316, Lane 7 – mixed spores (*G. mosseae* WY110 and *Gi. decipiens* AU102), Lane 8 – *Glomus versiforme* NT7, Lane 9 – *Glomus luteum* NT4. Fragment size determined by 100bp fragment ladder from Invitrogen (Burlington, Canada).

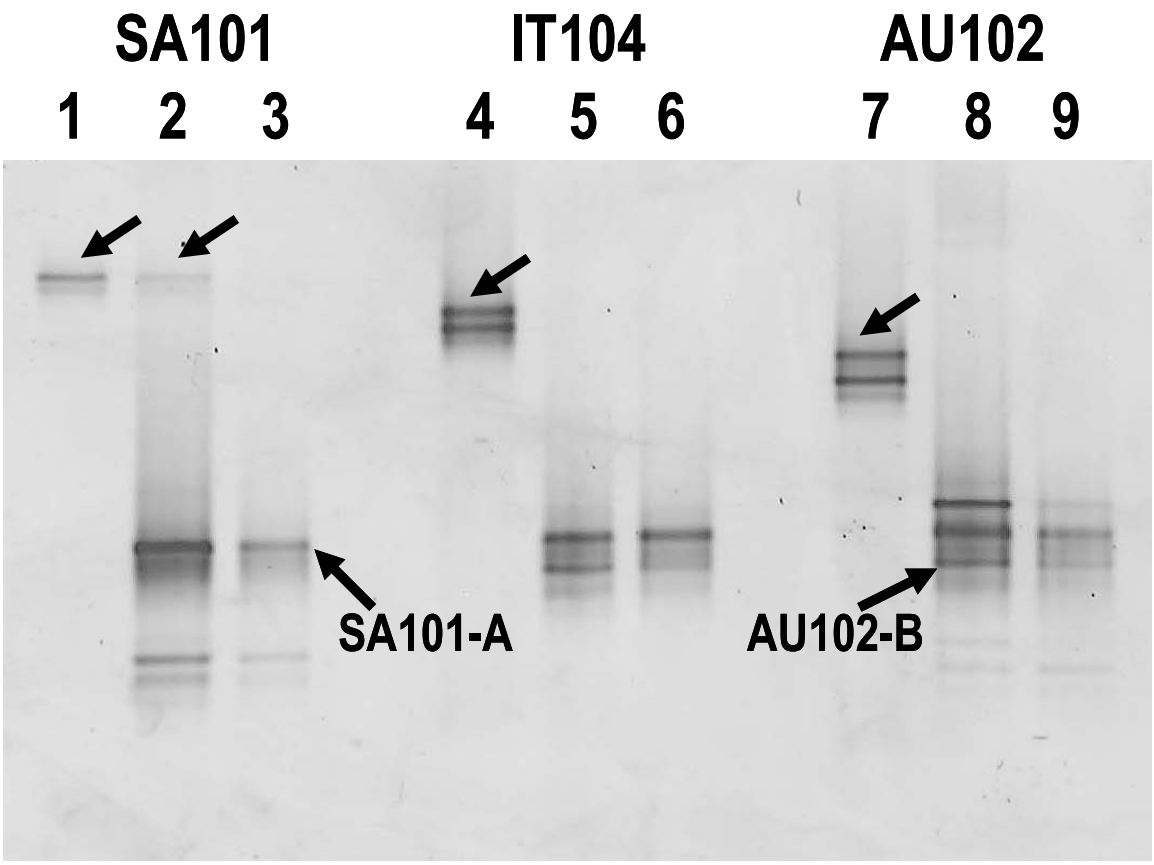

Figure 4.3. Reference AMF 18S rRNA gene fragments from reference root and soil samples were inconsistently detected by PCR-DGGE when a non-optimized DNA extraction method was used. Lanes 1, 4, 7 – reference AMF 18S rRNA gene DGGE signature generated from spore extracted DNA using the optimized procedure as in Figure 4.5 (*G. luteum* SA101, *G. versiforme* IT104, and *Gi. decipiens* AU102 are represented, respectively); Lanes 2, 5, 8 – 18S rRNA gene DGGE profile generated from root extracted DNA using the methods of Griffiths et al. (2000); Lanes 3, 6, 9 – 18S rRNA gene DGGE profile generated from soil extracted DNA using the methods of Griffiths et al. (2000). Arrows indicate bands that were excised and sequenced and identified as the respective reference AMF. SA101-A and AU102-B are sequences of non-AM soil fungi (see Table 4.1).

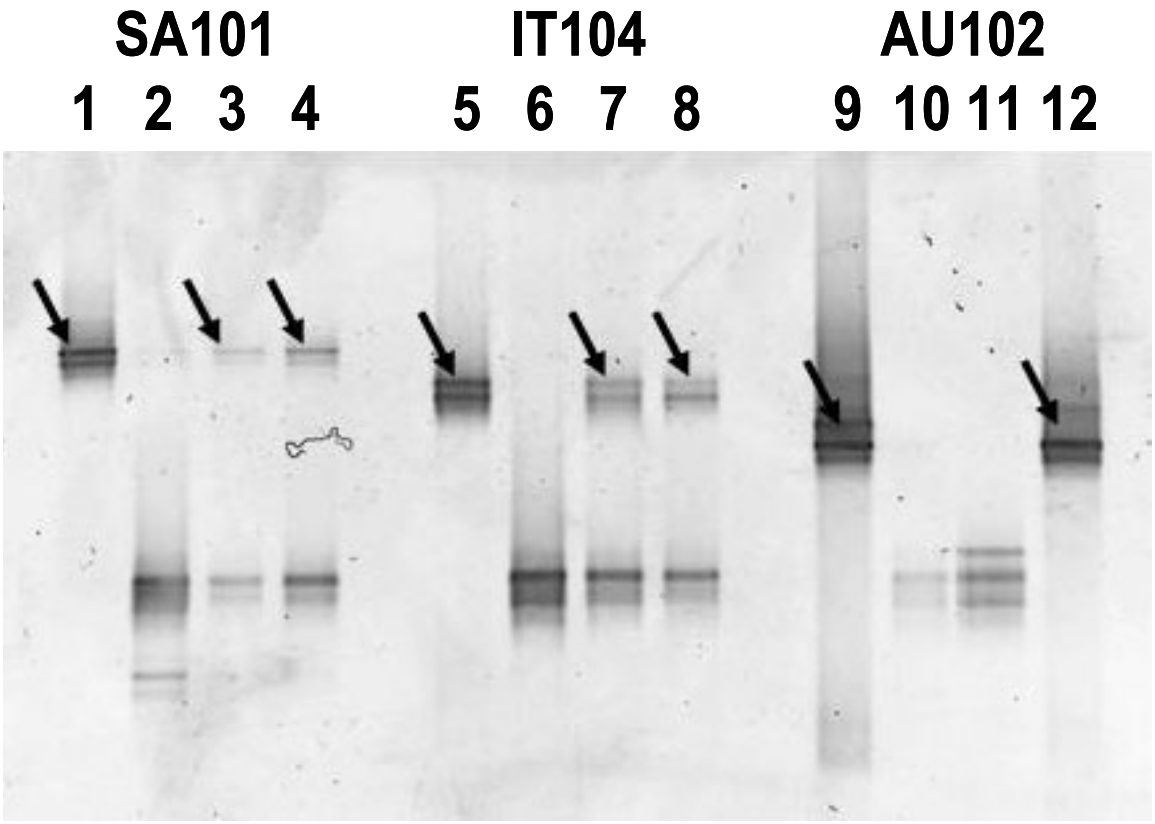

Figure 4.4. Reference AMF 18S rRNA gene fragments from reference soil samples were consistently detected by PCR-DGGE when the optimized DNA extraction method was used. Lanes 1, 5, 9 – reference AMF 18S rRNA gene DGGE signature generated from reference spore extracted DNA using the optimized procedure (*G. luteum* SA101, *G. versiforme* IT104, and *Gi. decipiens* AU102 are represented, respectively); Lanes 2, 6, 10 – 18S rRNA gene DGGE profile generated from reference soil extracted DNA using Griffiths et al.'s (2000) method; Lanes 3, 7, 11 – 18S rRNA gene DGGE profile generated from reference soil extracted DNA using optimized method; Lanes 4, 8, 12 – 18S rRNA gene DGGE profile generated from reference soil extracted DNA using the optimized method with extra spores (*G. luteum* SA101 = 30 spores , *G. versiforme* IT104 = 30 spores, *Gi. decipiens* AU102 = 11 spores) spiked into the soil prior to DNA extraction. Arrows indicate AMF band(s).

4.2 Analysis of Reference Spore Samples' DGGE Bands

Partial 18S rDNA sequences were obtained from the excised DGGE bands labelled in Figure 4.5 for the reference spore samples of *Glomus luteum* SA101, *Glomus versiforme* IT104, *Gigaspora decipiens* AU102, and *Glomus mosseae* WY110. Sequencing of DGGE bands for reference spore samples *Glomus etunicatum* UT316, *Glomus luteum* NT4, and *Glomus versiforme* NT7 was attempted but no usable sequences were obtained. Table 4.1 lists the designation of the sequences recovered and their most related isolate(s) determined by BLAST search of GenBank. BLAST searches yielded a minimum of 98% sequence similarity between INVAM's morphological classification of the reference isolates and the GenBank database.

The DGGE analysis of the NS31-GC/AM1 primed products yielded banding patterns within the range 39 to 40% denaturant under our conditions (Figure 4.5). Isolates *G. luteum* SA101, *G. versiforme* IT104, *Gi. decipiens* AU102, and *G. mosseae* WY110 were distinguishable from each other based upon DGGE mobility. Isolates SA101, *G. etunicatum* UT316, *G. versiforme* NT7, and *G. luteum* NT4 were visually indistinguishable. Isolates *G. versiforme* IT104 and *Gi. decipiens* AU102 produced a distinctive double-band DGGE signature, and, arguably, all reference species produced this doublet feature in the DGGE gel. BLAST results indicated bands IT104-2 and IT104-3 and AU102-4 and AU102-5 were18S rDNA sequences of *G. versiforme* and *Gi. decipiens*, respectively (Table 4.1). Positions of sequence variation between the two fragments for each species are highlighted in grey in Figure 4.6 and 4.7.

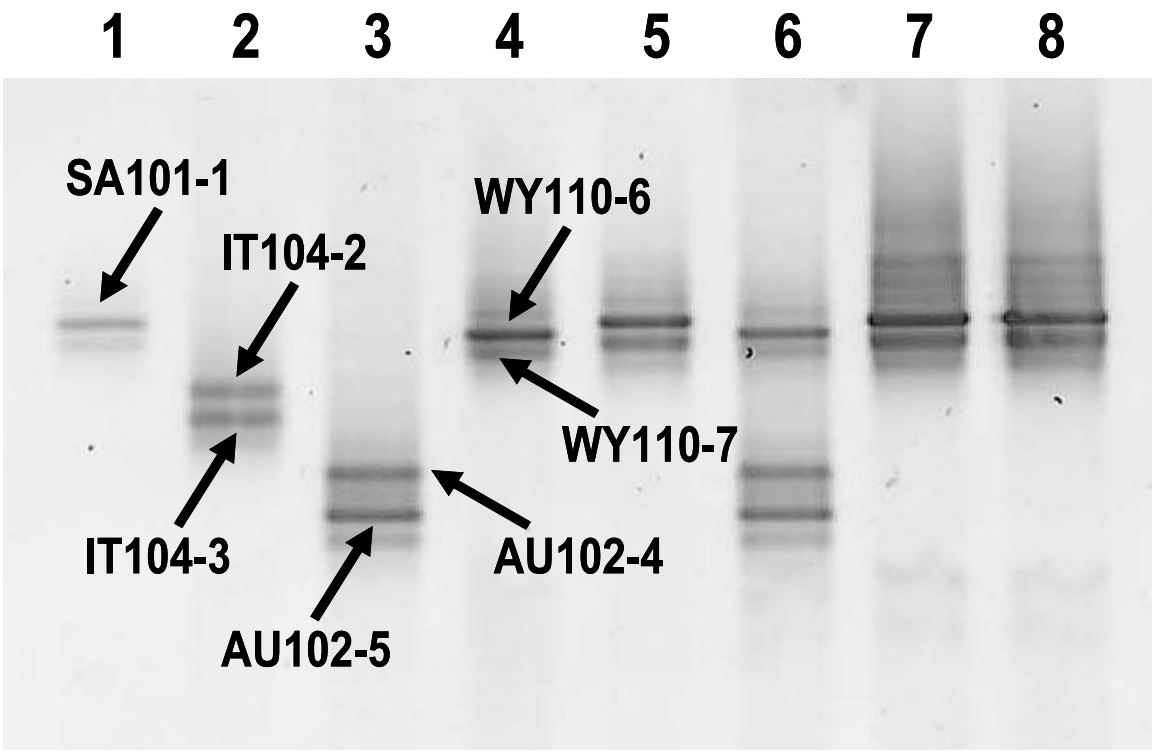

Figure 4.5. The DGGE profile of reference AMF species' partial 18S rRNA gene generated using nested PCR product amplified from reference spores. Lane 1 – *G. luteum* SA101, Lane 2 – *G. versiforme* IT104, Lane 3 – *Gi. decipiens* AU102, Lane 4 – *G. mosseae* WY110, Lane 5 – *G. etunicatum* UT316, Lane 6- mixed spores (of *G. mosseae* WY110 and *Gi. decipiens* AU102), Lane 7 – *G. versiforme* NT7, Lane 8 – *G. luteum* NT4. Each arrow locates a single band that was sequenced. Each band is labelled with the INVAM accession number (e.g. SA101) followed by a sequential designation (e.g., SA101-1 denotes the PCR-DGGE band from INVAM reference culture *G. luteum* SA101, and it was the first band excised from the gel). Sequencing of DGGE bands for reference spore samples *G. etunicatum* UT316, *G. luteum* NT4, and *G. versiforme* NT7 (Lanes 5, 7 and 8, respectively) was attempted but no usable sequences were obtained. Sequencing results for band SA101-1 (Table 4.1) indicated the sequence in this region of the 18S rRNA gene cannot distinguish between *G. luteum* (Lane 1) and *G. etunicatum* (Lane 5). Lanes 1 and 8 are the same isolate (*G. luteum*) cultivated at INVAM and locally, respectively. Lanes 2 and 7 are different isolates (IT104 and NT7, respectively) of the same species (*G. versiforme*).

Table 4.1. Sequences recovered from reference spore and reference soil DGGE bands for confirmation of reference culture identity.

INVAM accession no.	Species classification by INVAM	Sequence designation	Most related isolate(s) from GenBank (% sequence similarity by BLAST)[§]	GenBank accession no. for most related sequences
AU102	*Gigaspora decipiens*	AU102-4[†]	*Gigaspora decipiens* isolate BEG45 (98%)	U96146, GI:2073578
AU102	*Gigaspora decipiens*	AU102-5[†]	*Gigaspora decipiens* isolate BEG45 (99%)	U96146, GI:2073578
SA101	*Glomus luteum*	SA101-1[†]	*Glomus luteum* (99%) *Glomus etunicatum* isolate UT316 (99%)	AJ276089, GI:14270359 Y17639, GI:14275537
WY110	*Glomus mosseae*	WY110-6[†]/ WY110-7[†]	*Glomus mosseae* isolate BEG124 (100%)	AJ505618, GI:22293519
IT104	*Glomus versiforme*	IT104-2[†]	*Glomus versiforme* isolate BEG47 (99%)	X86687, GI:14018352
IT104	*Glomus versiforme*	IT104-3[†]	*Glomus versiforme* isolate BEG47 (100%)	X86687, GI:14018352
N/A	N/A	AU102-B[‡]	*Verticillium psalliotae* strain CBS 639.85 (98%)	AF339610, GI:15022605
N/A	N/A	SA101-A[‡]	*Phialophora verrucosa* (99%)	AJ232945, GI:15865216

[†] Sequence designations are as labelled on Figure 4.5.
[‡] Sequence designations are as labelled on Figure 4.3.
[§] 97% sequence similarity is minimum requirement for identity. Stackebrandt and Goebel (1994) demonstrated that at sequence similarity values below 97%, it is unlikely that two organisms will have more than 70% DNA-DNA reassociation after complete denaturation (the standard for species identity), and, hence, they are related at no more than the species level.

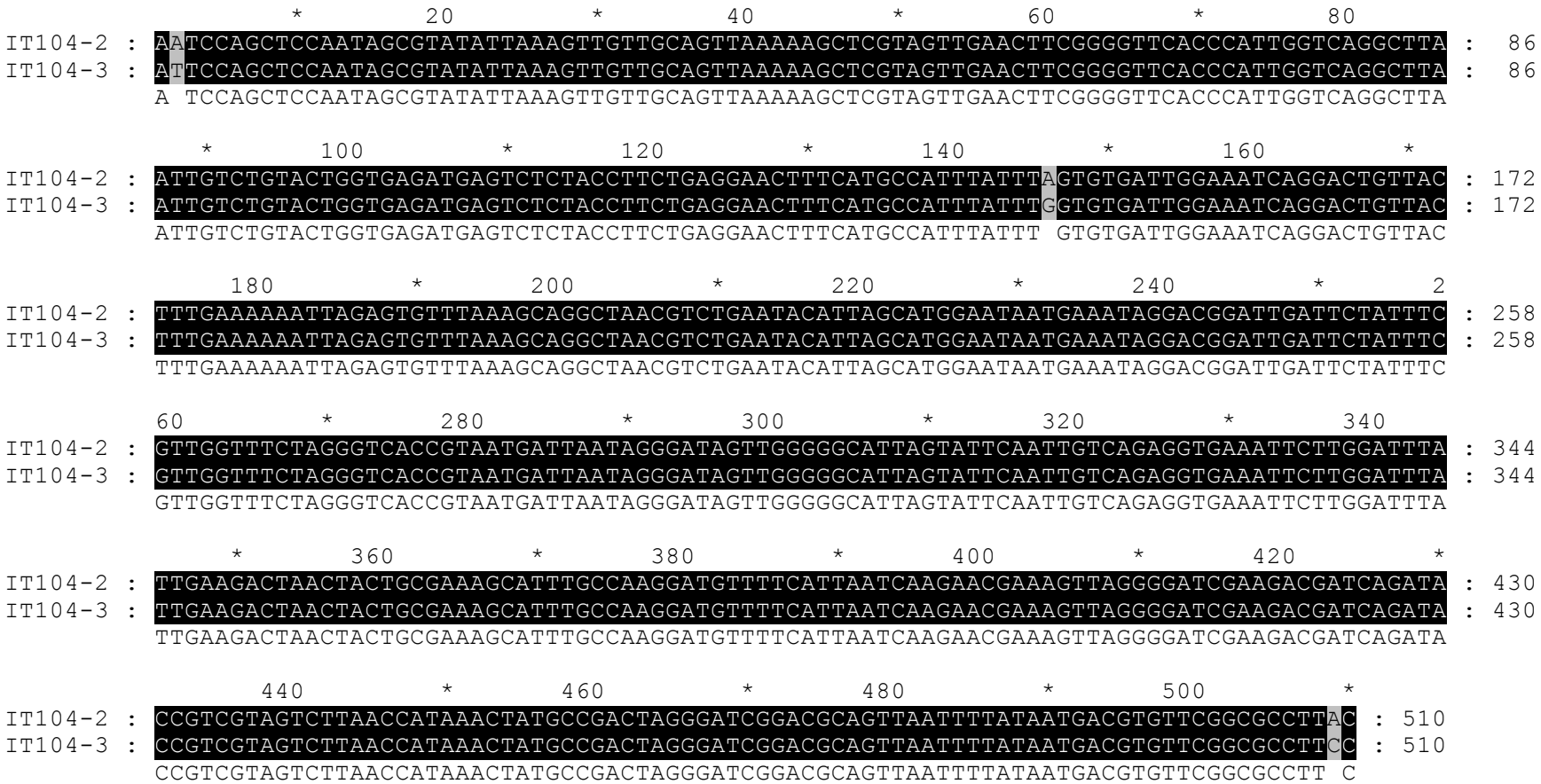

Figure 4.6. Partial 18S rDNA sequences recovered for *G. versiforme* IT104. Both sequences were identified as 18S rDNA of *G. versiforme.* Sequence designations are as labelled on Figure 4.5. Regions of consensus are highlighted in black. Positions of substitution are highlighted in grey.

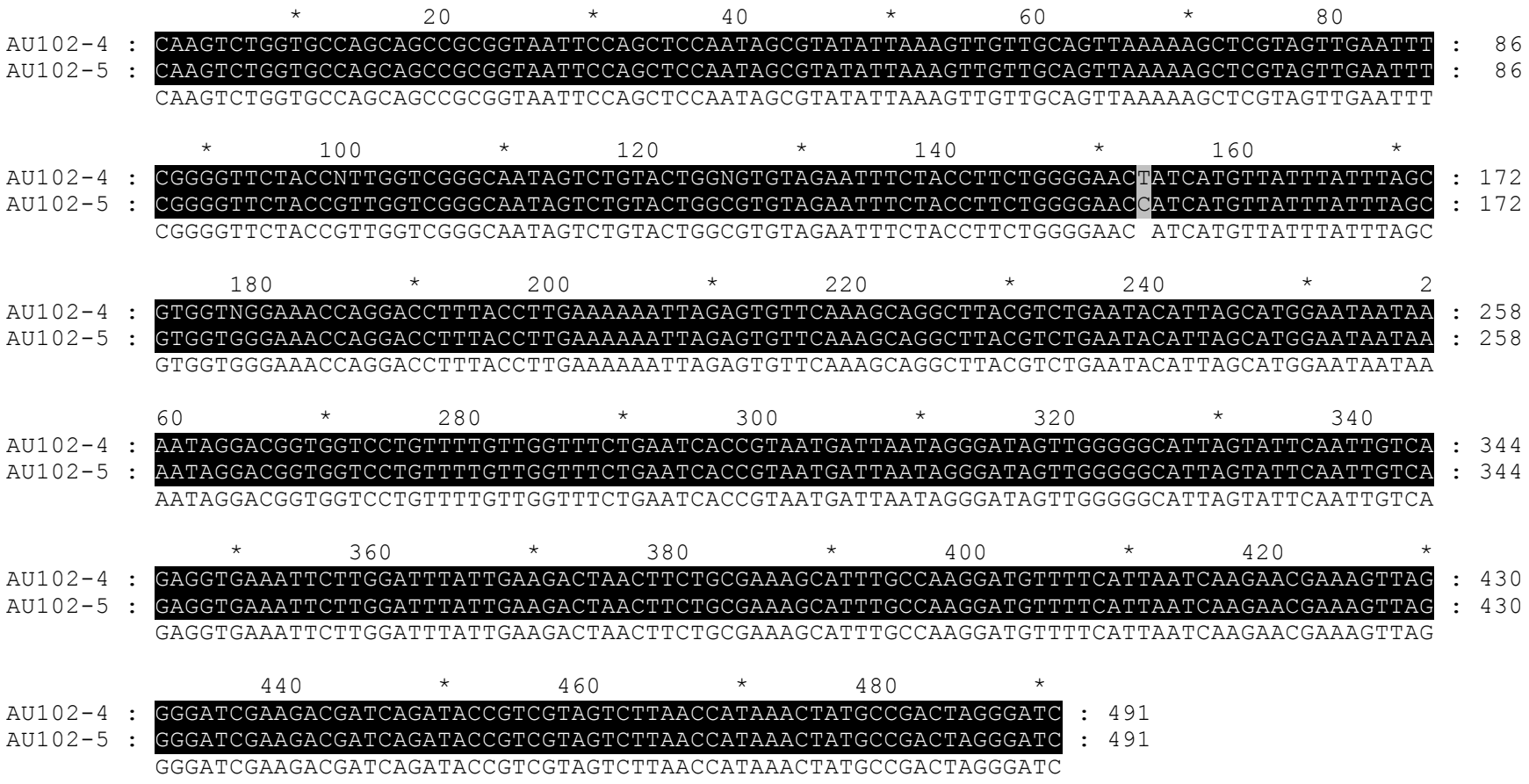

Figure 4.7. Partial 18S rDNA sequences recovered for *Gi. decipiens* AU102. Both sequences were identified as 18S rDNA of *Gi. decipiens.* Sequence designations are as labelled on Figure 4.5. Regions of consensus are highlighted in black. Positions of substitution are highlighted in grey.

4.3 Test of the Procedure's Ability to Segregate Members of a Spore Community

The procedure's ability to identify individual isolates from a mixture of spores was tested. Wet sieve and centrifugation extracted spores from reference cultures *Gi. decipiens* AU102 and *G. mosseae* WY110 were collected (three and ten spores, respectively) and subjected to the optimized extraction and PCR-DGGE procedures. Lane 6 of Figure 4.5 illustrated the result. Lane 6 contained bands with similar DGGE mobility as isolate *Gi. decipiens* AU102 (Lane 3) and isolate *G. mosseae* WY110 (Lane 4). Assuming DGGE band mobility is proof of presence of an isolate, this result demonstrated the procedure's ability to separate and identify individual isolates in a simple community of AMF.

4.4 Detection Limit and Primer Bias of the Developed Molecular Procedure When Applied to Reference Soils and Spores

The detection limit of the optimized procedure for isolate *Gi. decipiens* AU102 must be considered because of the absence of any *Gi. decipiens* AU102 band in Lane 11 of Figure 4.4. The spore density of *Gi. decipiens* AU102 in the reference soil was 2 spores per gram of soil (sp g^{-1}). Given the absence of an AMF band in Lane 11, the detection limit (x) in the reference soil must be greater than 2 sp g^{-1}. Figure 4.8 illustrated the detection limit test where 0, 4, 8, 12, and 16 sp g^{-1} were spiked into their respective soil sample prior to DNA extraction. *Gi. decipiens* AU102 bands were observed in soil extracts when the soil was spiked with four or greater sp g^{-1}. Therefore, the detection limit of the method for *Gi*. decipiens AU102 was $2 < x \leq 6$ (2 spores already in the soil plus 4 spores spiked into the sample) sp g^{-1}.

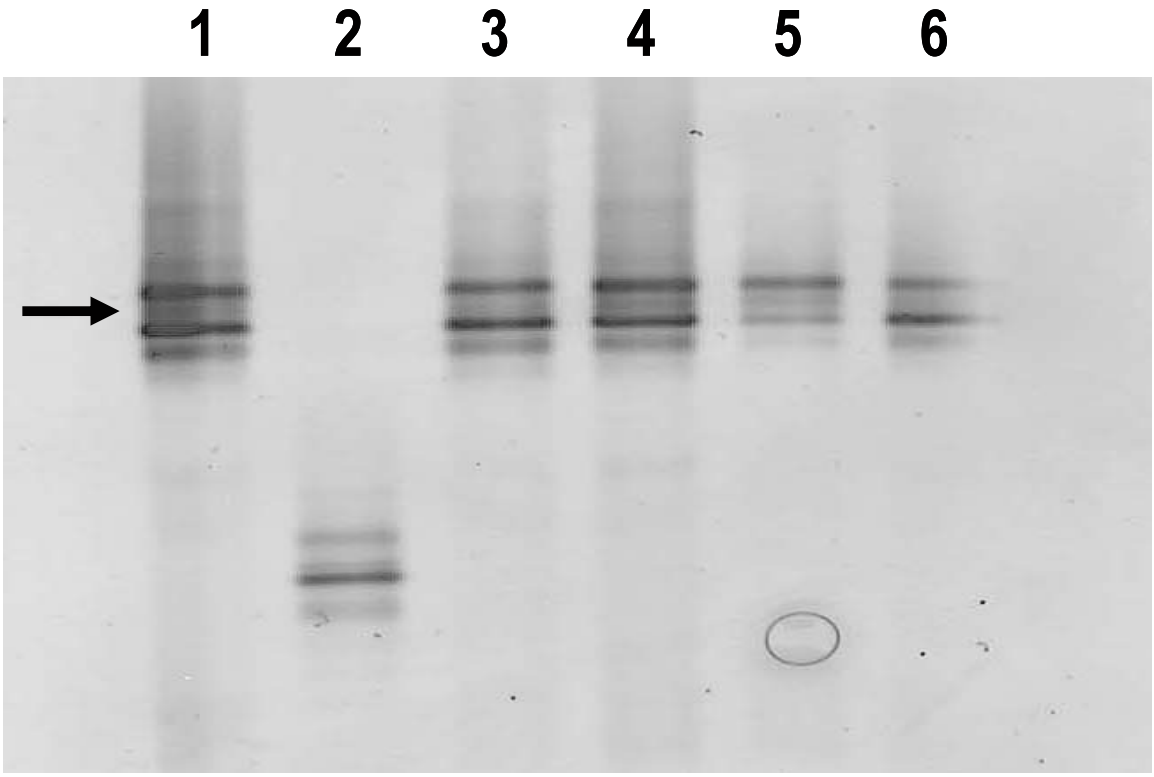

Figure 4.8. Denaturing gradient gel electrophoresis gel for the determination of the assay's detection limit for *Gigaspora decipiens* AU102 in reference soil samples. The detection limit is expressed as the number of spores per gram of soil (sp g^{-1}). Lane 1 – *Gi. decipiens* AU102's 18S rRNA gene DGGE signature generated from spore extracted DNA, Lanes 2, 3, 4, 5, 6 – 18S rRNA gene DGGE profiles generated from reference soil extracted DNA from *Gi. decipiens* AU102's reference culture using the optimized method with 0, 4, 8, 12, and 16 sp g^{-1}, respectively, spiked into the soil prior to DNA extraction. Arrow indicates the position of *Gi. decipiens* AU102 bands. The decline in band intensity in Lanes 5 and 6 was an artefact, rather than inhibition of PCR by increasing concentration of template, because the samples in these two lanes were accidentally flushed with the pipette during sample loading.

When all reference AMF templates were amplified together, the primer pair NS31-GC/AM1 demonstrated preferential amplification or primer bias (Figure 4.9). *Glomus luteum* SA101 and *G. mosseae* WY110 were the preferred templates because the detection limit (y) – expressed as the minimum percentage of the template in a mixed population required to produce a detectible PCR product by DGGE– for these two species was less than or equal to 1% of the total template concentration ($y \leq 1\%$; i.e., SA101 and WY110 bands were observed at 1%). *Gi. decipiens* AU102 and *G. versiforme* IT104 were the next preferred templates ($1\% < y \leq 5\%$; i.e., IT104 and AU102 bands were observed at 5% but not at 1%). All reference species were detectible in a community when all template concentrations were equal (25% each).

4.5 Detection, Identification, and Phylogenetic Analysis of PCR-DGGE -Isolated 18S rDNA Sequences from Organic Farm Field Soils

The success of the developed assay for detecting AMF was judged by its ability to detect AMF in soil samples with various physical, chemical, and biological properties. Twenty-three of the 38 samples processed had prominent DGGE bands of AMF origin (Figure 4.10 to 4.13). Fifteen (12 from Black, Brown, and Dark Brown soil zones) of the 38 samples had no detectable AMF bands, but non-AMF DGGE bands (those bands outside the DGGE mobility range of the reference species) were observed in these samples. The majority of the DGGE bands recovered were identified as *Glomus* sp. either by DGGE mobility (38 of 50 bands) or sequencing. Attempts were made to sequence all bands but some did not produce (by PCR) sufficient quantity for sequencing or no usable sequences were obtained. Bands that produced usable sequences are labelled in Figure 4.10 to 4.13 and their identity listed in Table 4.2.

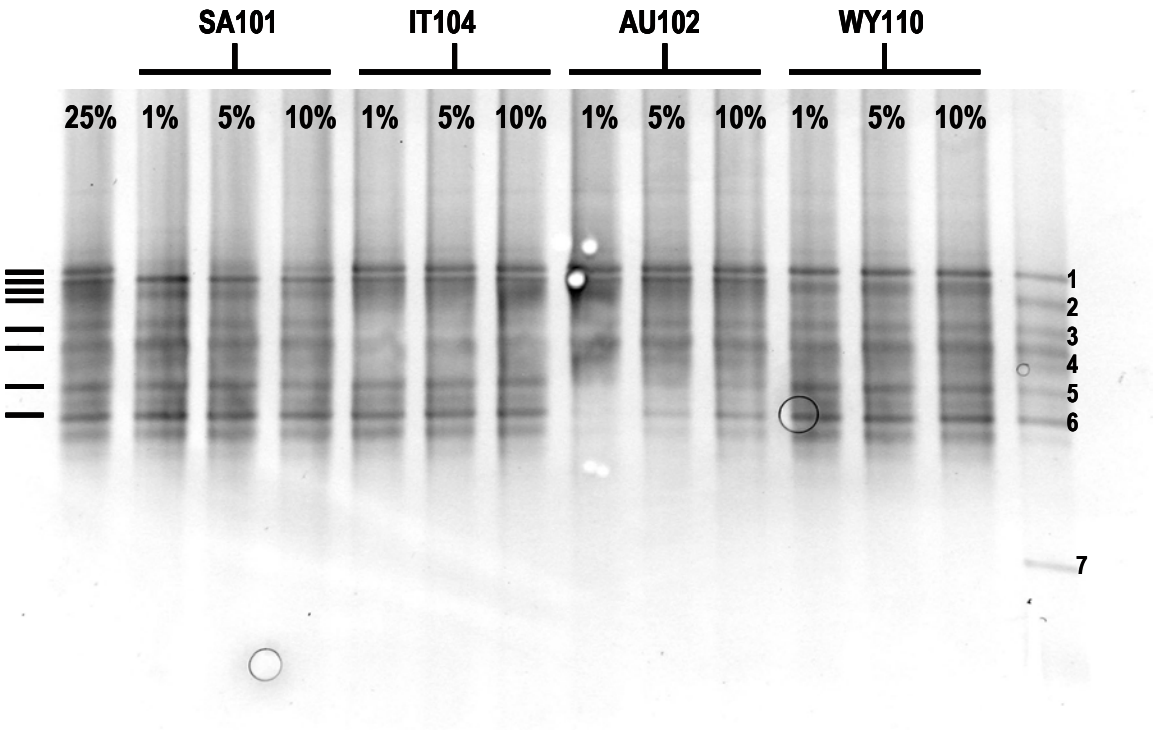

Figure 4.9. Denaturing gradient gel electrophoresis gel of the primer bias test performed on DNA templates of artificial communities constructed with reference AMF species. The sample for the 25% lane was amplified with equal concentration of template from each of the four reference species (i.e., 25% of each species). The sample for each 1% lane was amplified from a population of templates containing 1% template from the species in question and 33% from the other three species. The sample for each 5% lane was amplified from a population of templates containing 5% template from the species in question and 31.7% from the other three species. The sample for each 10% lane was amplified from a population of templates containing 10% template from the species in question and 30% from the other three species. Bands on the far left are illustrations of band positions. The band mobility sequence is as follow (from top to bottom): *G. luteum* SA101, *G. mosseae* WY110, *G. luteum* SA101, *G. mosseae* WY110, *G. versiforme* IT104, *G. versiforme* IT104, *Gi, decipiens* AU102, *Gi. decipiens* AU102. Unlabelled lane on the far right is a ladder constructed with PCR product of DGGE gel eluted DNA from Figure 4.3 and 4.5 (Band 1 = Band SA101-1, Band 2 = Band WY110-6, Band 3 = Band IT104-2, Band 4 = Band IT104-3, Band 5 = Band AU102-4, Band 6 = Band AU102-5, Band 7 = Band SA101-A). The slanted-appearance of this reference ladder is because of an artefact associated with the acrylamide gel polymerization.

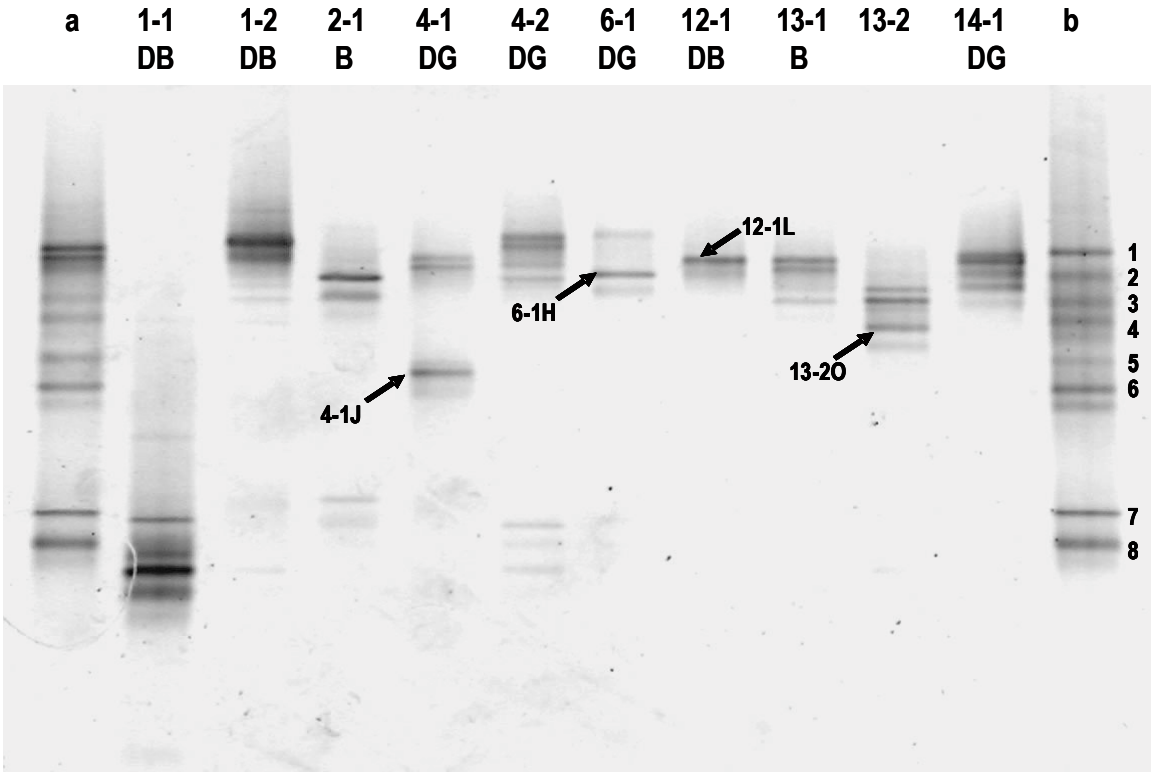

Figure 4.10. Denaturing gradient gel electrophoresis analysis of AMF communities in organic farm soil samples 1-1 to 14-1. Lane 'a' is a ladder constructed with reference spore PCR products of (from top to bottom) *G. luteum* SA101 (first set of two bands), *G. versiforme* IT104 (second set of two bands) and *Gi. decipiens* AU102 (third set of two bands). Lane 'b' is a ladder constructed with PCR product of DGGE gel eluted DNA from Figure 4.3 and 4.5 (Band 1 = Band SA101-1, Band 2 = Band WY110-6, Band 3 = Band IT104-2, Band 4 = Band IT104-3, Band 5 = Band AU102-4, Band 6 = Band AU102-5, Band 7 = Band SA101-A, Band 8 = Band AU102-B). Other lane designations refer to designation of the corresponding soil samples and B=Black Soil Zone, Br=Brown Soil Zone, DB=Dark Brown Soil Zone, DG=Dark Grey Soil Zone, G=Grey Soil Zone. Labelled bands were excised and sequenced and the BLAST results listed in Table 4.2. Bands with mobility greater than that of reference isolate *Gi. decipiens* were considered non-AMF. Bands in Lane 13-2 were excluded from summary in Table 4.3 because information pertaining to it was missing.

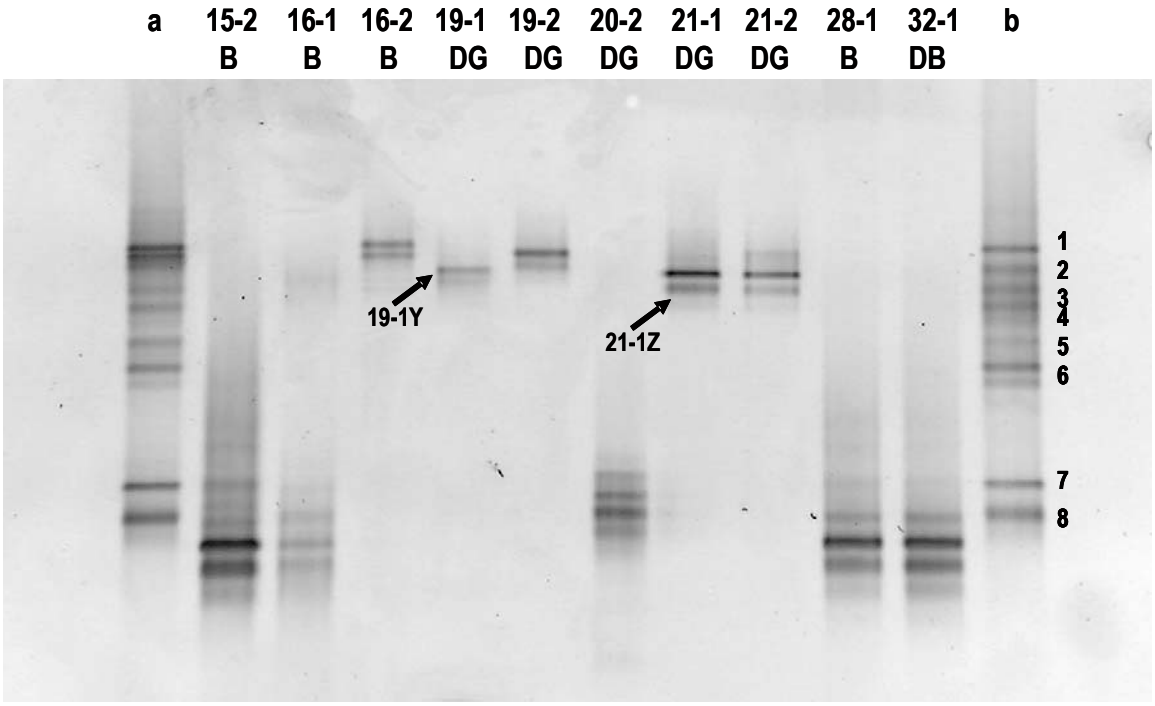

Figure 4.11. Denaturing gradient gel electrophoresis analysis of AMF communities in organic farm soil samples 15-2 to 32-1. Lane 'a' is a ladder constructed with reference spore PCR products of (from top to bottom) *G. luteum* SA101 (first set of two bands), *G. versiforme* IT104 (second set of two bands) and *Gi. decipiens* AU102 (third set of two bands). Lane 'b' is a ladder constructed with PCR product of DGGE gel eluted DNA from Figure 4.3 and 4.6 (Band 1 = Band SA101-1, Band 2 = Band WY110-6, Band 3 = Band IT104-2, Band 4 = Band IT104-3, Band 5 = Band AU102-4, Band 6 = Band AU102-5, Band 7 = Band SA101-A, Band 8 = Band AU102-B). Other lane designations refer to designation of the corresponding soil samples and B=Black Soil Zone, Br=Brown Soil Zone, DB=Dark Brown Soil Zone, DG=Dark Grey Soil Zone, G=Grey Soil Zone. Labelled bands were excised and sequenced and the BLAST results listed in Table 4.2. Bands with mobility greater than that of reference isolate *Gi. decipiens* were considered non-AMF.

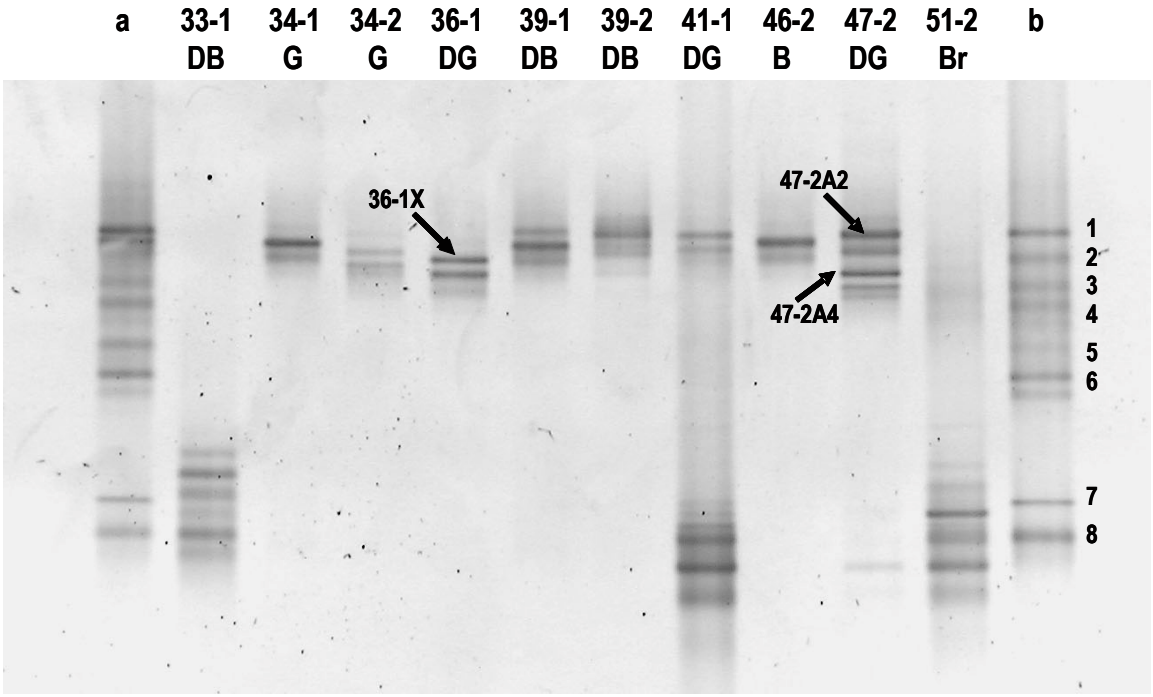

Figure 4.12. Denaturing gradient gel electrophoresis analysis of AMF communities in organic farm soil samples 33-1 to 51-2. Lane A is a ladder constructed with reference spore PCR products of (from top to bottom) *G. luteum* SA101 (first set of two bands), *G. versiforme* IT104 (second set of two bands) and *Gi. decipiens* AU102 (third set of two bands). Lane B is a ladder constructed with PCR product of DGGE gel eluted DNA from Figure 4.3 and 4.6 (Band 1 = Band SA101-1, Band 2 = Band WY110-6, Band 3 = Band IT104-2, Band 4 = Band IT104-3, Band 5 = Band AU102-4, Band 6 = Band AU102-5, Band 7 = Band SA101-A, Band 8 = Band AU102-B). Other lane designations refer to designation of the corresponding soil samples and B=Black Soil Zone, Br=Brown Soil Zone, DB=Dark Brown Soil Zone, DG=Dark Grey Soil Zone, G=Grey Soil Zone. Labelled bands were excised and sequenced and the BLAST results listed in Table 4.2. Bands with mobility greater than that of reference isolate *Gi. decipiens* were considered non-AMF.

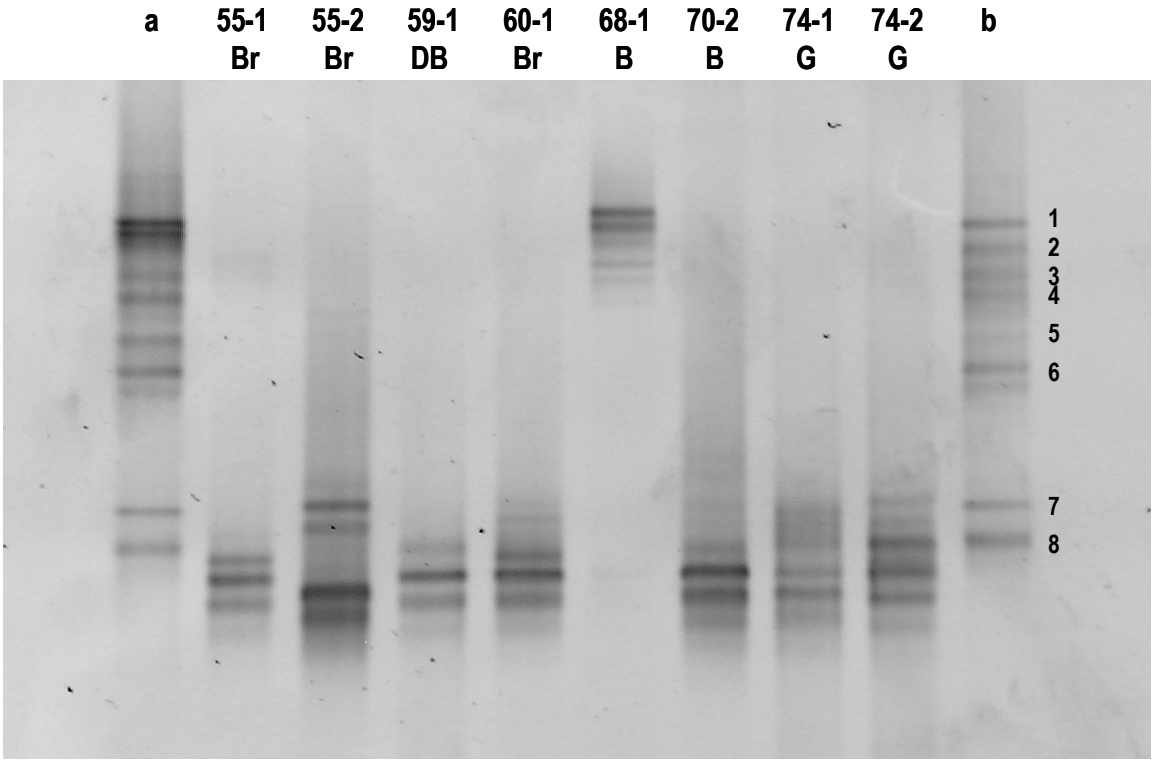

Figure 4.13. Denaturing gradient gel electrophoresis analysis of AMF communities in organic farm soil samples 55-1 to 74-2. Lane A is a ladder constructed with reference spore PCR products of (from top to bottom) *G. luteum* SA101 (first set of two bands), *G. versiforme* IT104 (second set of two bands) and *Gi. decipiens* AU102 (third set of two bands). Lane B is a ladder constructed with PCR product of DGGE gel eluted DNA from Figure 4.3 and 4.6 (Band 1 = Band SA101-1, Band 2 = Band WY110-6, Band 3 = Band IT104-2, Band 4 = Band IT104-3, Band 5 = Band AU102-4, Band 6 = Band AU102-5, Band 7 = Band SA101-A, Band 8 = Band AU102-B). Other lane designations refer to designation of the corresponding soil samples and B=Black Soil Zone, Br=Brown Soil Zone, DB=Dark Brown Soil Zone, DG=Dark Grey Soil Zone, G=Grey Soil Zone. Bands with mobility greater than that of reference isolate *Gi. decipiens* were considered non-AMF.

Table 4.2. Sequences recovered from organic farm field soil DGGE bands for identification.

Sequence designation[†]	Most related isolate from GenBank (% sequence similarity by BLAST)[‡]	GenBank accession no. of the most related sequences
4-1J	*Scutellospora calospora* (99%)	AJ306445, GI:15211856
6-1H	*Glomus* sp. Glo18 isolate (100%)	AY129625, GI:23092378
12-1L	*Glomus mosseae* isolate EEZ21 (100%)	AJ506089. GI:22474490
13 - 2O	*Glomus* sp. Glo4 isolate (99%)	AF074353, GI:3342472
19-1Y	*Glomus mosseae* BEG122 (99%)	AJ505616, GI:22293517
21-1Z	*Glomus* sp. Glo4 isolate (98%)	AF074353, GI:3342472
36-1X	*Glomus* sp. Glo18 isolate (99%)	AY129625, GI:23092378
47-2A2	Glomus sp. 5014b25.Llao5 (96%)	AF480158, GI:23451949
47-2A4	*Glomus* sp. Glo4 isolate (99%)	AF074353, GI:3342472

[†] Sequence designations are as labelled on Figure 4.10 to 4.13.
[‡] 97% sequence similarity is minimum requirement for identity.

Most of the bands sequenced were *Glomus* sp. In addition, *Scutellospora calospora*, a previously undetected AM fungus in Saskatchewan was found in this survey (Figure 4.10, Lane 4-1, band4-1J). Bands corresponding to all species represented by the reference cultures – except for *Gi. decipiens* AU102 – were detected by band mobility in the field samples. However, *G. mosseae* was the only sequence-confirmed reference species found in field soils.

A phylogenetic analysis of the recovered field soil and reference soil and spore sequences showed the sequences diverged into two main branches (Figure 4.14). One branch contained the non-AMF sequences. The other contained all AMF sequences. The AMF orders Diversisporales (surrounded by a solid box) and Glomerales (surrounded by a dotted box) were represented by the divergence of the AMF branch. A second phylogenetic analysis with the exclusion of the two non-AM fungal sequences provided better resolution of the Glomeromycota branch (Figure 4.15). An orthodox reassignment of *G. versiforme* from the Diversisporales to Glomerales branch and higher confidence (bootstrap values [out of 100] of 100 and 81 versus 70 and 69 for the Diversisporales and Glomerales branches, respectively) in the phylogenetic analysis was obtained.

No identifiable relationship between soil chemical properties (e.g., pH and levels of P and N) and AMF community composition was observed in this cursory survey. However, general trends between AMF community composition and soil zone were observed (Table 4.3) and it is relatable to AMF phylogeny. The two major branches of *Glomus* clade A (typified by *G. mosseae*; Figure 4.15) represent two ecological groups. The *G. mosseae*-like sequences (bands WY110-6, WY110-7, 12-1L, and 19-1Y) represented near one-half of the visualized bands (23 of 50 total bands) and were found

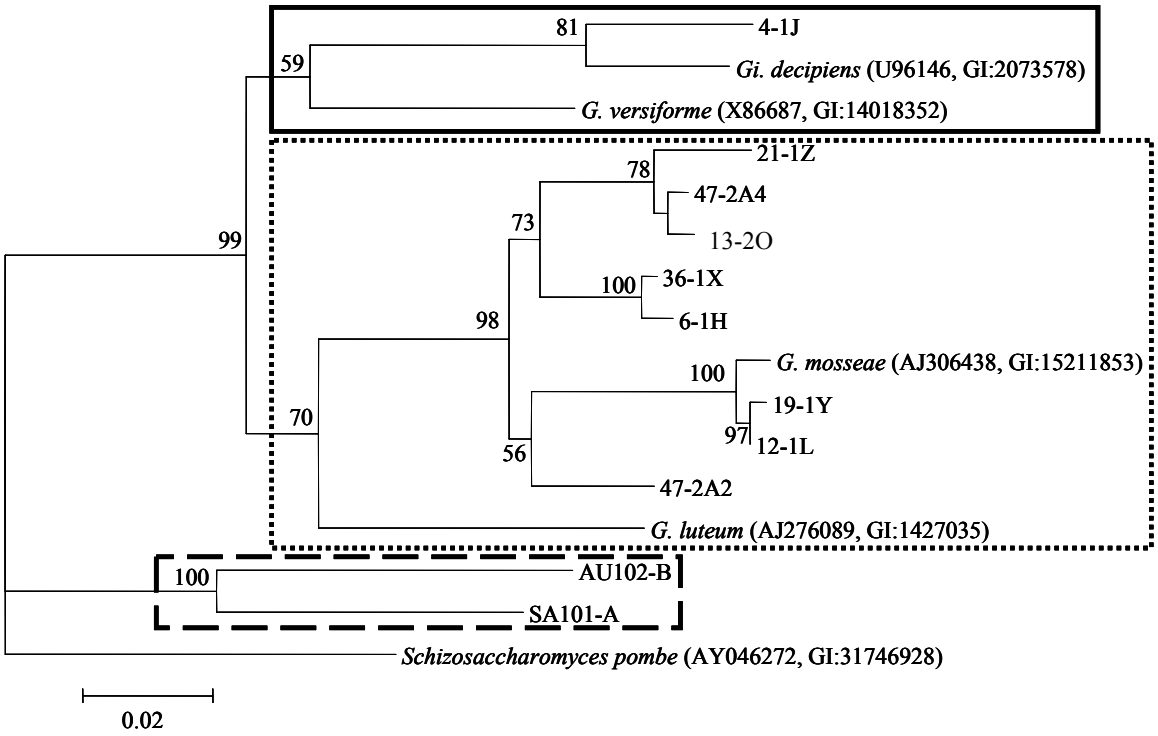

Figure 4.14. Phylogentic tree constructed with one outgroup (*Schizosaccharomyces pombe*), four reference spore, and 11 soil (two from reference soils, nine form field soils) 18S rRNA gene fragment sequences. Solid box highlights the AMF order Diversisporales (bootstrap = 59). Dotted box highlights the AMF order Glomerales (bootstrap = 70). The branch node hosting both AMF branches is supported by bootstrap of 99. Dashed box highlights the branch representing two non-AMF soil fungi amplified by the nested PCR (bootstrap = 100). A bootstrap value estimates the uncertainty of a statistic. In this case, the statistic is the topology or branching of the phylogentic tree. To generate a bootstrap uncertainty estimate for a given statistic from a set of data (nucleotide sequences), a sub-sample of a size less than or equal to the size of the data set is generated from the data, and the statistic is calculated. This sub-sample is generated *with replacement* so that any data point can be sampled multiple times or not sampled at all. This process is repeated for many sub-samples, typically between 500 and 1000 (depending on available computing time and size of data set) but in this case 100. The computed values for the statistic form an estimate of the sampling distribution of the statistic (Felsenstein, 1989; Felsenstein, 1997). For example, if the bootstrap for a branch node is 100, this means for each tree constructed from each of 100 sub-samples the branch node in question was present. Therefore, the uncertainty of a branch node increases with a lower bootstrap. See Table 4.1 and 4.2 for identity of the sequences. Scale bar represents the number of nucleotide substitutions required to convert one sequence into a neighbouring sequence.

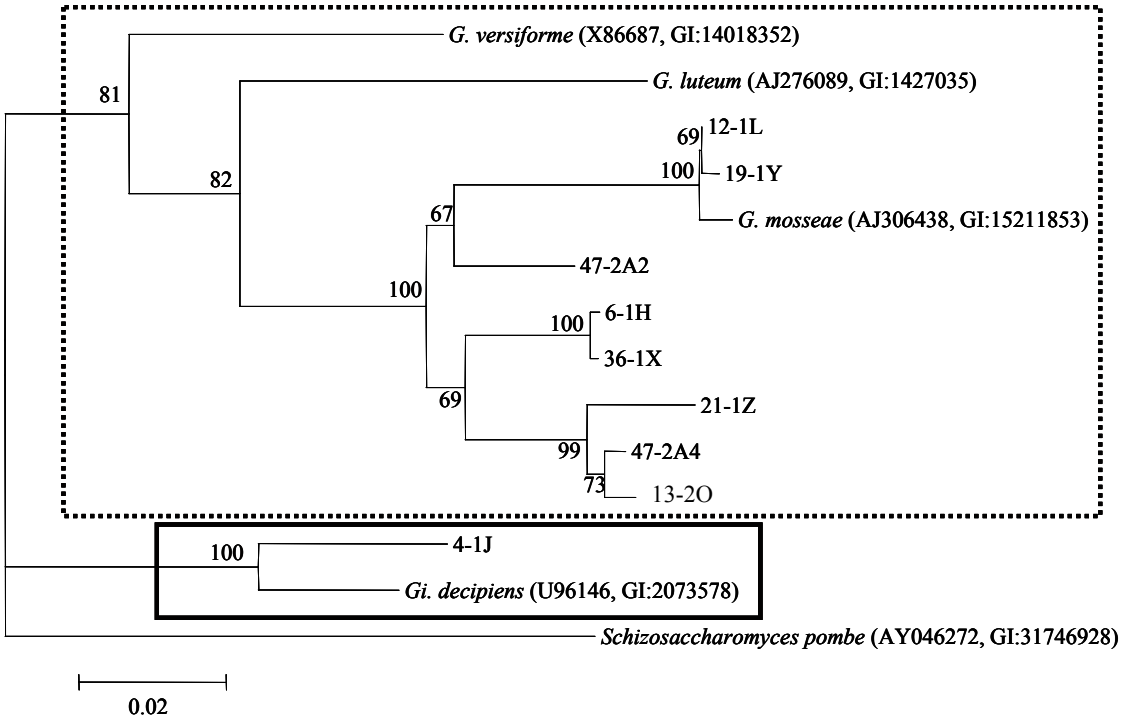

Figure 4.15. The phylogenetic tree is reconstructed with the exclusion of the two non-AMF sequences. Greater resolution of the Glomeromycota (reassignment of *G. versiforme* from the Diversisporales to Glomerales branch) and higher confidence (bootstrap values 100 and 81 versus 70 and 69 for the Diversisporales and Glomerales branches, respectively) in the phylogenetic analysis is obtained. See Table 4.1 and 4.2 for identity of the sequences. Scale bar represents the number of nucleotide substitutions required to convert one sequence into a neighbouring sequence.

Table 4.3. Summary of DGGE bands found in the soil zones of Saskatchewan.[†]

DGGE band[‡]	Soil zone					No. of bands
	Black (n = 9)	Brown (n = 4)	Dark Brown (n = 8)	Dark Grey[§] (n = 12)	Grey[§] (n = 4)	
1	1	0	1	3	0	5 ¶
2	3	0	3	5	1	12
3	2	0	0	7	2	11
4	3	0	0	4	0	7
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	2	0	1	2	0	5 #
9	1	0	0	2	0	3
10	0	0	0	1	0	1[††]
11	0	0	0	2	1	3 #
12	0	0	0	0	0	0
13	0	0	0	1	0	1
14	0	0	0	2	0	2
No. of bands	12	0	5	29	4	50

[†] Bands were tallied based on mobility, e.g., bands with similar mobility to band designations SA101-1 and 47-2A2 (both with same mobility but were identified as different by sequencing) were assigned to DGGE band 1 group.

[‡] DGGE band 1 = band designation SA101-1 or 47-2A2; band 2 = WY110-6 or 12-1L; band 3 = WY110-7, 6-1H, or 19-1Y; band 4 = IT104-2 or 21-1Z; band 5 = IT104-3 or 13-2O; band 6 = AU102-4; band 7 = AU102-5; bands 8, 9, 11, and 12 = unidentified bands with GC-content within *Glomus*; band 10 = 4-1J; band 13 = 36-1X; band 14 = 47-2A4. Band designations are as labelled on Figures 4.3 and 4.10 to 4.12.

[§] Majority (33 of 50) bands were recovered from the Dark Grey (n = 12) and Grey (n = 4) soil zones.

[¶] Thirty-five of 50 bands (encompassed by dotted box) had similar mobility to reference *Glomus* species. *G. mosseae*-like bands (DGGE band 2 and 3; 23 of 50 bands) were the majority, and these bands appear to be distributed throughout all soil zones Other AMF or AMF-like bands were found mostly in the Grey and Dark Grey soil zones.

[#] Eleven of 50 bands (encompassed by dashed box) were not identifiable by mobility (unlike any of the sequenced bands) or by sequencing (no usable sequences were obtained) but their mobility was within the range of the *Glomus* reference species.

[††] This was the only band definitively not of the genus *Glomus*.

distributed throughout all soil zones of Saskatchewan. Other *Glomus*-like bands (15 of 50 total bands) on the other branch were found dominantly (10 of 15 bands) in Grey to Dark Grey soil zone samples. Bands like *Glomus* clades B (*G. luteum*) and C (*G. versiforme*) were found with similar frequency in Black and Dark Grey soil zone samples. Bands unidentifiable as *Glomus* (11 of 50 total bands; no correspondence to known or reference band mobility and no usable sequence data) but were within the GC-content range of *Glomus* were found mostly (7 of 11 bands) in Grey and Dark Grey soil zone samples. The sequence recovered for *Scutellospora calospora* (Figure 4.10, band 4-1J) was the only Diversisporales (e.g., *Scutellospora* and *Gigaspora*) found, and it was in a sample from the Dark Grey soil zone.

5 DISCUSSION

The success of this PCR-DGGE procedure relied on the efficiency of DNA extraction from the samples. A number of published methods for fungal DNA extraction from spores and soils were attempted (Griffiths et al., 2000; Schwarzott and Schussler, 2001; Kowalchuk et al., 2002). The method of Griffiths et al. (2000) was the only one used successfully to extract amplifiable template from soil, though not from spores. The results attained with this method were inconsistent. This may arise from difficulties in disrupting spores in soils. The density and size of beads used in bead beating procedures can have an influence on the technique's ability to extract DNA (Cullen and Hirsch, 1998). In this case, 120 μm silica glass beads were used. It is possible that using larger and denser beads may increase the reliability of the procedure when treating soil samples. Alternatively, the mortar and pestle approach described here was more consistent in extracting amplifiable AMF template.

The adapted DNA extraction procedure and optimized nested PCR-DGGE technique was able to identify AMF isolates in reference soils. However, even under high stringency (67°C annealing temperature) the primers used in the PCR co-amplified non-AMF DNA from reference soil samples. Because PCR is an exponential reaction, even a small amount of competing non-AMF template will decrease the proportion of AMF DNA in the total amplified population (Innis and Gelfand, 1999). Hence, the extraction efficiency of AMF DNA relative to non-AMF DNA must be sufficiently high to overcome the detection limit of the PCR-DGGE assay (Clapp et al., 1995). This was

illustrated by the detection limit test performed on *Gi. decipiens* AU102 (Figure 4.8). Below the detection limit, non-AMF DNA is amplified by the PCR and its product visible by DGGE (Lane 2). Above the detection limit, *Gi. decipiens* AU102 DNA was amplified and visualized by DGGE whereas non-AMF bands were excluded (Lanes 3 to 5). Band detection by Sybr Green staining of DGGE gels requires the DNA population of interest to make up greater than 2% of the total DNA population (Kowalchuk et al., 2002). Therefore, an inefficient AMF DNA extraction results in inconsistent or no detection by PCR-DGGE. The adapted extraction procedure was more consistent and reproducible than Griffiths et al. (2000) when extracting from reference spores and oils.

The similar DGGE mobility of isolates *G. luteum* SA101 and *G. etunicatum* UT316 was unexpected (Figure 4.3 – Lanes 1 and 5, respectively); however, the high percentage of sequence similarity (Table 4.1) between *G. luteum* SA101 and *G. etunicatum* UT316 may indicate insufficient GC-content differences in the targeted 18S rDNA fragment to distinguish between *G. luteum* SA101 and *G. etunicatum* UT316 by DGGE. The combination of DGGE mobility and sequence information indicated in the fragment of the 18S rDNA analyzed there is no sequence difference between *G. luteum* SA101 and *G.* etunicatum UT316. The similar band pattern of Lane 8 (*G. luteum* NT4) to Lane 1 (*G. luteum* SA101) indicated isolate *G. luteum* NT4 was *G. luteum* SA101. This is logical because *G. luteum* NT4 was the voucher specimen trap-cultured and isolated by Talukdar and Germida (1993) and sent to INVAM where it was classified, archived, and given the accession *G. luteum* SA101. Lane 7 (*G. versiforme* NT7) had a similar band pattern to Lanes 1, 5, and 8. This indicated its sample was an isolate of *G. luteum* or *G. etunicatum*, but Talukdar and Germida (1993) putatively classified it by

morphology as isolate *G. versiforme* NT7 (analogous to isolate *Glomus versiforme* IT104). It is possible the *G. versiforme* NT7 culture was contaminated with *G. luteum* NT4 inoculum through successive regeneration or a *G. luteum* NT4 inoculum was mislabelled as *G. versiforme* NT7. Misidentification of the original NT7 culture is unlikely because it too was submitted to INVAM for identification, archive, and research purposes. However, *G. versiforme* NT7 was lost during successive culturing at INVAM.

Operon heterogeneity appeared to play an important role in DGGE banding patterns. A double-band pattern was observed for all reference isolates repeatedly (e.g., Figure 4.3 and 4.5). Intra-species and intra-spore operon heterogeneity has been reported by others (Sanders et al., 1995; Clapp et al., 1999; Jansa et al., 2002b). Whether the two variants of the 18S rRNA gene observed are from a single spore or different spores are unknown because the reference spore PCR-DGGE profile was generated with extractions from multiple spores. This observation complicates the interpretation of field DGGE profiles because any AMF species may be represented by two or more bands. Therefore, the actual number of AMF species in a field DGGE profile may be less than half of what is visually detected by DGGE.

However, operon heterogeneity alone may not be the only phenomenon to explain the doublet appearance for all reference AMF observed. Observable sequence variation cannot account for the greater band separation observed for *Gi. decipiens* AU102 (with one base pair substitution) versus *G. versiforme* IT104 (with three base pair substitutions). In addition, sequencing results for reference bands WY110-6 and WY110-7 (as labelled on Figure 4.5) indicated the two bands' sequences were identical (over 509 usable nucleotides). These sequences may have variations in the remaining

(non-usable sequences) part of the amplified fragment (Wartiainen et al., 2003), and it is the location of the base pair variation in conjunction with the number of variations that determines the mobility of the DNA fragment in the DGGE. Alternatively, Kocherginskaya et al. (2001) demonstrated that single-stranded and double-stranded molecules from the same template have different mobility during DGGE. Regardless of the origin for the doublet feature, the double band pattern is consistently observed and will complicate DGGE analysis of AMF.

Polymerase chain reaction problems such as chimera formation, DNA polymerase error, and primer bias may decrease the accuracy of the procedure – especially when dealing with a population of related amplicons (Speksnijder et al., 2001). The formation of chimeric DNA molecules during the PCR has been recognized as a source of sequence infidelity (Wang and Wang, 1997). The RDP Chimera Check program used on the recovered sequences was inconclusive for chimera formation because of the relatively small fungal 18S rRNA gene database. However, high similarity indices obtained for the recovered sequences (as determined by BLAST) provide a degree of confidence in their fidelity. The error rate of the proprietary *Taq* used in the Master Mix is unknown, but non-proofreading polymerases have reported error rates ranging from 4.0×10^{-2} to 2.2×10^{-4} (Innis and Gelfand, 1999). For the 30-cycle reaction used to amplify a fragment of ~550bp, the number of potential mismatch insertions range from one to 40 bases. The potential error is doubled because of the nested PCR strategy used. This makes suspect the operon heterogeneity interpretation of the double banding pattern observed for the reference spore DGGE gel (Figure 4.5). However, the repeated observation of the pattern within the reference cultures and the sequence results supported the operon heterogeneity

hypothesis (Table 4.1 and Figure 4.6 and 4.7). Primer bias in simulated communities was observed (Figure 4.9). This will contribute to the underestimation of the number of AMF species in a community.

If we assume each DGGE band represented an individual species in the community, this cursory AMF community composition survey of 38 field soils from organic farms by molecular techniques found zero to four species per site (Figure 4.10 to 4.13). This is similar to Talukdar and Germida's (1993) identification of three to six species by trap culture techniques for conventional farm sites across the province. Both results are low when compared to the number of AM fungal species detected at other temperate low input sites (26 species) (Oehl et al., 2003), conventional cultivated sites (13 species) (Hamel et al., 1994), and native sites (37 species) (Bever et al., 2001). It is possible that similar migration behaviour of fragments with different origin but same GC content may have underestimated the number of species in a sample (Kowalchuk et al., 2002). The sampling strategy used may not be optimally suited to assess community composition of AMF in agricultural soils. For example, the 0 to 15 cm sample used for DNA extraction may miss species present at greater depth (Douds et al., 1995). Or, simply, there are fewer AMF species in Saskatchewan soils. More work is needed to address these possibilities.

The phylogenetic analysis of all DGGE recovered sequences support the current AMF phylogenetic taxonomy (Morton and Redecker, 2001; Schussler et al., 2001a; Schussler et al., 2001b; Schwarzott et al., 2001). The Diversisporales branch is distinct from the Glomerales branch in both analyses, but the inclusion of *G. versiforme* in the Diversisporales was supported by a moderate bootstrap value of 59 (Figure 4.14).

Glomus versiforme's reassignment to the Glomerales (Figure 4.15) strengthened the bootstrap value (100) for the Diversisporales. The moderate bootstrap values (81 and 82) for the first two nodes of the Glomerales branch in Figure 4.15 support the hypothesis that the Glomerales is polyphyletic (Schussler et al., 2001b; Schwarzott et al., 2001). Specifically, *G. versiforme* may be assigned to the Diversisporales as in Figure 4.14 (Schwarzott et al., 2001). The majority of the recovered sequences belong to "*Glomus* Group A" (GlGrA) as represented by *G. mosseae* (bootstrap value of 100 at the third node in Figure 4.15). This group is the largest sub-clade in the Glomerales and include many well-studied species (e.g., *G. mosseae, G. intraradices, G. fasciculatum, G. clarum,* etc.) that are prevalent in agricultural soils (Morton and Benny, 1990; Schwarzott et al., 2001). Schwarzott et al. (2001) noted the phylogenetic distance between GlGrA and GlGrB (represented by *G. luteum* in Figure 4.15) is greater than the distance between the families Gigasporaceae and Acaulosporaceae (both in the order Diversisporales); thereby, giving greater support to the polyphyly assessment of Glomerales.

The dominant number of *Glomus*-like bands observed and recovered was not a surprise (Table 4.3). In particular, the large number and wide distribution of *G. mosseae*-like bands recovered in Saskatchewan agrees with the literature's general assessment of *G. mosseae* as a common AM fungus found in a variety of cultivated field soils (Sylvia and Schenck, 1983). The near absence of members from the Diversisporales (i.e., *Gigaspora* and *Scutellospora*) concurs with the correlation of *Gigaspora* and *Scutellospora* population decline with cultivation (Douds et al., 1993). From the high proportion of bands recovered from the Grey to Dark Grey soil zones (33 of 50 bands) it is arguable cropping history and management (data not shown) in relation to soil type and

climate promoted or inhibited AMF establishment and maintenance. For example, rotation of poorly mycorrhizal crops (e.g., wheat) with fallowing and tillage in the southwestern part of the province (Brown and Dark Brown soil zones) will select for specific AMF species and diminish soil inoculum levels (see section 2.5.1). In contrast, producers in the Grey Dark Grey soil zones generally grew strongly mycorrhizal leguminous crops (e.g., pea and lentil) with no till or maintained forage cover such as alfalfa. These conditions could promote AMF diversity and build up soil inoculum levels. The aforementioned problems with DNA extraction and PCR are exacerbated by the likely lower diversity and inoculum levels as a function of the agriculture practiced in the areas of the Dark Brown and Brown soil zones

6 CONCLUSIONS

A diverse AMF population is a key factor to improve the sustainability of low input and organic agricultural systems (Mader et al., 2002; Oehl et al., 2003). To increase our ability to optimize management of AMF in field situations, there is a need for more information on how agricultural practices influence the variation in AMF community development and function in different crop species. The first step is to fully characterize the AMF community composition. The reliance on spore morphology to characterize AMF communities is subjective and provides an incomplete interpretation of their *in situ* reality (Bever et al., 2001). The PCR-DGGE technique described here is one tool for objective characterization of complex AMF communities in agro-ecosystems.

Because many molecular methods for microbial ecology depend on PCR, users of this method must be aware of its shortcomings (Speksnijder et al., 2001; Kowalchuk et al., 2002). Proper controls and optimized reactions must be used to maintain the fidelity of the information gathered. Though not without its problems, PCR's technical flexibility and speed make it an attractive tool. The method described here was qualitative, but a competitive or real time PCR approach targeting a single-copy gene can make the procedure quantitative (Edwards et al., 2002; Landeweert et al., 2003). A reverse transcription PCR for mRNA transcripts of the rRNA operon or metabolic genes can monitor the functional diversity and metabolic activity of the AMF population (Rhody et al., 2003). The usefulness of molecular techniques is limited only by our ingenuity to their application and vigilance to their limitation.

The PCR-DGGE method described found zero to four AMF species in the field soil samples. Given the inherent technical limitations, this is a possible under-estimation of the true species numbers in Saskatchewan field soils. However, this result is comparable to Talukdar's (1993) finding of three to six species. Besides identifying individual AMF species in the community, sequence data obtained by this molecular approach was useful for phylogenetic analysis between isolated sequences. However, sequence data alone is insufficient for absolute phylogenetic taxonomy determination (Schwarzott et al., 2001). In the foreseeable future, morphological and molecular techniques will complement one another in AMF community studies to gather different but complementary information (Clapp et al., 1995; Helgason et al., 1998; Kowalchuk et al., 2002).

What is the future for this presented work? Firstly, a number of technical issues require resolution. For example, the low number of AMF bands recovered from the Black, Dark Brown, and Brown soil zones suggests the procedure is not robust enough to overcome possible reaction inhibitors or sensitive enough to detect AMF in these samples. Therefore, further sample purification and reaction optimization is required. In addition, there is a need to determine how to identify DGGE bands with same mobility but different origin. An additional procedure such as cloning or restriction analysis is needed. Second, a complementary quantitative procedure must be developed. The identity of the members in an AMF community is important, but without the knowledge of population size, conclusions concerning the impact of management on changes in AMF community dynamics cannot be determined. Third, an empirical trend in AMF bio-geographic distribution was observed. That is, *G. mosseae*-like bands were distributed

throughout Saskatchewan but other *Glomus* species-like bands were limited to the Dark Grey soil zone. Appropriate statistical analysis (multivariate statistics or principle component analysis) must be performed to validate this observation. By resolving these three issues, this PCR-DGGE procedure is a good tool for AMF biodiversity studies or for monitoring the effect o single or combination of agricultural practices on the mycorrhizal population to promote crop growth.

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