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Characterisation and identification of arbuscular mycorrhizal fungi species by PCR/RFLP analysis of the rDNA internal transcribed spacer (ITS)

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Abstract - The polymerase chain reaction coupled with restriction fragment length polymorphism analysis (PCR/RFLP) was applied to distinguish arbuscular mycorrhizal fungi (AMF) species from an impacted semiarid soil. The ITS1-5.8S-ITS2 region was amplified with the primers ITS1 and ITS4 and the products of amplification were digested with the restriction enzymes HinfI, MboI and AluI. The obtained banding pattern, except for the AluI, allowed the distinction at the molecular level of the AMF species: *Paraglomus occultum, Glomus mosseae, Glomus intraradices* and *Glomus etunicatum*. The results showed that this technique has a potential to be used as a marker to differentiate AMF species with high phylogenetic affinity.

Key words: AM fungi, ITS, *Glomus* and *Paraglomus* species.

INTRODUCTION

Mycorrhizal fungi occur in highly diverse communities, representing the widest association between plants and fungi found in nature (Dickie and FitzJohn, 2007). Mycorrhized plants show higher capacity of nutrient uptake, and resistance to biotic and abiotic stresses than non mycorrhized plants (Smith and Read, 1997; Renker *et al.*, 2003). The identification and characterisation of these fungi are the first steps for study of this association. The morphological identification of arbuscular mycorrhizal fungi (AMF) species relies mainly on the characteristics of soil-borne spores and it also difficult in environmental samples due to developmental variation or parasitism of spores. Because factors controlling sporulation and morphological differentiation of AMF taxa are poorly understood, and occurrence of taxa in the spore community and in roots do not necessarily coincide, community studies based solely on spores appear to be of little ecological relevance (Gamper and Leuchtmann, 2007).

Sporocarp production is only loosely related to belowground community patterns and many fungi produce cryptic and/or hypogeous sporocarps. Because of these difficulties, there has been an increasing reliance on molecular methods for identifying species from belowground structures (Horton and Bruns, 2001; Dickie and FitzJohn, 2007).
Many molecular techniques have been used to study the AMF, improving the knowledge on phylogenetic, cytogenetic, functional and ecological aspects and the selection of one or more techniques is a function of the research objective (Renker *et al.*, 2003, 2006; Dickie and FitzJohn, 2007; Gamper and Leuchtmann, 2007).

Approaches polymerase chain reaction (PCR) - based on seem to offer the best current prospects for detecting most of the AM fungi present in an ecosystem. However, the results of PCR-based analyses may be based by differential amplification (or non-amplification) of target DNA at different concentrations, bearing the risk that PCR analyses focusing exclusively on spores, roots or extra-radical mycelium, could will fail to detect some important components of the AM community that are weakly represented in the sampled material (Hempel *et al.*, 2007).

PCR/RFLP has been applied in mycorrhizal research to identify strains of introduced or naturally occurring mycorrhizal fungi or of economically important species and also to differentiate and identify mycorrhizal symbionts unambiguously. The PCR/RFLP polymorphism of the ITS region is generally regarded as appropriate to differentiate AMF at

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the species level (Renker *et al.*, 2003, 2006; Hempel *et al.*, 2007).

The fragment of rDNA which will be amplified for analysis of intra-specific diversity or between groups of AMF isolates with high phylogenetic affinity should include the internally transcribed spacer (ITS1, 5.8S, ITS2), a region that presents high variability in both, the composition of bases and in the fragment size (Grifoni *et al.*, 1995; Renker *et al.*, 2003, 2006; Gamper and Leuchtmann, 2007).

Semiarid soils have a high diversity of plant and AMF but the identification of AMF which based on morphological character of spores is difficult, mainly when spores are in low amounts as occurs in impacted areas. Thus alternative techniques should be employed to allow identification. Further studies with AMF can be useful for helping establishment of plants and revegetation of impacted semiarid areas.

The aim of this study was to differentiate between AMF species with high phylogenetic affinity by comparison of their ITS (ITS1, 5.8S, ITS2) restriction patterns.

MATERIALS AND METHODS

Soil samples were collected from an impacted gypsite mining area in Araripina, Pernambuco State. Spores of AMF, present in the samples, were multiplied in successive cycles of greenhouse pot, culturing in soil with sorghum and peanut as hosts. After three months, spores of AMF, extracted from soil by wet sieving (Gerdemann and Nicolson, 1963) and sucrose centrifugation (Jenkins, 1964) were selected on a stereomicroscope. Similar spores were grouped, mounted its glass slides with PVLG or Melzer reagent + PVLG (1:1). The species were identificated with the help of light microscope (Schenck and Pérez, 1990; http://invam.caf.wvu.edu).

After identification, spores of four species (*Paraglomus occultum* Morton & Redecker, *Glomus mosseae* Gerdemann & Trappe, *Glomus etunicatum* Becker & Gerdemann and *Glomus intraradices* Schenck & Smith) were separated and the DNA was extracted using the method described by Lanfranco *et al.* (2001). Approximately 150 to 200 spores of each AMF species were sonicated (3 to 4 cycles of 30 s) and broken with the aid of a micropestle in 50 µL of 1X reaction buffer (LABTRAD do Brasil LTDA). With 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, they were incubated at 95 ºC for 15 min and centrifuged at 10.000 x g for 5 min. The supernatant was stored at -20 ºC.

The PCR reaction consisted of 3 µL of total DNA added to the mix to receive a final volume of 25 µL, containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 10 mM of each dNTPs, 25 ng of each primer ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATC3') (White *et al*., 1990) and 2 units of Taq DNA polymerase (Invitrogen). The amplification reactions were carried out in termocycler M.J. Research, Inc. (USA) PCR, model PTC 100, using a program with initial denaturation at 95 ºC 3 min, forty cycles of 95 ºC 45 s, 50 ºC 45 s, 72 ºC 1 min and a final extension at 72 ºC for 5 min. After the thermal cycling, an aliquot of the PCR products was digested with the restriction enzymes MboI, HinfI and AluI, according to the recommendation of the manufacturer (Invitrogen). The restriction products were separated by electrophoresis, at 3V/cm for 2 h, in 1.5% agarose gel, in TBE buffer which was stained with Sybr Gold. The banding patterns generated with the restriction of the ITS region were observed and photographed in a photodocumentation system with UV light. The 100 bp DNA Ladder (Invitrogen) was used as the fragment size marker.

RESULTS AND DISCUSSION

Using the primers ITS1 and ITS4, the amplified product of the locus ITS1-5.8S-ITS2 of the rDNA presented fragments of about 600 bp for *G. mosseae* and *G. etunicatum* and 700 bp for *P. occultum* and *G. intraradices* (Fig. 1). Similar results were observed by Redecker *et al.* (1997), who mentioned that the amplification products generated for species of Glomus presented approximately 600 bp. The amplified products of the ITS1 and ITS2 regions from the rDNA, using the ITS1 and ITS4 primers, also generated fragments of about 600 bp for *Gigaspora margarita* Becker & Hall, *Gigaspora decipiens* Hall & Abbott and *Scutellospora* sp.

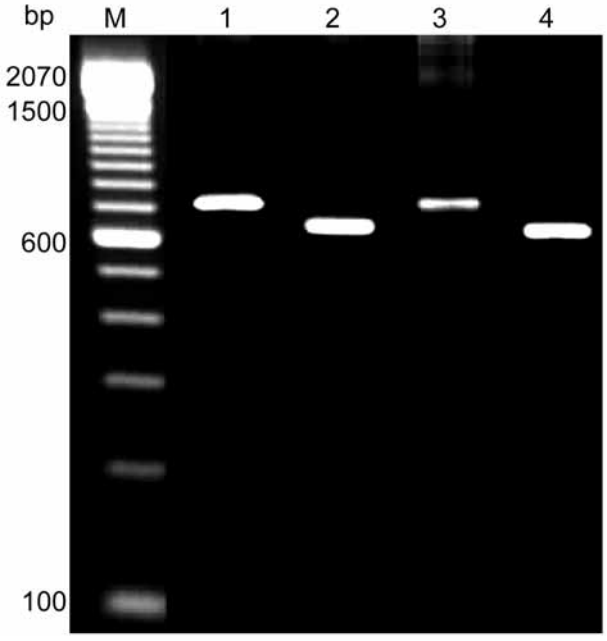

FIG. 1 - PCR amplification of the ITS regions of AMF isolates using primers ITS1/ITS4. M: 100 bp DNA ladder, 1: *Paraglomus occultum*, 2: *Glomus mosseae*, 3: *Glomus intraradices*, 4: *Glomus etunicatum*.

TABLE 1 - ITS length and fragment after restriction of ITS

Arbuscular mycorrhizal fungi	ITS - length class	Fragment (bp)		
		HinfI	MboI	AluI
Glomus etunicatum	600	500 300	590 210	-
Glomus mosseae	600	550 300	300 250 180	-
Glomus intraradices	700	320 250	590 250	-
Paraglomus occultum	700	500	500 300	-

(Lanfranco *et al.*, 1999). Our results showed two polymorphic patterns: one comprising *P. occultum* and *G. intraradices* and the other including the species *G. mosseae* and *G. etunicatum* (Fig. 1). By polymerase chain reaction and sequencing of the ITS region members of seven different families and species, groups within Glomeromycota were identified. The revealed data showed remarkable differences in the composition of AMF taxa (Hempel *et al.*, 2007).

The amplification of the ITS region allowed the distinction of three AMF species at the generic level: *Scutellospora calospora* Walker & Sanders, *Acaulospora laevis* Gerdemann & Trappe and *Glomus deserticola* Trappe; Bloss & Menge using the primer pairs ITS1/ITS2 and ITS1/ITS Harney *et al.* (1997). The spores of these species have enough variation in the ITS region, allowing their genetic distinction in field samples (Harney *et al.*, 1997). Within the rDNA, the non-coding ITS displays a higher polymorphism than the SSU and LSU (White *et al.*, 1990). It is routinely used to identify ectomycorrhizas (Buscot *et al*., 2000) and was demonstrated to be also adequate for AMF identification (Redecker *et al.*, 1997, 2000; Renker *et al.*, 2003). In most of the studies of ITS on AM spores ITS1/ITS4 were used (Antoniolli *et al.*, 2000; Hildebrandt *et al.*, 2001; Renker *et al.*, 2003; Dickie and FitzJohn, 2007).

In our work the ITS1/ITS4 amplification products did not differentiate all the AMF at the species level. But with the help of the restriction enzymes HinfI and MboI digestion (180 to 590 bp) fragments (Table 1) differentiate between the four AMF species (Fig. 2 and 3). The restriction banding patterns showed 2 to 3 fragments per restriction enzyme, with 5 profile for the enzyme HinfI and 6 for MboI (Fig. 2 and 3). No restriction sites were observed for the enzyme AluI (Table 1). Consequently, no variability among these four AMF species was detected with this enzyme. Conversely, the combination of the restriction patterns of the other enzymes allowed the molecular distinction of all AMF species, mainly the profiles obtained with endonuclease MboI. The analysis of the restriction patterns of the amplified

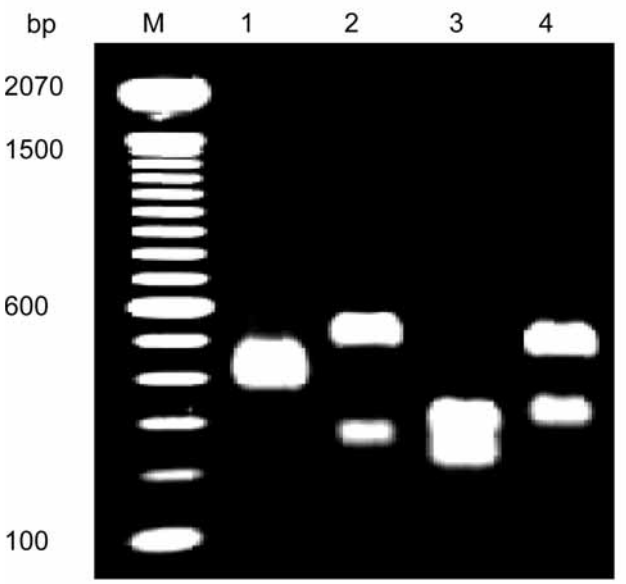

FIG. 2 - HinfI restriction profile of the ITS fragments amplified from AMF spores DNA. M: 100 bp DNA ladder, 1: *Paraglomus occultum*, 2: *Glomus mosseae*, 3: *Glomus intraradices*, 4: *Glomus etunicatum*.

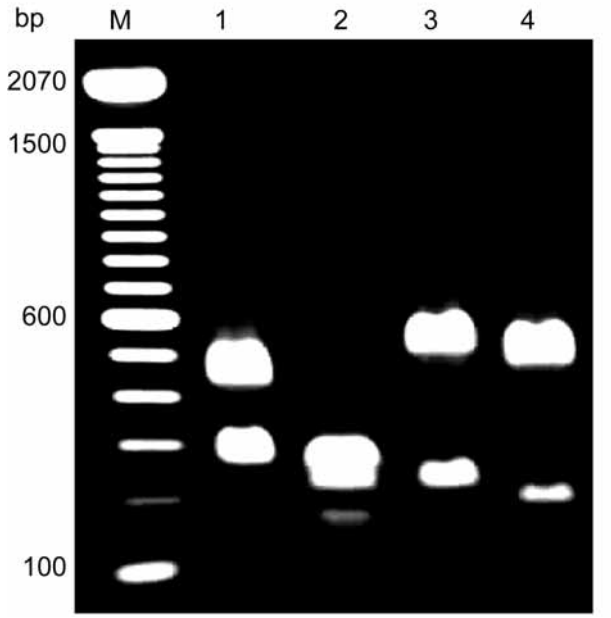

FIG. 3 - MboI restriction profile of the ITS fragments amplified from AMF spores DNA. M: 100 bp DNA ladder, 1: *Paraglomus occultum*, 2: *Glomus mosseae*, 3: *Glomus intraradices*, 4: *Glomus etunicatum*.

rDNA internally transcribed spacer allowed the differentiation of the strains at the intraspecific level due to the easily analysable and reproducible banding patterns generated from ITS region.

Different restriction patterns detected genetically between species, confirm different species. However, if differences are not observed, the species may or may not be identical that depends on the specific restriction site of the restriction enzyme used in the fragment analysis. The lack of restriction sites may have occurred with all AMF digested with the AluI. Redecker *et al.* (1997) also observed a lack of restriction sites for the AluI endonuclease in a great variety of *Glomus* species. *Glomus* sp. strain S328 is an exception. Analysis of the ITS sequence of *G. mosseae* BEG12 (GenBank access nº X84232) reported by Sanders *et al.* (1995) confirmed the absence of the AluI site. A search among ITS sequences available in GenBank (http://www.ncbi.nlm.nih.gov/) indicated that for most members of the Glomeromycota, the ITS displays contain no restriction site for AluI (Renker *et al.*, 2003).

The digestion of the ITS fragment with HinfI and MboI was effective for the inter- and intra-specific separation of AMF, constituting an additional technique to study these fungi, and may be used to resolve controversies in the taxonomy of the group. The molecular markers are as important as the morphological and physiological methods. Molecular methods contribute to increase the knowledge of identity and variability of the AMF. The results confirm that the PCR/RFLP technique, using the MboI and HinfI enzyme, has a potential for utilization as a marker to differentiate between species of AMF with high phylogenetic affinity.

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