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Characterisation of arbuscular mycorrhizal fungi in roots by means of epifluorescence microscopy and molecular methods

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Abstract - In this work we characterised different species of the arbuscular mycorrhizal (AM) fungal genus *Glomus* by using Restriction Fragment Length Polymorphisms (RFLPs), combining epifluorescence microscopy and nested PCR, for the easy retrieval of AM fungal DNA *in planta*. Epifluorescence microscopy allowed us to select highly colonised root segments for DNA extraction, enhancing the chance of fungal DNA amplification by PCR. The primer pair used in nested PCR after a first reaction performed by using NS31/AM1 primers, showed a high specificity for *Glomus* DNA amplification and yielded enough fungal DNA for RFLP analyses. RFLP patterns of PCR products from colonised roots matched with reference profiles obtained from spores and discriminated the four *Glomus* species tested. The method described may be useful for checking the establishment of mycorrhizal colonisation by the inoculated fungal species in controlled experimental conditions.

Key words: arbuscular mycorrhizal fungi, autofluorescence, nested PCR, RFLP patterns, detection *in planta.*

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are distributed worldwide and establish symbioses with the roots of about 80% of plant species. They are fundamental for soil fertility and plant nutrition as they produce large mycelial networks which uptake and transfer phosphate and other mineral nutrients from the soil to host plants (Smith and Read, 1997). AM fungi play different functional roles in different environmental conditions, whose knowledge provides keys to understand the complex plant/fungal interactions affecting plant productivity and the stability of plant community structure (van der Heijden *et al*., 1998a, 1998b). Such high functional diversity was reported to be correlated with taxonomic, phenotypic and genetic variation among isolates and species of the AM fungal symbionts (Koch *et al*., 2004; Munkvold *et al*., 2004; van der Heijden *et al*., 2004). About 200 different species of AM fungi (Phylum Glomeromycota) were described on the basis of morphological characteristics of spores, which lead to the recognition of orders, families and genera (Gerdemann and Trappe, 1974; Walker, 1983; Morton, 1988), whose taxonomic and phylogenetic consistency was evaluated by molecular studies (Schüßler *et al*., 2001). Though, when spores are not available, identification *in planta* represents the only reliable method to describe AM fungi actively colonising host plant roots. Since morphological characters of intraradical fungal structures are able only to discriminate at the family level, PCR-based detection methods, mainly targeted to the ribosomal DNA gene regions containing sequences of different variability, were recently introduced to identify AM fungi *in planta*.

Different molecular strategies have been proposed, such as the development of a set of PCR primers targeted at five major phylogenetic subgroups of Glomeromycota, designed for the specific amplification of internal transcribed spacers (ITS) and small ribosomal subunit (SSU) from roots (Redecker, 2000). Another strategy to characterise AM fungi within plant roots in the field is represented by a combination of nested PCR and *AluI* digestion of the ITS region (Renker *et al*., 2003).

Taxon-discriminating molecular primers, based on variability of the large ribosomal subunit (LSU, 25S rDNA) were developed to detect AM fungal strains or groups of species within plant roots (van Tuinen *et al*., 1998; Kjoller and Rosendhal, 2000; Geue and Hock, 2004; Gollotte *et al*., 2004; Rosendahl and Stuckenbrock, 2004; Farmer *et al*., 2007).

The small subunit of the ribosomal DNA gene (SSU, 18S rDNA) represented the target of different specific primers designed to identify AM fungi from both spores and roots (Simon *et al*., 1992, 1993; Helgason *et al*., 1998). Moreover, the new phylum of *Glomeromycota* was proposed on the basis of sequence data of the SSU (Schüßler *et al*., 2001), and a large database of sequences is now available. A fundamental step towards the characterisation of AM fungal diversity in the field is the utilisation of the primer AM1, designed to exclude plant DNA sequences after extraction from roots. Such primer, utilised in combination with NS31, allowed the differentiation of AM fungal

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TABLE 1 - Geographic origin and inoculum source of AM fungal isolates used in this study

Glomus isolates	Local code	IBG[a] code	Geographic origin	Collector	Original inoculum supplier[b]
G. mosseae	IMA1	BEG 12	Kent (UK)	B. Mosse	Rothamsted Exp. St. (UK)
G. mosseae	IN101C	-	Indiana (USA)	R. Kemery	INVAM, Morgantown, W. Va.
G. *intraradices*	IMA5	-	Liguria (I)	M. Giovannetti	IMA, Pisa (I)
G. *intraradices*	IMA6	BEG 141	Burgundy (F)	A. Trouvelot	INRA, Dijon (F)
G. viscosum	IMA4	BEG 27	Tuscany (I)	M. Giovannetti	IMA, Pisa (I)
G. coronatum	IMA3	BEG 28	Tuscany (I)	M. Giovannetti	IMA, Pisa (I)
Glomus sp. AD-1	AD-1	BEG 139	Abu Dhabi (UAE)	J. Dodd	IIB, Canterbury (UK)

[a] IBG: International Bank of Glomeromycota; [b] Exp. St.: Experimental Station; INVAM: International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi; IMA: International Microbial Archives; INRA: Institut National de la Recherche Agronomique; IIB: International Institute of Biotechnology.

taxa colonising the roots of plants collected from woodland and from adjacent arable sites (Helgason *et al*., 1998).

These primers have successfully been used in many works to study the structure of AM fungal communities growing in natural and agricultural ecosystems, since the variability of the region amplified allowed the resolution of taxonomic units at approximately the species level (Opik *et al*., 2006). Such variability could be exploited in lab and greenhouse experiments to monitor the establishment of mycorrhizal colonisation by different AM fungal species without the need of designing taxon-discriminating primers. To this aim, we combined epifluorescence microscopy and a nested PCR method based on NS31/AM1 primers in order to amplify AM fungal DNA only from root segments showing colonisation, and to discriminate different *Glomus* species by RFLP analysis.

MATERIALS AND METHODS

AM fungal isolates. The experiments were carried out on *Glomus mosseae* (Nicolson et Gerdemann) Gerdemann et Trappe (two isolates), *Glomus intraradices* Schenck et Smith (two isolates), *Glomus coronatum* Giovannetti, *Glomus* sp. AD-1, *Glomus viscosum* Nicolson, colonising the roots of *Petroselinum crispum* L. and *Allium porrum* L. plants, maintained in the pot culture collection of the Department of Crop Plant Biology, University of Pisa, Italy. Details of the isolates are given in Table 1.

Spores of *Glomus* isolates were recovered from pot-cultures by wet-sieving and decanting, down to a mesh size of 100 µm (Gerdemann and Nicolson, 1963). Root samples were recovered from the 400 µm sieve. Root pieces of 1-2 cm were mounted on slides in water and observed under the fluorescence microscope before DNA extraction. Infected root pieces were blotted dry with paper towels, frozen with liquid nitrogen and stored at –80 °C until used.

Fluorescence test. Root pieces mounted on slides were observed under a Reichert-Jung Polyvar (Wien, Austria) microscope equipped with Nomarski interference contrast optics and with epifluorescence optics (HBO 200 mercury lamp; Osram, Munchen, Germany). Autofluorescence of whole roots mounted in water was assessed with B1 filter combination: BP 450-495, LP 520, DS 510. Root samples showing detectable level of autofluorescence were selected for DNA extraction.

DNA extractions.

Extraction from roots. DNA was extracted from 1-2 cm of plant roots using the potassium ethyl xanthate method (Edwards *et al*., 1997). Plant roots were reduced to powder using liquid nitrogen, re-suspended in extraction buffer (6.25 mM potassium ethyl xanthate, 100 mM Tris-HCl pH 7.5, 700 mM NaCl, 10 mM EDTA pH 8) and incubated at 65 °C for 1 h. Plant and fungal DNA was extracted twice (1v:1v) with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with isopropanol (1v:1v) and 3M sodium acetate pH 5.2 (1v:1/10v) for 1 h at room temperature and centrifuged at 12000 rpm for 25 min. The pellet was washed with 70% ethanol, dried under vacuum (Concentrator 5301 Eppendorf, Hamburg, Germany) and re-suspended in 50 µl of ultrapure water; 10 ng of DNA were used for PCR amplifications.

Extraction from spores. Intact, healthy spores of the seven fungal isolates were manually collected with a capillary pipette under a dissecting microscope (Wild, Leica, Milano, Italy). They were sonicated two times (1 min each) in a B-1210 cleaner (Branson Ultrasonics, Soest, The Netherlands) and washed three times in sterile bidistilled water. Spores were selected once more under the dissecting microscope and transferred in Eppendorf tubes before DNA extraction, according to the protocol described by Redecker *et al*. (1997). Briefly, 5 spores were crushed using a glass pestle in 10 µl of 0.25 M NaOH, then incubated in boiling water (1 min). After adding 5 µl of 0.5 M Tris-HCl (pH 8) and 10 µl of 0.25 M HCl the Eppendorf tubes were dipped again in boiling water (2 min). Extracts (1 µl) were directly used for PCR amplifications.

PCR amplifications. Partial SSU rDNA sequences from both spores and plant roots were amplified in 25 µl of a primary PCR reaction mix using 0.625 U of AmpliTaq Gold DNA Polymerase (Applied Biosystem, Milan, Italy), 10 pmol of the primers NS31 (Simon *et al*., 1992) and AM1 (Helgason *et al*., 1998), 0.2 mM (each) dNTPs, 1.5 mM $MgCl_2$ and the manufacturer's reaction buffer. A thermal cycler (Eppendorf Mastercycler® personal, Eppendorf, Milan, Italy) was programmed as follows: 95 °C for 9 min, 10 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, 25 cycles at 95 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min, 1 cycle at 72 °C for 10 min.

A nested PCR reaction was performed by diluting (1/10-100-1000) the first PCR amplification and using 2 µl of dilutions as template for the second reaction in a final volume of 50 µl. The newly designed primer pair SSU-1f (5'-AGC AGC CGC GGT AAT TCC A-3')/ SSU-2r (5'-AAG GCG CCG AAT GAG TCA TTA-3') was added in the PCR mix (10 pmol for each primer). Taq DNA polymerase, dNTPs and $MgCl_2$ concentrations were the same described above. Amplification conditions were as follows: 95 °C for 5 min, 32 cycles at 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min, 1 cycle at 72 °C for 10 min.

PCR products were examined by 1.5% agarose (Bio-Rad, Milano, Italy) gel electrophoresis containing ethidium bromide (0.5 µg/ml).

Restriction enzyme analysis. Nested amplified DNA (10 µl) from spores and roots was digested with *MboI, HinfI* (Takara, Madison, Wi, USA), *TaqI* (Biolabs, Hitchin, UK), *RsaI, TasI* (Fermentas UAB, Lithuania) restriction enzymes, according to manufacturer's instructions. Digested DNA was electrophoresed through 2% MetaPhor agarose (BMA, Rockland, ME USA) containing ethidium bromide (0.5 µg/ml) and a 1 kb Plus DNA Ladder (Invitrogen, Milano, Italy) was used as a molecular weight marker. DNA profiles were acquired with the ImageMaster VDS system and analysed using ImageMaster 1D Elite software (Amersham Bioscience, Cologno Monzese, Italy).

RESULTS

Fluorescence test on roots

Plant root pieces were observed under the fluorescence microscope, using the B1 filter, in order to optimise a protocol for the retrieval of AM fungal DNA from plant roots. The establishment of mycorrhizal symbiosis was shown by a strong increase in autofluorescence of plant roots inoculated with all *Glomus* isolates. Arbuscules were the fungal structures showing the highest level of autofluorescence, appearing bright yellow when excited with blue light (Fig. 1). The intensity of fluorescence varied among root pieces and only root samples showing more than 50% of colonised root length were selected for DNA extraction.

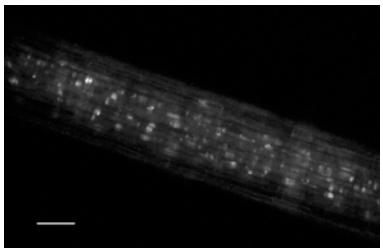

FIG. 1 - Autofluorescence of *Glomus mosseae* arbuscules within fresh roots of *Allium porrum*. *Bar* 125 µm.

Nested PCR on roots

Fungal DNA from colonised root segments was amplified with the couple of primers NS31/AM1 (Fig. 2A). The amplification product was ~550 bp, the expected size for the V3-V4 variable regions of SSU of the AM fungi tested, *G. mosseae*, *G. coronatum*, *G. intraradices, G. viscosum*, *Glomus* sp. AD-1. A nested PCR was performed to enhance the efficiency of amplification and to increase the amount of DNA available for further analyses. Primers SSU-1f/SSU-2r, which were designed from alignments of the 18S rDNA sequences of Glomeraceae, Gigasporaceae and Acaulosporaceae retrieved from database, allowed the amplification of our *Glomus* species. The first amplification product diluted 10, 100 and 1000 times always gave a band of the expected size (520 bp) (Fig. 2B). Occasionally, no bands corresponding to fungal rDNA were obtained from mycorrhizal root fragments after the first PCR, while a specific amplification product was always detected after the second amplification, demonstrating the high sensitivity of the nested PCR. In addition, we always observed that DNA from uncolonised leek roots was amplified by NS31/AM1 primers, generating a fragment of 210 bp. Nevertheless, nested PCR performed with the internal primers SSU-1f/SSU-2r specifically amplified *Glomus* fungal DNA and did not produce any aspecific band (data not shown).

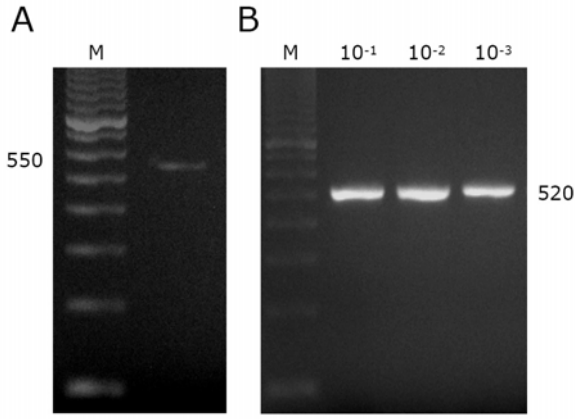

FIG. 2 - A: Amplification products obtained by using NS31/AM1 primers on DNA from leek roots colonised by *Glomus mosseae* IMA1. B: Second amplification of nested PCR using SSU-1f/SSU-2r primers on serially diluted NS31/AM1 products. M (100 bp ladder, Amersham). Sizes of amplifications products are indicated (bp).

RFLP analysis

In order to characterise the different *Glomus* species, the SSU-1f/SSU-2r PCR products, both from spores and roots, were digested with five restriction enzymes. The RFLP patterns obtained from spores matched with those from roots (Figs. 3, 4) and corresponded to the expected theoretical profiles worked out from the database sequences of the different *Glomus* species analysed. Theoretical and actual fragment sizes are reported in Table 2.

TaqI and *MboI* identified two different profiles, discriminating *G. intraradices* from the other species (Table 2). *RsaI* and *HinfI* produced three patterns (Fig. 3, Table 2) and were able to distinguish *G. intraradices* from *G. viscosum* and from the *G. mosseae*/*G. coronatum*/*Glomus* sp. AD-1 group. Interestingly, *RsaI* evidenced the occurrence of two different sequences in *G. viscosum* IMA4 (Fig. 3A), one lacking restriction sites - in accordance with the sequence Y17652 of *G. viscosum* isolate BEG27, a sibling of IMA4 - and the other showing one restriction site, occur-

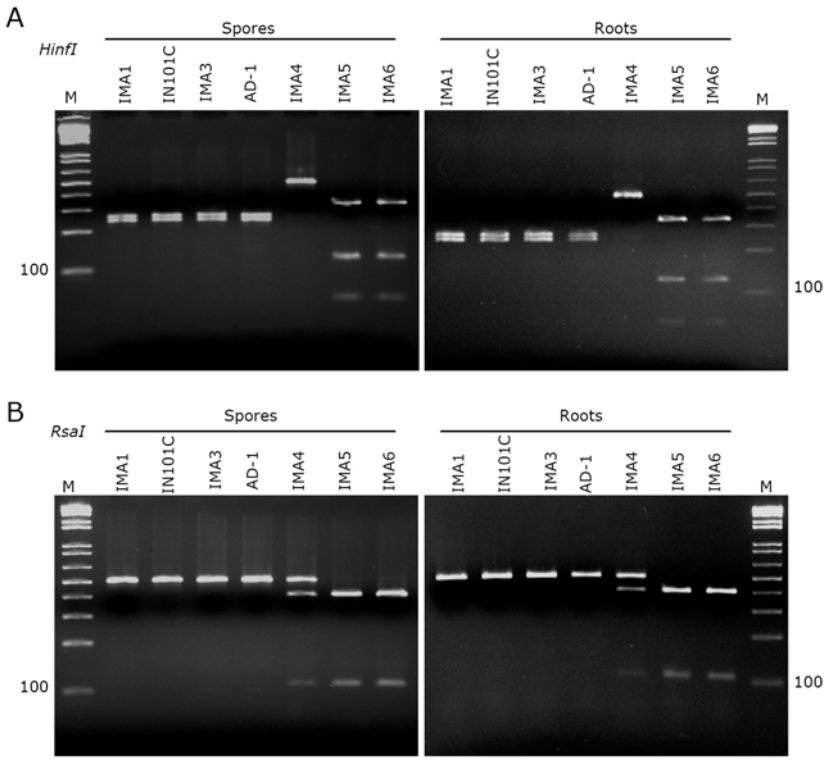

FIG. 3 - *HinfI* (A) and *RsaI* (B) restriction patterns of PCR products from spores and root segments colonised by different *Glomus* isolates. M (1 kb Plus DNA Ladder, Invitrogen); IMA1 and IN101C (*Glomus mosseae*), IMA3 (*Glomus coronatum*), AD-1 (*Glomus* sp.), IMA4 (*Glomus viscosum*), IMA5 and IMA6 (*Glomus intraradices*).

ring in the sequences AJ505620, AJ505812, AJ505813 of the isolates EEZ20, BEG126 and EEZ34, respectively.

The restriction enzyme *TasI* was selected since it was able to discriminate 15 *G. mosseae* sequences of different isolates present in GenBank from one sequence of an Australian *G. coronatum* isolate. Actually, RFLP analysis produced four patterns: one for *G. mosseae* isolates, one for the Italian *G. coronatum* and *Glomus* sp. AD-1, one for *G. viscosum* and one for *G. intraradices* isolates (Fig. 4; Table 2).

DISCUSSION

In this work we characterised different species of the AM fungal genus *Glomus* by using RFLPs, combining epifluorescence microscopy and nested PCR for the retrieval of fungal DNA *in planta*. Plant roots colonised by AM fungi cannot be distinguished from non-mycorrhizal roots without specific staining. This fact entails the use of a large amount of plant material to increase the probability of obtaining infected root pieces for AM fungal DNA recover-

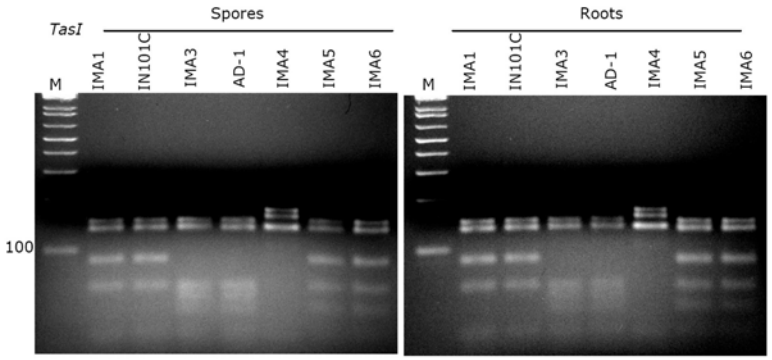

FIG. 4 - *TasI* digests of PCR products from spores and root segments colonised by different AM fungi. M (1 kb Plus DNA Ladder, Invitrogen); IMA1 and IN101C (*Glomus mosseae*), IMA3 (*Glomus coronatum*), AD-1 (*Glomus* sp.), IMA4 (*Glomus viscosum*), IMA5 and IMA6 (*Glomus intraradices*).

TABLE 2 - Restriction fragment lengths of SSU-1f/SSU-2r PCR products both from spores of the different AMF isolates and from colonised roots

Restriction enzyme	Isolates	Profile fragment lengths (bp)	Total length (bp)
TaqI	IMA1, IN101C, IMA3, AD-1, IMA4	285, 141, 93	520
	IMA5, IMA6	257, 168, 93	520
MboI	IMA1, IN101C, IMA3, AD-1, IMA4	206, 141, 76, 45, 41, *10*[a]	520
	IMA5, IMA6	258, 164, 45, 41, *10*	520
RsaI	IMA1, IN101C, IMA3, AD-1	520	520
	IMA4	520, 411, 109	520
	IMA5, IMA6	411, 109	520
HinfI	IMA1, IN101C, IMA3, AD-1	260, 244, *16*	520
	IMA4	504, *16*	520
	IMA5, IMA6	334, 120, 49,*16*	520
TasI	IMA1, IN101C	150, 134, 85, 56, *27, 23, 17, 15, 12*	520
	IMA3, AD-1	150, 134, 56, 47, 38, *27, 23, 17, 15, 12*	520
	IMA4	177, 157, 134, *23, 15, 13*	520
	IMA5, IMA6	150, 134, 84, 56, 40, *27, 15, 13*	520

[a] Fragments under 40 bp size (italics) are theoretical and deduced on database sequences.

ing, since the risk of selecting uncolonised roots is quite high in the presence of a low AM fungal colonisation (Kowalchuk *et al*., 2002). One simple and rapid method for the detection of mycorrhizal infection relies on the autofluorescence of arbuscules under ultraviolet light (Ames *et al*., 1982). This non-destructive approach has proven to be a highly effective method for the assessment of metabolically active fungal structures in host roots (Gange *et al*., 1999), and allowed us to select only colonised root segments for DNA extraction, enhancing the chance of fungal DNA amplification by PCR, even with a limited amount of plant material. Other authors used different strategies to verify the occurrence of AM fungi within plant roots, which involved the amplification of DNA either from root pieces stained with Trypan blue or from the alkaline extraction mixture obtained during root staining (van Tuinen *et al*., 1998; Redecker, 2000).

In combination with autofluorescence, we used a nested PCR protocol which was successfully utilised for the detection of AM fungi in roots, because of its ability to improve specificity and sensitivity (van Tuinen *et al*., 1998; Redecker, 2000; Turnau *et al*., 2001; Geue and Hock, 2004). Nested PCR enables to analyse very small root sectors. This reduces the risk to have too complex mix of target regions in the DNA extracts, which may give biased picture of the AMF species colonising roots due to competitive inhibition during PCR. The newly designed SSU-1f/SSU-2r primers allowed us to amplify *Glomus* fungal DNA also when aspecific sequences occurred in the first PCR step performed with NS31/AM1 primers.

We extracted DNA from root segments, whose total length did not exceed 1-2 cm, and obtained enough fungal DNA to be used for RFLP analyses. Such molecular method was used for the identification of ectomycorrhizal fungi colonising root tips (Horton and Bruns, 2001) and for the characterisation of AM fungi (Redecker, 2000). Actually, RFLP patterns may produce as informative data as the more expensive and time consuming DNA sequencing, and could help to characterise AM fungal taxa, once deposited in available databases (Redecker *et al*., 2003). RFLP profiles of the V3-V4 region of 18S rDNA were considered enough variable as to separate taxonomic units at approximately the species level (Opik *et al*., 2006). In this work we discriminated *Glomus* species by means of RFLP patterns of PCR products from colonised roots, which matched with reference profiles obtained from spores. *TasI* endonuclease produced different patterns for the different *Glomus* species, differentiating *G. mosseae* from *G. coronatum*, which could not be discriminated by any of the other restriction enzymes. *Glomus* sp. AD-1 and *G. coronatum*, which share most morphological features, showed the same RFLP patterns, confirming previous reports on their taxonomical relatedness (Dodd *et al*., 1996). Interestingly, *TasI*, chosen on the basis of the unique available 18S sequence of the Australian isolate of *G. coronatum* (AJ276086), was able to recognise the same restriction sites, which are highly conserved in isolates originating from Australia, Europe and Near East Asia. *RsaI* evidenced the occurrence of multiple sequences in our isolate of *G. viscosum*, confirming previous data on sequence variability of 18S rDNA, detected in *Glomus clarum* and *Scutellospora* sp. (Clapp *et al*., 1999; Kowalchuk *et al*., 2002). Sequence variability, even in single spores, was previously demonstrated in AM fungi, both for repetitive sequences, such as ITS (Sanders *et al*., 1995; Hijri *et al*., 1999; Jansa *et al*., 2002; Giovannetti *et al*., 2003; Koch *et al*., 2004), 25S rDNA (Rosendhal and Stukenbrock, 2004) and for single copy genes, such as DNA polymerase a (POL1-like) (Pawlowska and Taylor, 2004).

Our data show that RFLP analysis is a powerful diagnostic tool - sometimes more useful than sequencing - even in the presence of multiple sequences, providing a wide and direct picture of sequence variability within the same isolates and spores (Redecker *et al*., 1997; Kuhn *et al*., 2001). Accordingly, it could be utilised as a standard method to check the establishment of mycorrhizal colonisation by the inoculated fungal species in controlled experimental conditions.

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