

A combined morphological and molecular approach to characterize isolates of arbuscular mycorrhizal fungi in *Gigaspora* (Glomales)

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Summary

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- Morphological features of resting spores and information from nucleotide sequences of ribosomal RNA were used to characterize seven mycorrhizal fungal isolates in *Gigaspora* from different geographical areas.
- Detailed observations were made under the light microscope on single spores mounted in Melzer's reagent and polyvinyl alcohol-lactic acid-glycerol medium to resolve size, colour and cell wall structures. Neighbour-joining analyses were carried out on a portion of the 18S gene and on the internal transcribed spacer (ITS) region amplified by PCR from multispore DNA preparations.
- Combined data allowed us to design oligonucleotides that unambiguously distinguished *Gi. rosea* from *Gi. margarita* and *Gi. gigantea* and also identified two isolates as *Gi. rosea* that had been previously diagnosed as *Gi. margarita*. ITS sequences revealed substantial genetic variability within clones of a single isolate of *Gi. rosea* as well as among geographically disjunct *Gi. rosea* isolates.
- The results show how complementary morphological and molecular data can clarify relationships among species of low morphological divergence. Sequence information allowed the extent of genetic divergence within these species to be investigated and provided useful PCR primers for detection and identification.

Key words: *Glomales*, *Gigaspora*, ribosomal sequences, primers, identification, mycorrhizal roots.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophic organisms, whose growth and hyphal development depend on the symbiotic association with plant roots. AM mycorrhizas are an example of an evolutionarily stable and compatible plant–microorganism interaction. Regardless of whether research is basic or applied, correct identification of isolates is an important requisite. Similarly, isolates deposited in public collections responsible for the preservation of fungal biodiversity and distribution of reference strains (Morton *et al.*, 1993; Dodd *et al.*, 1994) must be identified with certainty.

Grouping and ranking isolates of AM fungi as taxonomic units has been possible using morphological traits of spores and other fungal structures (Walker, 1992; Bianciotto & Bonfante, 1999). Mycorrhizal structures such as abundance of coiled and swollen hyphae together with arbuscules that have swollen hyphal trunks, spores formed singly in soil from a distinctive sporogenous cell, and extraradical thin-walled auxiliary cells resolve the family Gigasporaceae. The two genera in this family, *Gigaspora* and *Scutellospora*, are separated by absence and presence, respectively, of hyaline flexible inner (or 'germinal') spore walls (Morton & Benny, 1990). In *Scutellospora*, germination is from a germination shield on the innermost of these flexible walls and hence is

spatially and temporally independent of spore wall development (Morton, 1995). The resultant absence of constraints on spore wall development may explain the greater range of possible wall phenotypes (and thus species) in *Scutellospora* (Morton *et al.*, 1995). By contrast, germination originates from the inner layer of the spore wall in *Gigaspora*, and the low number of species in this genus suggests strong constraints on the breadth of possible spore wall phenotypes. Only one–three characters (size, colour, possibly thickness) are recognized in *Gigaspora*, and they are continuous rather than discrete (Bentivenga & Morton, 1995). As a result, the five species described to date may not be easily distinguished under field conditions, where age or degradation causes a merging of phenotypes and difficulty in identification (Bago *et al.*, 1998; Merryweather & Fitter, 1998). Fresh spores most abundantly recovered from active glasshouse pot cultures provide the clearest distinction between morphological traits.

Simon *et al.* (1993) were the first to target nuclear ribosomal RNA genes of AM fungi as a means to study the relationship between morphological and molecular divergence in an evolutionary context. Other conserved gene sequences, differential primers, and cloning techniques (Sanders *et al.*, 1995; Redecker *et al.*, 1997; van Tuinen *et al.*, 1998; Clapp *et al.*, 1999; Lanfranco *et al.*, 1999) have provided additional information on genetic diversity among nuclei within spores

and among spores of different isolates. Wider investigations on this genetic diversity are an important prerequisite to develop specific molecular tools to be used for diagnostic purposes.

The aim of the present study is to apply both morphological and molecular criteria to differentiate geographically separated isolates of *Gigaspora*. Since a simple ITS-RFLP analysis does not seem useful in *Gigaspora* because of the few polymorphic sites (Redecker *et al.*, 1997), sequencing of a portion of the 18S rDNA and the ITS region was used to investigate the diversity within the genus and to develop species-specific primers. Combined data allowed us to design oligonucleotides able to unambiguously recognize *Gi. rosea* isolates from *Gi. margarita* and *Gi. gigantea* and also to verify identity of two isolates as *Gi. rosea* morphologically diagnosed as *Gi. margarita*.

Materials and Methods

Biological material

All of the fungal isolates compared in this study (Table 1), except the *Scutellospora* species from Porto Caleri, were propagated in glasshouse pot cultures. Spores of *Scutellospora* were collected along Italian coastal sand dunes situated on the east (Porto Caleri, near Rovigo) coast.

Morphologically identified as	Origin	Isolate code and relative bank	Provided by
<i>Gigaspora margarita</i> Becker & Hall	West Virginia USA	WV205A-5 INVAM	J. Morton
<i>Gigaspora margarita</i> Becker & Hall	New Zealand	BEG34	V. Gianinazzi-Pearson
<i>Gigaspora margarita/rosea</i> Nicolson & Schenck	Unknown	194757 DAOM	D. Douds and G. Bécard
<i>Gigaspora margarita/rosea</i> Nicolson & Schenck	Brazil	E29 HC/F	M. Souza
<i>Gigaspora rosea</i> Nicolson & Schenck	USA	BEG 9	V. Gianinazzi-Pearson
<i>Gigaspora rosea</i> Nicolson & Schenck	Florida USA	FL185 INVAM	D. Douds
<i>Gigaspora gigantea</i> (Nicol. & Gerd.) Gerdemann & Trappe	Pennsylvania USA	E30 HC/F	D. Douds
<i>Gigaspora gigantea</i> (Nicol. & Gerd.) Gerdemann & Trappe	USA	E31 HC/F	G. Bécard
<i>Gigaspora gigantea</i> (Nicol. & Gerd.) Gerdemann & Trappe	USA	MA453A-7 INVAM	INVAM
<i>Scutellospora persica</i> Walker & Sanders	Porto Caleri (Rovigo) Italy	E28 HC/F	V. Bianciotto

Table 1 List of arbuscular mycorrhizal fungi (AMF) isolates analysed in this study

BEG, European Bank of Glomales; DAOM, Department of Agriculture, Ottawa, Mycology; INVAM, International culture collection of arbuscular and Vesicular–Arbuscular Mycorrhizal fungi. HC/F, Herbarium Cryptogamicum Fungi, Department of Plant Biology, Turin, Italy.

Morphological description

Spores were separated by hand, broken and mounted in Melzer's reagent and polyvinyl alcohol-lactic acid-glycerol medium (PVLG) (Koske & Tessier, 1983) and observed under a Zeiss microscope (Carl Zeiss, Oberkochen, Germany). To check for the presence of intracellular bacteria the sporal cytoplasm was stained with the Live/Dead *Bac*-Light bacterial viability kit (Molecular Probes, Inc., Eugene, OR, USA) and observed as previously described in Bianciotto *et al.* (1996).

DNA extraction from pool of spores and mycorrhizal roots

AM spores were cleaned by sterilization with cloramine T (4%) and streptomycin (0.004%) and by four rounds of sonication. Five to 10 spores were crushed in 30 µl of 1X REDTaq PCR Reaction Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM MgCl₂ and 0.01% gelatine) and incubated at 95°C for 15 min, centrifuged for 5 min at 10 000 g, and the supernatant stored at -20°C. Genomic DNA was extracted from mycorrhizal roots of *Trifolium repens* and *Araucaria angustifolia* as described by Lanfranco *et al.* (1999).

PCR amplifications

A number of DNA dilutions (undiluted, 1 : 10, 1 : 50) were tested for optimum amplification. Reactions were carried out in a final volume of 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM MgCl₂, 0.01% gelatin, 200 µM each dNTPs, 1 µM of each primer NS1-NS2 or ITS1-ITS4 (White *et al.*, 1990) and 2 units of REDTaqTM DNA polymerase (SIGMA) (Sigma-Aldrich, S.r.l. Milan, Italy). The PCR programme was as follows: 95°C 3 min (1 cycle), 95°C 45 s, 50°C 45 s, 72°C 1 min (35 cycles), 72°C 5 min (1 cycle). PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. In all PCR experiments, negative controls consisted of reaction mixture without template DNA.

Cloning and sequencing

The amplified products were purified on agarose gel using the QIAEX II Gel Extraction Kit (Qiagen S.p.A., Milan, Italy) and directly cloned into pGEM-T vector with a cloning kit from Promega (Madison, WI, USA). XL-2 Blue ultracompetent cells (Stratagene) were transformed and plated on selective medium following the manufacturer's instructions. Plasmid DNAs were prepared with the Qiagen Plasmid Mini Kit (Qiagen). The sequences were determined by the Service de Séquençage, Université Laval, Québec (Canada) from both strands using T7 and Sp6 primers. The sequences have been deposited at the EMBL data library under the accession numbers shown in Fig. 3 and Fig. 4.

Sequence analysis

DNA sequence analyses were performed with PC/gene software (IntelliGenetics, Inc. Mountain View, CA, USA). Sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1997) and manually corrected to optimize aligned sites with GeneDoc (Nicholas *et al.*, 1997). Neighbour-joining analysis, with Kimura 2-parameter model correction for multiple substitutions, was carried out with CLUSTAL X and the trees were visualized with TreeView (Page, 1996). To investigate node supports, 1000 bootstrap replications were performed. Maximum parsimony analysis was carried out with all characters weighted equally and gaps treated as missing. The analysis was performed with the heuristic search algorithm with branch swapping made by the tree-bisection-reconnection algorithm; input order bias for the same dataset was minimized by performing 100 replicates with random addition of taxa.

Generation of ITS specific primers

From sequence alignments, two new specific primers GiR2 (5'-ATCACGAAGTAAATTACTTGG-3') and GiR3 (5'-ATCACCACCTACTTGGTAG-3') were designed and used in different combinations with primers GiITS1 and GiITS2 (Lanfranco *et al.*, 1999). PCR conditions were as described above, except the annealing temperature was 55°C.

Results

Morphological analysis

Upon examination of fungal isolates belonging to *Gigaspora* from public or private collections, isolates *Gi. margarita* DAOM 194757 and *Gi. margarita* E29 appeared to share similar features with *Gi. rosea*. Spore size ranged from 160 to 280 µm for both isolates, whereas *Gi. margarita* (BEG34, as reference strain) spores ranged from 260 to 400 µm (shown at the same magnification in Figs 1 and 2). In addition, spores were white to cream and rose-pink, as expected for *Gi. rosea*, while *Gi. margarita* spores generally are white to pale yellow. A more detailed observation under the light microscope showed that the two species differed in their cell wall structures. In particular, young spores of *Gi. margarita* possessed a spore wall with laminate layers that merged and resembled 'waves' without sharp transitions from ridge to trough (Fig. 1). By contrast, this property was not observed in the laminate spore wall layer of isolates E29 (Fig. 2a,b) and DAOM 194757 (Fig. 2c,d) as well as in the spore wall of the reference isolate *Gi. rosea* FL185 (Fig. 2e,f). When observed by fluorescent confocal microscopy after staining with the Live/dead *Bac*-Light kit (Molecular Probes), isolates DAOM 194757 and E29 did not contain intracellular bacteria, a feature common to all isolates so far analysed of *Gi. margarita* (Bianciotto *et al.*, 2000).

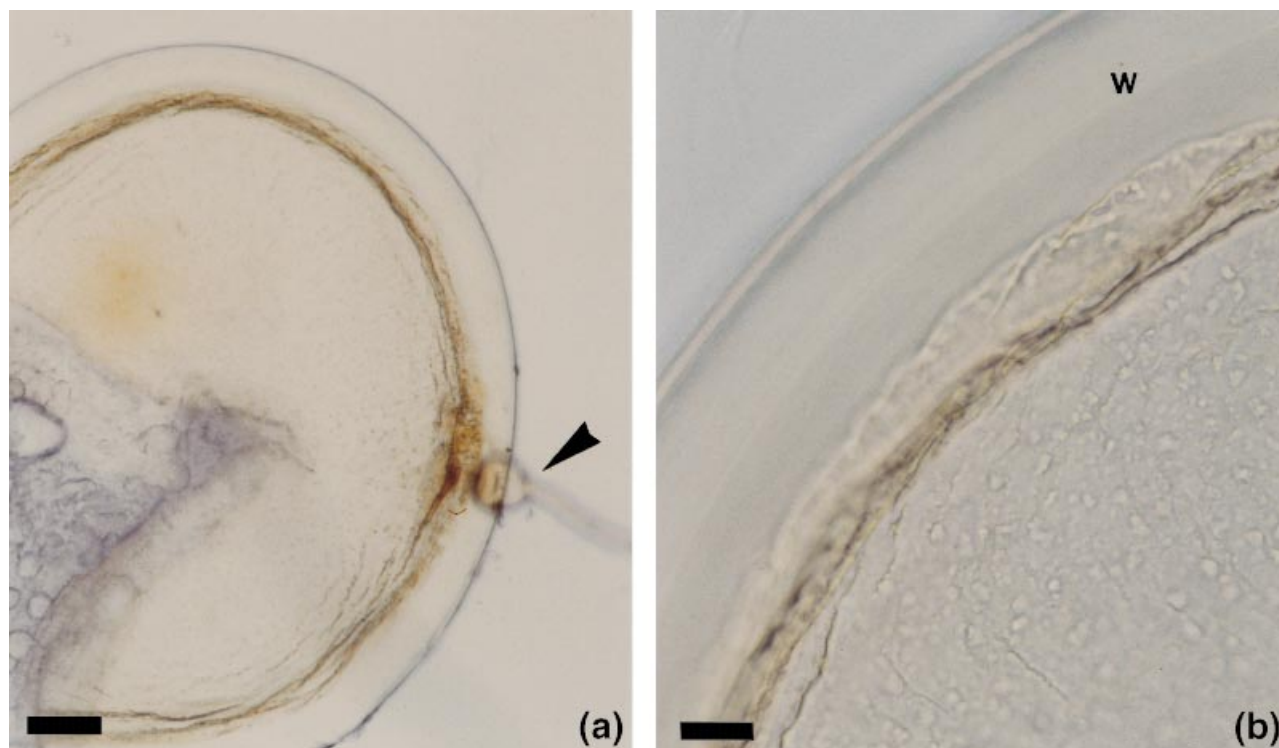


Fig. 1 Crushed spore of *Gigaspora margarita* BEG 34 mounted in polyvinyl alcohol-lactic acid-glycerol medium (PVLG). (a) Note the spore size and the bulbous sporogenous cell (arrow). Bar, 50 μ m. (b) Morphological features of the spore wall (w). *Gi. margarita* possess a spore wall with laminate layers that merged and resembled 'waves' without sharp transitions from ridge to trough. Bar, 9 μ m.

Spores belonging to Gigasporaceae collected from a Mediterranean Italian site (Porto Caleri) were also included in this study. The isolate was identified as *Scutellospora persica*: spores of this species are very similar to those of *S. castanea* in all properties, except that *S. castanea* has a smooth outer spore wall layer (Walker *et al.*, 1993).

Analysis of ribosomal sequences

To solve the ambiguity concerning the *Gigaspora* isolates, a portion of 550 bp from the 5' end of the 18S ribosomal gene was amplified from seven isolates, cloned into a plasmid and sequenced. All sequences were aligned with those from Gigasporaceae currently available in GenBank. A tree was constructed using the Neighbour-joining method, with *Glomus etunicatum* as outgroup (Fig. 3). For some isolates (*Gi. gigantea* E30, *Gi. gigantea* E31, *Gi. margarita/rosea* E29) different clones were sequenced. The only well supported cluster (95% bootstrap) consisted of species in *Gigaspora*. Within this cluster the *Gi. margarita/rosea* DAOM 194757 and *Gi. margarita/rosea* E29 isolates grouped with *Gi. rosea* isolate BEG9. Two *Gi. gigantea* isolates grouped with *Gi. gigantea* WV932 and *Gi. margarita*

BEG34. The *Scutellospora* sequences clustered together although with a very low bootstrap support.

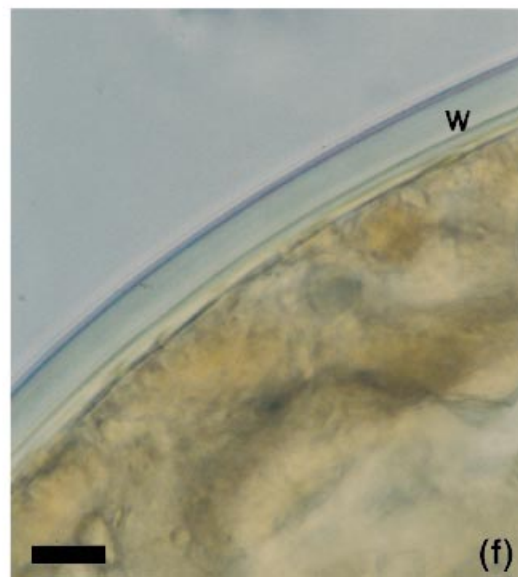
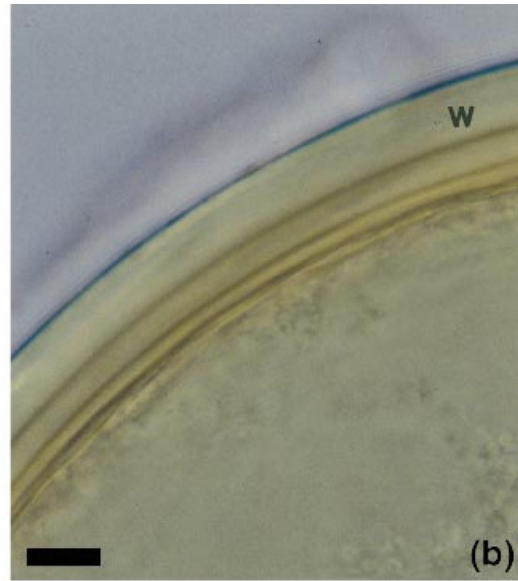
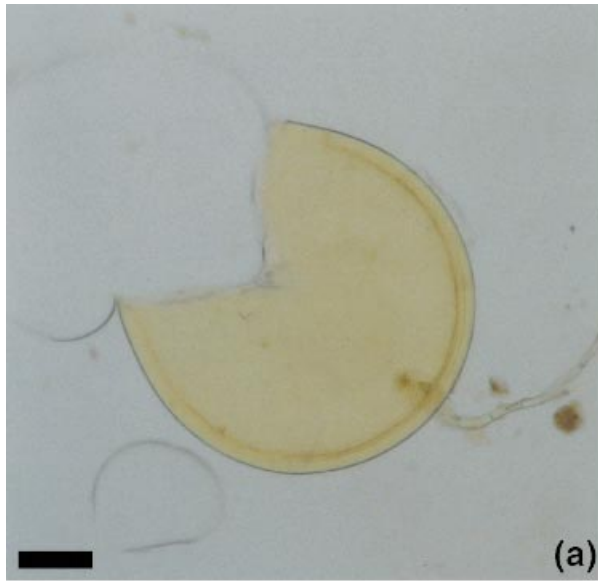
A similar approach was used to analyse the 5.8S gene and the flanking ITS1 and ITS2 regions. After cloning, two or three recombinant plasmids were sequenced for each isolate. The alignment of the sequences revealed substantial differences among clones from the same isolate (up to 8.5% divergence between clones 1–2 of the BEG9 isolate). Sequence data were analysed with Neighbour-joining and Parsimony, using as outgroup *Glomus mosseae*. The same tree topology was obtained, and only the Neighbour-joining tree is shown in Fig. 4.

The *Scutellospora* genus represented a well defined cluster supported by high bootstrap value (100%). Both clones from *S. persica* clustered with *S. castanea* (100% bootstrap), confirming morphological observations. *S. pellucida* and *S. heterogama* formed a separated group (100% bootstrap).

All *Gigaspora* isolates clustered together with high bootstrap support (99%). Within this group, a well supported cluster (100% bootstrap) comprised all sequences from two isolates of *Gi. margarita*.

Another cluster, supported by 71% bootstrap, comprised all *Gi. rosea* and *Gi. gigantea* isolates, as well as E29, DAOM

Fig. 2 Comparison, at the same magnification, of crushed spores of the three *Gigaspora rosea* isolates mounted in polyvinyl alcohol-lactic acid-glycerol medium (PVLG). (a, b) *Gi. rosea* isolate E29. (c, d) *Gi. rosea* isolate DAOM 194757. (e, f) *Gi. rosea* isolate FL185. Note the similar spore wall (w) laminations in the three isolates. (a, c, e) Bars, 50 μ m. (b, d, f) Bars, 9 μ m.



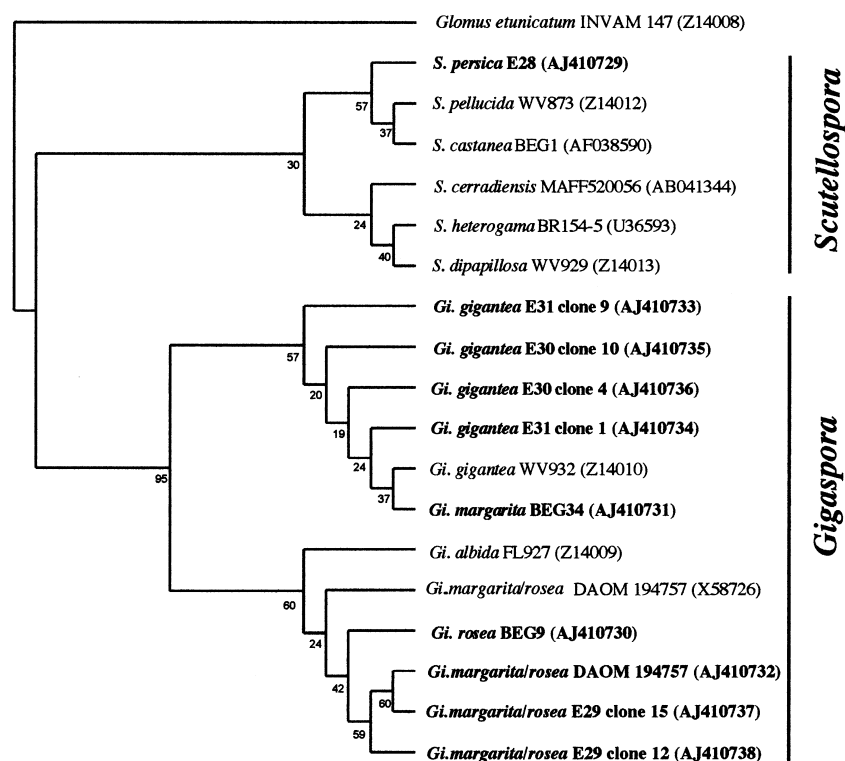


Fig. 3 Neighbour-joining tree obtained from the alignment of a portion of the 18S rDNA. Percentage bootstrap support (out of 1000 trials) is indicated. Sequences obtained in this work are in bold. Isolate and accession number (the latter in parentheses) are indicated.

194757 and *Gi. albida* BR203. Parsimony analysis gave for this cluster a 79% bootstrap value. Within this complex, all *Gi. gigantea* sequences (both those obtained in this study and those retrieved from data banks) formed a distinct cluster supported by 68% bootstrap value. A lower bootstrap value (52%) was obtained with Parsimony. *Gi. rosea* isolates and isolates E29 and DAOM 194757 segregated in several groups. A cluster supported by 100% bootstrap comprised *Gi. rosea* FL185 (AF004700) and clone 2 of isolate E29. Another cluster supported by high bootstrap value (94%) comprised *Gi. rosea* FL105, *Gi. rosea* FL 185 (AF004701), *Gi. rosea* BEG 9 (clone 2), isolates DAOM 194757 (clone 3) and E29 (clone 8). *Gi. albida* BR203 clustered with *Gi. rosea* FL105 clones (89 and 79% bootstrap, respectively).

Estimation of percentage of sequence identity indicated that, in all three species of *Gigaspora* analysed, the extent of intraisolate genetic variation was similar to the variation found among isolates. This can also be visualized in the tree (Fig. 4) where, for example, clone 2 of *Gi. rosea* BEG 9 clustered with other isolates (bootstrap 94%) rather than with clones 1 and 3 of the same isolate. Similar patterns were observed among other isolates in this study (E29, DAOM 194757, WV205-A, E30, E31) and from data banks (FL105, FL185).

PCR amplification with specific primers

When tested on nine isolates belonging to different *Gigaspora* species, the primer pair GiITS1/GiITS2 successfully amplified only DNA from *Gi. margarita* samples, thus confirming the

species-level specificity already described (Lanfranco *et al.*, 1999) (Fig. 5a).

To develop molecular probes which take into account the genetic relationships occurring in the genus *Gigaspora*, two new primers (GiR2 and GiR3) were designed on the basis of sequence alignment. They were used in combination with the primer GiITS1. Two different primers combinations were used to assess species specificity within *Gigaspora*: GiITS1/GiR2 and GiITS1/GiR3.

When GiITS1 and GiR2 were tested on the same nine isolates, an amplified product of the expected size (385 bp) was obtained from *Gi. gigantea* isolates as well as from *Gi. rosea* isolates (Fig. 5b). By contrast, the GiITS1/GiR3 primer combination was more specific as it gave an amplified product of 375 bp only from spores of *Gi. rosea* isolates (Fig. 5c). All *Gi. rosea* isolates tested were positively amplified. These primers were also tested on roots of herbaceous or woody plant colonized by *Gi. margarita* or *Gi. rosea* isolates. Specificity of the primers pairs (GiITS1-GiITS2, and GiITS1-GiR3, Fig. 6) and GiITS1-GiR2 (data not shown) was confirmed.

Discussion

Ribosomal sequences as a tool for the identification of Glomales

Macro- and microanatomy yield characters that 'form the historical bedrock in fungal taxonomy' (Kohn, 1992). Genomic sequences also are being used to generate phylogenetic trees to

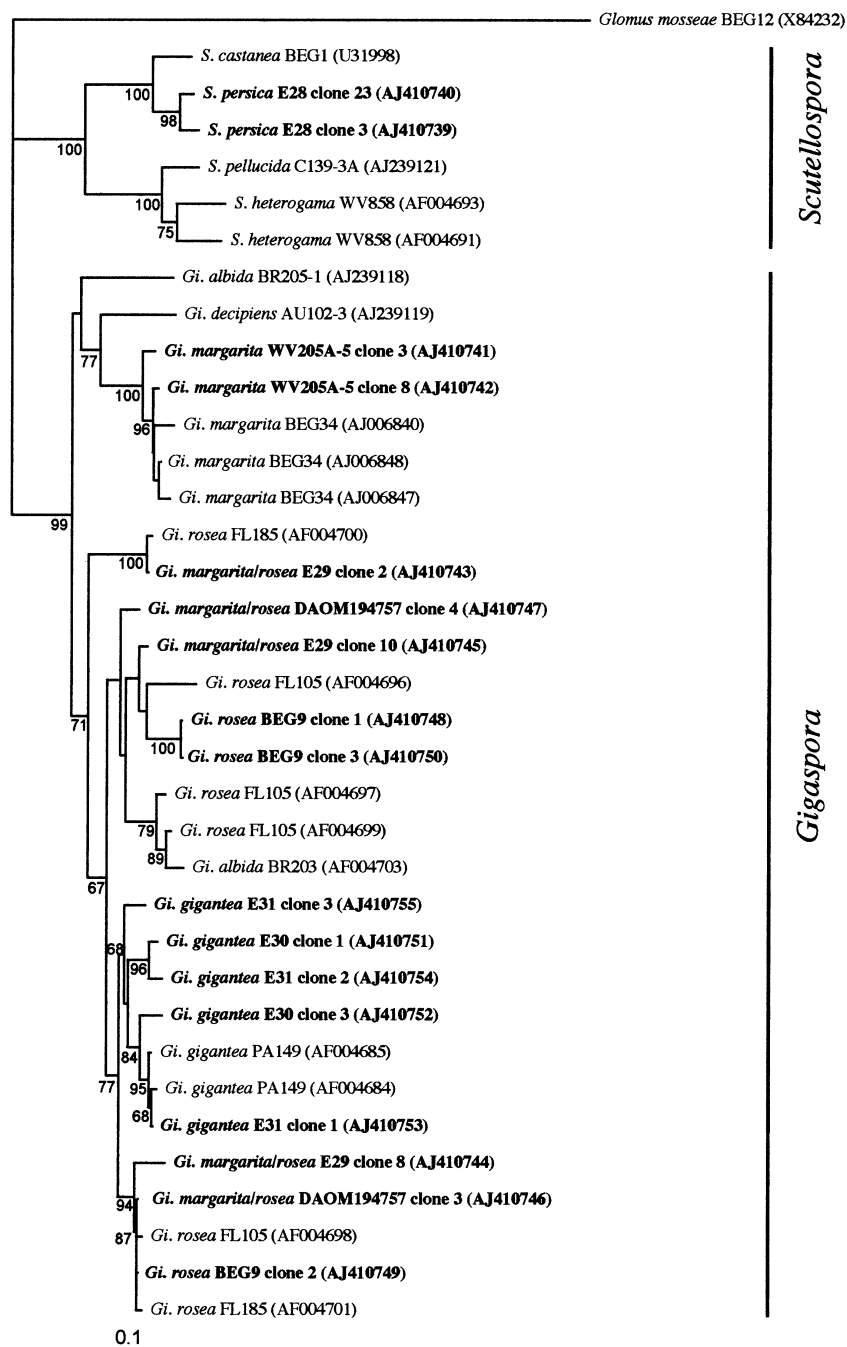


Fig. 4 Neighbour-joining tree obtained from the alignment of the internal transcribed spacer (ITS) region. Percentage bootstrap support (out of 1000 trials) is shown when it exceeds 65%. Sequences obtained in this work are in bold. Isolate and accession number (the latter in parentheses) are indicated.

complement these data and assess degree of congruence or conflict (Berbee & Taylor, 1999; Tehler *et al.*, 2000). Combined morphological and molecular data have been recently used to define relationships among ancient species within *Glomales* (Redecker *et al.*, 2000a,b) as well as to provide diagnostic primers important in the classification of these species (Morton & Redecker, 2001).

Species discrimination based on morphological criteria can be difficult within the genus *Gigaspora* (Bentivenga & Morton, 1995). Our investigation revealed that two isolates

previously identified as *Gi. margarita* (DAOM 194757 and E29) should be transferred to *Gi. rosea*. Based on a short sequence of the 18S rDNA, Bago *et al.* (1998) have already suggested that DAOM 194757 belongs to the *Gi. rosea*. Moreover, *Gi. margarita* isolates normally harbour intracellular bacteria, but DAOM 194757 and E29, like all other isolates of *Gi. rosea* examined thus far (Bianciotto *et al.*, 2000), lack such bacteria. Although a larger number of isolates should be investigated, this feature may represent an additional taxonomic tool to discriminate *Gi. rosea*.

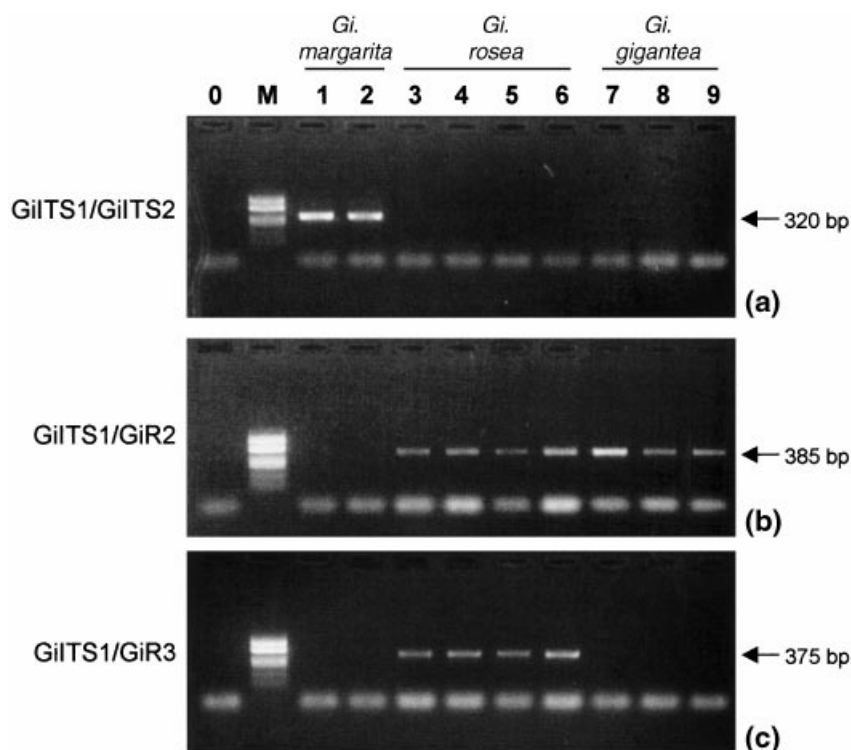


Fig. 5 Gel electrophoresis of PCR products obtained from spore DNA of the following isolates: *Gigaspora margarita* (BEG34 lane 1, WV205A lane 2), *Gigaspora rosea* (DAOM 194757 lane 3, FL185 lane 4, BEG9 lane 5, E29 lane 6) and *Gigaspora gigantea* (E30 lane 7, E31 lane 8, MA453A-7 lane 9). Different primer combinations were used: (a) GiITS1/GiR2; (b) GiITS1/GiR3; (c) GiITS1/GiITS2. M, pUC18 digested with *Hae*III. Lane 0: no DNA control.

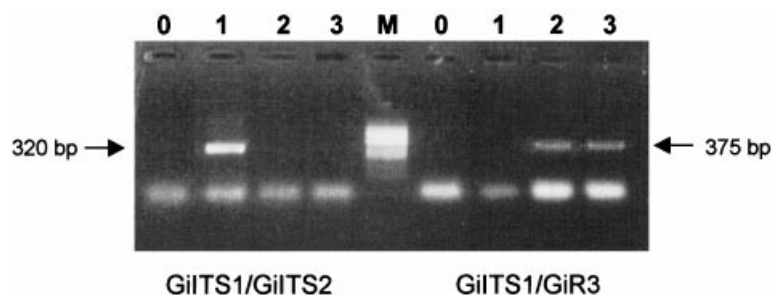


Fig. 6 Gel electrophoresis of PCR products obtained with primers GiITS1/GiR3 or GiITS1/GiITS2 from the following samples: *Trifolium repens* roots colonized with *Gigaspora margarita* BEG 34 (lane 1) or *Gigaspora rosea* BEG9 (lane 2); *Araucaria angustifolia* roots colonized with *Gigaspora rosea* E29 (lane 3). Lane 0: no DNA control. M, pUC18 digested with *Hae*III.

Correct identification of AM fungal isolates is becoming more important for basic and applied research. Isolates propagated in pot cultures for many years and routinely used in different laboratories for studies on biology and physiology of the mycorrhizal symbiosis need to be correctly identified. An accurate identification of reference fungal isolates deposited in public collections is also required. Sequences derived from improperly identified isolates would result in erroneous phylogenetic trees. For example, isolate DAOM 194757 was treated as *Gi. margarita* in the phylogenetic tree proposed by Simon *et al.* (1993), but it clearly belongs in *Gi. rosea*. Similarly, it may be possible that the isolate *Gi. albida* BR203 (INVAM) might have been misidentified, since the corresponding ITS sequence clustered with the *Gi. rosea* sequences (Fig. 3). The continuum of few morphological traits between species in *Gigaspora* necessitates complementation of molecular characters. *Gigaspora* is a genus in which spores of the few extant species do not diverge

greatly in morphological traits and the traits present tend to overlap.

ITS: a useful target DNA region to investigate genetic variability?

The partial 18S rDNA sequences analysed in this study were not suitable to resolve species but can be useful to discriminate higher taxa in Glomales as described by Schüßler *et al.* (2001).

Within the ITS region, the highly conserved 5.8S rRNA gene resolves taxa at the family level and above, as evidenced by the discovery of ascomycetous fungal sequences in AM spores (probably present as contaminants) (Redecker *et al.*, 1999). A tree based on the 5.8S gene confirmed that all ITS sequences of fungi this study belong to Glomales (data not shown).

The ITS spacers are generally considered to be appropriate to discriminate at species level, provided that genetic variability

among isolates of the same species is lower than variability among species. In Glomales the evaluation of intraspecific variation is complicated by the presence of several different ribosomal variants within the same isolate (Sanders *et al.*, 1995; Lloyd-MacGilp *et al.*, 1996). Clapp *et al.* (2001) estimated the magnitude of sequence variation in the D2 region of the 28S rDNA gene, which should contain sufficient inter-species polymorphisms to discriminate AM fungi (van Tuinen *et al.*, 1998). The analysis of isolates from three species (*G. mosseae*, *G. coronatum* and *G. constrictum*) revealed several sequence variants within the same isolate in all species. A cluster analysis of these sequences showed that some sequences from *G. mosseae* and *G. constrictum* clustered with *G. coronatum*. Sequence variability among isolates therefore obscured species level resolution. Sequence variability within isolates was also found in the ITS region of *Glomus* species (Lloyd-MacGilp *et al.*, 1996) and for the three *Gigaspora* species examined in this study (*Gi. rosea*, *Gi. margarita* and *Gi. gigantea*).

At least two ITS variants were obtained from a multisporal preparation of DNA from each isolate used in this study. ITS variants have already been described in AM fungi; in some cases the ITS variants were found within single spores (Sanders *et al.*, 1995; Lloyd-MacGilp *et al.*, 1996; Hijri *et al.*, 1999; Lanfranco *et al.*, 1999; Antonioli *et al.*, 2000; Pringle *et al.*, 2000; Redecker *et al.*, 2000a). Sequence polymorphism within single spores was also reported in the 18S rDNA gene of a *Scutellospora* sp. (Clapp *et al.*, 1999).

Spores of species in Gigasporaceae possess thousands of nuclei (Bianciotto & Bonfante, 1992) and genetically different nuclei have been found within single spores (Trouvelot *et al.*, 1999). Given that AM fungal hyphae are coenocytic and the nuclei migrate, an individual of AM fungi actually might be considered a pool of genetically heterogeneous nuclei.

Intra-isolate ITS variation was as great as that of geographically disjunct isolates of the *Gigaspora* species examined. Similar patterns have been described within and among isolates of *Glomus mosseae* (Lloyd-MacGilp *et al.*, 1996). Bootstrap support was not very high as result of this variability, but sequences obtained from the same species still clustered together.

From the results of this study, ITS sequences appear, at least, to be appropriate to distinguish *Gi. margarita* from *Gi. rosea*. By contrast, morphological features seem to be more informative than molecular data to discriminate *Gi. gigantea* from *Gi. rosea*. As described by Bentivenga & Morton (1995), morphological and histochemical features are sufficient to distinguish *Gi. gigantea* from *Gi. rosea*. As an additional feature, the *Gi. gigantea* isolates analysed in this study contain intracellular bacteria which are not present in *Gi. rosea* (Bianciotto *et al.*, 2000). Molecular analysis showed that *Gi. gigantea* sequences were nested within the *Gi. rosea* cluster and formed a separate group with both Parsimony and Neighbour-joining analyses. However, bootstrap supports were low to moderate,

thus suggesting that the ITS region may be inadequate to clarify their phylogenetic relationships. Sequences from other loci may provide additional information.

New specific primers to identify *Gigaspora rosea*

PCR-based detection and diagnostic methods require suitable primers and several authors have developed oligonucleotides that discriminate AM fungi at different taxonomic ranks (Simon *et al.*, 1992; van Tuinen *et al.*, 1998; Lanfranco *et al.*, 1999; Redecker, 2000; Schübler *et al.*, 2001). Due to its variability, the ITS region is a very useful target not only to investigate genetic polymorphisms, but also to design species-specific primers. The primers described in this work discriminated *Gi. rosea* from *Gi. gigantea* and *Gi. margarita*. Although they have not been tested for cross-hybridization with *Gi. decipiens* and *Gi. albida*, they represent the first molecular tool to discriminate species within the genus *Gigaspora*. van Tuinen *et al.* (1998) also described two primers which selectively amplify a region of the 28S rDNA gene for *Gi. rosea* (BEG 9) in mycorrhizal roots. However, cross-amplification of those primers with other *Gigaspora* species was not assessed and the specificity was only tested against *Glomus mosseae*, *Glomus intraradices* and *Scutellospora castanea*.

It may be surprising that, despite the variability of the ITS sequence observed within spores and among isolates of the same species, species-specific primers can be designed. Possibly, the DNA polymerase may accept some mismatches of the primer with the target sequence. Alternatively, target sites perfectly annealing the primer may be present among the several ITS variants in the spore (Lanfranco *et al.*, 1999). Since no signatures characteristic for the *Gi. rosea* ITS sequences were identified in the alignment, the GiR3 primer was designed on a region that was found in most, but not all, clones. This supports the hypothesis that the primer selects variants that are present in the spore.

The major results obtained in this investigation show how complementary morphological and molecular data can clarify relationships among species of low morphological divergence. Sequence information allowed us to investigate the extent of genetic divergence within these species and to provide useful PCR primers for detection and identification.

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