Biology, ecology and evolution of the family Gigasporaceae, arbuscular mycorrhizal fungi (Glomeromycota)

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Chapter 6

PCR-Denaturing Gradient Gel Electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity is an accurate and sensitive method to assess species diversity of arbuscular mycorrhizal fungi of the genus *Gigaspora*

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ABSTRACT

Despite the importance of arbuscular mycorrhizal fungi in the majority of terrestrial ecosystems, their ecology, genetics, and evolution are poorly understood, partly due to difficulties associated with detecting and identifying species. We explored the inter- and intraspecies variations of the 18S rRNA genes of the genus*Gigaspora* to assess the use of this marker for the discrimination of *Gigaspora* isolates and of *Gigasporaceae* populations from environmental samples. Screening of 48 *Gigaspora* isolates by PCR-denaturing gradient gel electrophoresis (DGGE) revealed that the V3-V4 region of the 18S rRNA gene contained insufficient variation to discriminate between different *Gigaspora* species. In contrast, the patterns of 18S ribosomal DNA (rDNA) heterogeneity within the V9 region of this marker could be used for reliable identification of all recognized species within this genus. PCR-DGGE patterns provided insight into some putative misidentifications and could be used to differentiate geographic isolates of *G. albida*, *G. gigantea*, and *G. margarita* but not *G. rosea*. Two major clusters were apparent based upon PCR-DGGE ribotype patterns, one containing *G. albida*, *G.candida*, *G. ramisporophora*, and *G. rosea* and the other containing *G. decipiens* and *G. margarita*. Dissection of the DGGE patterns by cloning, DGGE screening, and sequencing confirmed these groupings and revealed that some ribotypes were shared across species boundaries. Of the 48 isolates examined, only two displayed any spore-to-spore variation, and these exceptions may be indicative of coisolation of more than one species or subspecies within these cultures. Two Brazilian agricultural soils were also analyzed with a *Gigasporaceae*specific nested PCR approach, revealing a dominance of *G. margarita* within this family.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) form one of the most common symbioses with plants (Smith & Read 1997), and their importance to natural and man-made ecosystems is well established (Sieverding 1991, Smith & Read 1997, van der Heiden et al 1998). The AMF form a monophyletic group of obligate plantsymbiotic fungi belonging to the phylum *Glomeromycota* (Schüßler et al 2001). Unfortunately, their ecology, genetics, and evolution are as yet poorly understood (Gianinazzi-Person et al 2001, Sanders 2002). The main hurdles to AMF research are the inability to obtain axenic cultures and the difficulties associated with identifying AMF, especially in planta (Clapp et al 2002, Gianinazzi-Person et al 2001). However, the recent application of molecular biological techniques for

characterization of AMF has led to important advances in our understanding of the phylogeny (Schüßler et al 2001, Schwarzott et al 2001), ecology (Helgason et al 1998, 2002, Husband et al 2002a, 2002b, Kowalchuk et al 2002), genetics (Gianinazzi-Person et al 2001, Harrison 1999), and evolution (Gandolfi et al 2003, Sanders 2002) of this group of obligatory symbiotic fungi. rRNA genes have become the most widely used targets for detection of AMF in environmental samples (Clapp et al 2002). Several PCR-based strategies targeting rRNA genes have recently been developed to detect AMF in DNA extracted from roots, soil, or spores (Helgason et al 1998, Kjoller & Rosendahl 2000, Kowalchuk et al 2002, van Tuinen et al 1998). Such strategies have provided new insights into AMF diversity by circumventing the need for trap cultures and morphological identifications, which can be highly biased, time-consuming, and inaccurate. Despite these advances, the operational taxonomic units obtained in most of these works can only be identified precisely to genus level or above. Thus, little progress has been made in species characterization and identification per se, which are still strongly dependent on morphological analysis and the investigator's level of expertise. Few studies have actually used the rRNA genes to identify species of AMF (Redecker et al 1997), with most analyses being limited to the detection of defined species of interest (Lanfranco et al 2001, van Tuinen et al 1998).

Molecular analyses have revealed that a single AMF isolate or even individual spores may contain substantial heterogeneity among rRNA gene copies (Antoniolli et al 2000, Clapp et al 1999, Kuhn et al 2001, Lanfranco et al 1999, Lloyd-MacGilp et al 1996, Sanders et al 1995; for a recent review, see reference Sanders 2002), which may be unevenly distributed in the heterokaryotic nuclei of AMF spores (Kuhn et al 2001, Trouvelot et al 1999). Intraspecific rRNA heterogeneity seems to be a common phenomenon in AMF as well as in other groups of organisms, such as bacteria (Amann et al 2000, Nübel et al 1996), plants (Buckler et al 1997), insects (Tang et al 1996), and crustaceans (Gandolfi et al 2001). However, little progress has been made in the interpretation of this heterogeneity. Such heterogeneity may lead to overestimations of the number of species when interpreting clone libraries of rRNA recovered from the environment (Dahlof et al 2000). However, if the heterogeneity is consistent within a species, intraisolate heterogeneity might be used as an advantage to generate species-specific rDNA fingerprints for AMF detection and identification.

The genus *Gigaspora* represents an ecologically and economically (Balota & Lopes 1996, Santos et al 2000) important group within the *Glomeromycota*, and numerous studies have been dedicated to identify species within this genus and to study their ecology. The taxonomy of the genus *Gigaspora* has recently been revised by morphological (Bentivenga & Morton 1995), fatty acid methyl ester (Bentivenga & Morton 1996), molecular (Bago et al 1998), and combined (Lanfranco et al 2001) approaches. Among the eight *Gigaspora* species described to date, Bentivenga & Morton (1995)

considered five to be valid species based on spore morphology: *G. albida*, *G. decipiens*, *G. gigantea*, *G. margarita*, and *G. rosea.* Two species were considered synonymous with previously described *Gigaspora* species (*G. candida G. rosea*, and *G. ramisporophora, G. margarita*), and one species, *G. tuberculata*, was considered synonymous with *Scutellospora persica*. However, there are few useful morphological characters for *Gigaspora* species determination, and character ranges such as spore size and color often overlap between species (Bentivenga & Morton 1995). Bago et al. (1998) used molecular signatures within the V9 region of the 18S rRNA gene as diagnostic characters for *Gigaspora* spp. identification. These authors were able to identify three distinct groups among the currently recognized species of *Gigaspora*: the *Gigaspora rosea* group (*G. rosea* and *G. albida*), *Gigaspora margarita* group (*G. margarita* and *G. decipien*s), and *Gigaspora gigantea*. Their analysis also revealed intraspore heterogeneity, which caused ambiguities in the signatures they found. Lanfranco et al. (2001) continued the molecular characterization of selected *Gigaspora* species and proposed a number of species-specific primer sets, and Yokoyama et al. (2002) developed primers based on satellite fragments to identify specific isolates of *G. margarita*. Thus, while important strides have been made recently, rapid and reliable methods to assess *Gigaspora* diversity are still lacking. Furthermore, little is known about the establishment, distribution, diversity, and competitiveness of *Gigaspora* spp. in the field (Balota & Lopes 1996, Santos et al 2000).

PCR-denaturing gradient gel electrophoresis (DGGE) was initially developed to study mutations. Nowadays, it has become one of the most applied culture-independent techniques to study the community structure of microorganisms (Muyzer & Smala 1998). Separation in DGGE is based on differences in sequence composition that affect the melting behavior of the amplicons, causing a decrease in the electrophoretic mobility of a partially melted DNA molecule in a polyacrylamide gel containing a linearly increasing gradient of DNA denaturants (for more information, see reference Muyzer & Smala 1998). Recently, Kowalchuk et al. (2002) successfully applied PCR-DGGE to study the community structure of AMF associated with *Ammophila arenaria*. This study had three main goals: (i) to develop a rapid and reliable method to characterize and identify *Gigaspora* species, based upon PCR-DGGE analysis of variable regions of the 18S rRNA gene; (ii) to assess the level of intraspore and intraisolate 18S rDNA heterogeneity within the genus *Gigaspora* and evaluate PCR-DGGE as a method for studying this phenomenon; and (iii) to test the application of a *Gigasporaceae*-specific PCR-DGGE strategy for the assessment of *Gigaspora* diversity in environmental samples.

MATERIAL AND METHODS

AMF strains

The AMF strains used as controls for standardization of PCR DGGE protocols and to evaluate the level of discrimination between and withinspecies are listed in Table 1. All *Gigaspora* species were represented by a reference isolate and as many additional isolates as we could collect from various sources (Table 1). The accession (catalogued) strain *G. gigantea* MN453A-7 was obtained from C. Walker, who received the material from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) in the form of spores dispersed in quartz sand since 9 October 1997. All *Gigaspora* strains used were characterized as pure cultures by morphological analysis. The curator of the INVAM collection sent us a blind test among the *G. rosea* strains we bought to test the capacity of our technique to differentiate between species.

Soil samples

Samples were taken from an 8-year-old grassland field in a cattle farm in Brazil. The farm, Agropecuária Lopes, was located in Santo Antônio, Goiás State (16°28 00 S, 49°17 00 W, at 823 m above sea level), Brazil. The grassland was dominated by *Brachiaria decumbens*, which had replaced the native vegetation in the Cerrado (savannah) biome. Intact soil cores (7.5 cm in diameter; 8.0 cm deep) were collected with polyvinyl chloride cylinders. The soil, a clayey dark red oxissol, had a pH of 5.5 (soil/water ratio, 1:2.5 [vol/vol]). It contained 0, 2.4, and 0.7 cmol of charge per kg (dry weight) of soil (cmolc) of Al, Ca, and Mg, respectively, dm^3 in a 1 N KCl extraction and 0.16 and 1 mg of P and K, respectively, dm^3 in a Mehlich I extraction. The samples were transported to Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil, and used to establish 10 trap cultures to assess the diversity of AMF. *Brachiaria decumbens* was used as the host plant. The trap cultures were sent to the Netherlands for further analysis. Two of these trap cultures were selected: one contained large numbers (sample A) and the other contained small numbers (sample B) of *Gigaspora* spores (Table 2). Spore identification and counting as well as DNA extraction were performed with three replicates of 30 g of soil inoculum each. The procedure for morphological identification of spores is described in de Souza et al. (1999).

Spore extraction and preparation for DNA extraction

Spores were extracted directly from the material received from the collections with the standard wet sieving technique, followed by centrifugation in water and subsequently in 50% sucrose solution (for details of spore extraction, see reference 15). After extraction, the spores were carefully

Table 1. Species, strains, contributors or sources, origins, and germ plasm collections of the *Gigaspora*, *Scutellospora*, and *Glomus* isolates used in this study[a]

N°	Species	Code	Contributor/Source	Origin	Germoplasm Bank[a]
1	*Gigaspora albida**	BR607A	J. Morton	Brazil	INVAM
2	*Gigaspora albida*	BR601	J. Morton	Brazil	INVAM
3	*Gigaspora albida*	UFLA24	J.O. Siqueira	Brazil	UFLA
4	*Gigaspora albida*	CL151	J. Morton	USA	INVAM
5	*Gigaspora albida*	FL713	J. Morton	USA	INVAM
6	*Gigaspora albida*	INVAM927	L. C. Maia	USA	CNPAB
7	*Gigaspora candida**	BEG17	V. Gianninazzi-Person	Taiwan	BEG
8	*Gigaspora decipiens**	AU102	J. Morton	Australia	INVAM
9	*Gigaspora decipiens*	W3516	L. Abbott/ C. Walker	Australia	Walker
10	*Gigaspora gigantea*	VA105C	J. Morton	USA	INVAM
11	*Gigaspora gigantea*	UFLA872	J.O. Siqueira	Brazil	UFLA
12	*Gigaspora gigantea**	MN453A-7	C. Walker	USA	INVAM
13	*Gigaspora gigantea*	MA453A	J. Morton	USA	INVAM
14	*Gigaspora gigantea*	MN414D	J. Morton	USA	INVAM
15	*Gigaspora gigantea*	MN922A	J. Morton	USA	INVAM
16	*Gigaspora gigantea*	NC110A	J. Morton	USA	INVAM
17	*Gigaspora gigantea*	NC150	J. Morton	USA	INVAM
18	*Gigaspora gigantea*	CUT	D.D. Douds	USA	USDA-ARS
19	*Gigaspora gigantea*	CUT	G.Bécard	USA	CNRS
20	*Gigaspora margarita**	WV205A	INVAM	USA	INVAM
21	*Gigaspora margarita*	CNPAB1	F. A. de Souza	Brazil	CNPAB
22	*Gigaspora margarita*	CNPAB16	F. A. de Souza	Brazil	CNPAB
23	*Gigaspora margarita*	BEG34 Fr	V. Gianninazzi-Person	New Zealand	BEG
24	*Gigaspora margarita*	BEG34 It	V. Bianciotto	New Zealand	Torino
25	*Gigaspora margarita*	IES32	R. HerreraPeraza	Cuba	IES
26	*Gigaspora margarita*	UFLA36	J.O.Siqueira	Brazil	UFLA
27	*Gigaspora margarita*	TARI SM 478	M. Saito	Taiwan	MAFF
28	*Gigaspora margarita*	K - 1 - 520052	M. Saito	Japan	MAFF
29	*Gigaspora margarita*	C – 520054	M. Saito	Japan	MAFF
30	*Gigaspora margarita*	Ni A	M. Saito	Nepal	MAFF
31	*Gigaspora ramisporophora**	CNPAB22	F. A. de Souza	Brazil	CNPAB
32	*Gigaspora rosea**	FL105	J. Morton	USA	INVAM
33	*Gigaspora rosea*	BR151A	J. Morton	Brazil	INVAM
34	*Gigaspora rosea*	BR227B	J. Morton	Brazil	INVAM
35	*Gigaspora rosea*	BR235	J. Morton	Brazil	INVAM
36	*Gigaspora rosea*	FL219A	J. Morton	USA	INVAM
37	*Gigaspora rosea*	FL676	J. Morton	USA	INVAM
38	*Gigaspora rosea*	KS885	J. Morton	USA	INVAM
39	*Gigaspora rosea*	MA457C	J. Morton	USA	INVAM
40	*Gigaspora rosea*	NB103D	J. Morton	USA	INVAM
41	*Gigaspora rosea*	NC178	J. Morton	USA	INVAM
42	*Gigaspora rosea*	NY328A	J. Morton	USA	INVAM
43	*Gigaspora rosea*	UT102	J. Morton	USA	INVAM
44	*Gigaspora rosea*	WV187	J. Morton	USA	INVAM
45	*Gigaspora rosea*	BEG9	V. Gianninazzi-Person	Unknown	BEG
46	*Gigaspora rosea*	IES19	R. HerreraPeraza	Brazil	IES
47	*Gigaspora rosea*	CI – 520062	M. Saito	Japan	MAFF
48	*Gigaspora rosea*	INVAM185	D.D.Douds	USA	USDA-ARS
49	*Gigaspora rosea*	DAOM194757	D.D.Douds	Canada	USDA-ARS
50	*Gigaspora rosea*	DAOM194757	G.Bécard	Canada	CNRS
51	*Gigaspora* sp.	TW1-1	M. Saito	Taiwan	MAFF
52	*Scutellospora gregaria*	CNPAB7	F. A. de Souza	USA	CNPAB
53	*Scutellospora heterogama*	CNPAB2	F. A. de Souza	Brazil	CNPAB
54	*Scutellospora reticulata*	CNPAB11	F. A. de Souza	Brazil	CNPAB
55	*Glomus clarum*	CNPAB21	F. A. de Souza	Brazil	CNPAB

a *, accession strains considered type or ex-type materials.

b BEG, European Bank of Glomales, Dijon, France; CNPAB, Embrapa Agrobiologia, Rio de Janeiro, Brazil; CNRS, Centre National de la Recherche Scientifique, Toulouse, France; IES, Instituto Ecologia y Sistematica, Havana, Cuba; INVAM, International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, Morgantown, W.V.; MAFF, Ministry of Agriculture, Forest and Fisheries, Ibaraki, Japan; Torino, Dipartimento Biologia Vegetale, Universita di Torino, Tonna, Italy; UFLA, Universidade Federal de Lavras, Minas Gerais, Brazil; USDA-ARS, U.S. Department of Agriculture Agricultural Research Service; Walker, C. Walker, personal collection, New Milton, England.

selected under a binocular microscope, further cleaned by ultrasonication for 15 s, and rinsed in autoclaved ultrapure water (Millipore B.V., Etten-Leur, The Netherlands). This procedure was repeated four times. Individual clean and healthy-looking spores were selected and transferred to 1.5-ml microcentrifuge tubes at either 1 or 60 spores per tube and stored at 80°C until required. Individual spores were used to ensure purity and to compare the results with DNA extracted from multiple spores.

Table 2. Arbuscular mycorrhizal fungi spore number, in trap cultures obtained from soil samples collected in an 8-year-old *Brachiaria decumbens* grassland field used for cattle in Goiás State, Brazil

Species	Sample A	Sample B
	Number of spores/30g of dry soil	
Acaulospora mellea	-[a]	8.3
A. morrowiae	23.3	-
A. rehmii	8.3	19.0
A. tuberculata	1.7	36.7
A. scrobiculata	4.3	-
Archaeospora gerdemannii	95.3	-
Gigaspora decipiens/margarita	63.0	5.0
Glomus macrocarpum	57.3	2.3
Glomus N.1.	17.0	-
Glomus N.2.	128.0	28.0
Glomus N.3.	-	4.7
Scutellospora coralloidea	0.7	-
S. heterogama	1.3	-
Total	400.2	104.0

a —, not detected.

Control experiments with multiple target and nontarget AMF species

In order to evaluate the effect of different target and nontarget species combinations on the detection limits and reproducibility of PCR-DGGE banding patterns, we combined DNA from strains *Scutellospora heterogama* CNPAB2 and *G. margarita* CNPAB16 at different ratios. The ratios used were *Scutellospora heterogama* CNPAB2 to *G. margarita* CNPAB16 at 1:1; 1:5; 1:10; 1:25; 1:50, and 1:100 and *G. margarita* CNPAB16 to *S. heterogama* CNPAB2 at the same ratios. In addition, we also combined nontarget DNA obtained from *Glomus clarum* CNPAB5 in ratios ranging up to 100:1 with *G. margarita* CNPAB16. Three replicas were performed for each combination.

Greenhouse experiment

To ensure that the PCR-DGGE approach was sensitive enough to detect multiple species of *Gigaspora*

and *Scutellospora* in root samples, we performed a greenhouse experiment. Clover plants (*Trifolium pratense*) were inoculated or not with a mixture of soil inoculum containing *G. margarita* (CNPAB16), *S. gregaria* (CNPAB7)*, S. heterogama* (CNPAB2), and *S. reticulata* (CNPAB11). To ensure nodulation, the clover plants were also inoculated with soil filtrate containing rhizobia collected from clover field plots. The plants were grown in plastic cone pots containing 250 ml of a mixture of clay soil and sand (1:1, vol/vol) at pH 6.2 (soil/water ratio, 1:2.5). The pots were fertilized intermittently with 1/10th-strength nutrient solution (Hoagland & Aron 1950) without N and P. After 2 months of growth, the pot contents were harvested. The soil was carefully removed from the roots with tap water, and the root system was cleaned by ultrasonication (60 W; B-2200 E1; Bransonic) twice for 3 min each in autoclaved water, followed by a final wash with autoclaved water. The roots from each pot were divided into subsamples for either DNA extraction or assessment of colonization rate by the method of Giovannetti and Mosse (1980).

DNA extraction from spores, roots, and soil samples

Spores were removed from the freezer (80°C) and crushed with a micropestle (Treff AG, Degersheim, Switzerland) in 40 l of 10 mMTris-HCl buffer, pH 8.0, with 10 l of 20% Chelex 100 (Bio-Rad Laboratories, Hercules, Calif.), for single spores. The same procedure was used with multiple spores except that the reagent concentrations were 80 and 40 l for buffer and Chelex, respectively. The tubes were then incubated at 95°C for 10 min, chilled on ice, and centrifuged at 10,000 *g* for 2 min. The supernatant was carefully transferred to a new tube and stored at 20°C until use. Samples from multiple spore isolations were treated with RNase before being stored. DNA extractions from trap plants with bulk soil and root material were performed with the UltraClean soil DNA isolation kit according to the manufacturer's instructions (MoBio Laboratories, Solana Beach, Calif.). Prior to DNA extraction, the samples (10 g of soil or 2 g of root) were homogenized and ground under liquid N2 with a mortar and pestle. A subsample of 0.5 g of bulk soil or root material was used for each DNA extraction. After extraction, the soil- and root-derived DNA was purified once more with the Wizard DNA purification kit (Promega, Madison, Wis.) as described by the manufacturer. For the greenhouse experiment, we extracted DNA from plant roots with the protocol described by Edwards et al. (1997) with 50 mg of liquid nitrogen-powdered roots.

Nested PCR conditions for amplification from spore DNA

The DNA from spores was first amplified with the forward primer NS1 in combination with reverse primer ITS4, covering the region from the beginning of the 18S rRNA gene through the 5 end of the 25S rRNA gene (White et al 1990). Primer positions are given in Fig. 1, and primer sequences,

references, and PCR conditions are provided in Table 3. These reactions were performed in a final volume of 15 l, with 5 l of template DNA. The PCR mixture was composed of 200 M each of the four deoxynucleoside triphosphates, 1.5 M $MgCl_2$, a 0.4 M concentration of each primer, and 1 U of Expand high-fidelity DNA polymerase (Roche Diagnostics, Nederland B.V., Almere, The Netherlands) according to the manufacturer's recommended buffer conditions. All reactions were performed in a PTC200 thermal cycler (MJ Research; Waltham, Mass.). The product of this first PCR amplification was diluted 1:1,000, and 2 l of this dilution was used as the template in a second round of PCR (reaction volume, 25 l) designed to target either the V3-V4 or V9 region of the 18S rRNA gene (Fig. 1, Table 3). In each case, one of the primers contained a GC clamp to stabilize the amplicon's melting behavior for DGGE analysis (Sheffield et al 1987).

Gigasporaceae-specific PCR conditions for the analysis of environmental samples

To obtain *Gigasporaceae*-specific products from soil, roots, and spores from trap cultures and the greenhouse experiment, the first step of the nested PCR combined primers FM6 (this study) and GIGA5.8R (Redecker 2000) (see Fig. 1 and Table 3 for primer positions and sequences). PCR mix was prepared as described above, and the DNA extracted from soil and root samples was diluted 1:50 to 1:100 and used as template. The product of this first PCR amplification was diluted 500- to 1,000-fold, depending on product concentration, and used as the template for a second PCR with the primer pair NS7 (White et al 1990) with GC clamp in combination with primer F1Ra (this study) as described above.

DGGE analysis

All DGGE analyses were performed with the D-Gene system (Bio-Rad), with gradients of 25 to 40% and 32 to 42% denaturant and running conditions of 75 V for 16 h for the NS31 and GC/AM1 and 95 V for 17 h for the NS7 and GC/F1Ra primer combinations, respectively. Gels were run in 0.5 TAE (Tris-acetate-EDTA) buffer at a constant temperature of 60°C. Gels were stained for 20 min in MilliQ water containing 0.5 mg of ethidium bromide liter 1 and destained twice for 15 min in MilliQ water prior to UV transillumination. Gel images were digitally captured with the ImaGo system (B & L, Maarssen, The Netherlands). DGGE banding patterns were assessed by cluster analysis with a Jaccard similarity coefficient, and similarities between profiles were depicted as a dendrogram constructed by the unweighted pair group method with arithmetic average (UPGMA) within the Bionumerics program, version 2 (Applied Maths, Kortrijk, Belgium). The banding pattern of *G. rosea* FL105 was used as a marker to standardize different gels.

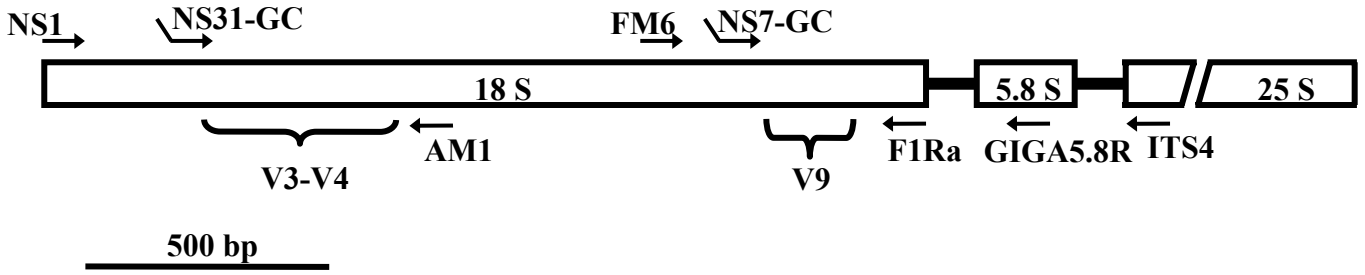

FIG. 1. Cartoon focusing on the 18S rRNA gene. Approximate positions of primers (arrows not to scale) and variable regions targeted by PCR-DGGE analyses are shown. Bent tails on primers indicate the presence of a GC clamp.

Table 3: rDNA primers, primer combinations, GC clamp, and PCR conditions used in this study[a]

Primer[a]	Sequence	Partner primer	Target group	PCR conditions	Product size (bp)
NS1	5'-GTAGTCATATGCTTGTCT C-3'	ITS4	Eukaryote Univ.	94 °C for 60s, 55 °C for 60s, 68 °C for 240s × 30	2,300
NS31-GC[b]	5'-TTGGAGGGCAAGTCTGGTGCC-3'	AM1	Eukaryote Univ.	94 °C for 60s, 61 °C for 60s, 68 °C for 40s × 30	600
FM6	5'-ACCTGCTAAATAGTCAGGCTA-3'	GIGA5.8R	*Gigasporaceae*	94 °C for 60s, 59 °C for 60s, 68 °C for 45s × 30	700
NS7-GC[b]	5'-GAGGCAATAACAGGTCTGTGATGC-3'	F1Ra	Eukaryote Univ.	94 °C for 60s, 60 °C for 60s, 68 °C for 28s × 30	400
ITS4	5'-TCCTCCGCTTATTGATATGC-3'		Eukaryote Univ.		-
AM1	5'-GTTTCCCGTAAGGCGCCGAA-3'		Fungi		-
GIGA5.8R	5'-ACTGACCCTCAAGCAKGTG-3'		*Gigasporaceae*		-
F1Ra	5'-CTTTTACTTCCTCTAAATGACC-3'		Fungi		-

a Primer sources: NS1, NS7, and ITS4, White et al. (1990); NS31, Simon et al. (1992); AM1, Helgason et al. (1998); GIGA5.8R, Redecker (2000); FM6 and F1Ra, this study. The GC clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3') was attached to the 5' end of primers NS31 and NS7.

Tests for reproducibility

All the PCR amplifications and DGGE analyses were performed with three independent single-spore DNA isolates and compared with multiple-spore isolates. This was done to detect potential artifacts due to spore contamination (Schüßler 1999) and to evaluate the reproducibility of the method. For soil and root DNA, three subsamples were compared for each trap culture.

Recovery of DNA from DGGE gels

The most prominent bands obtained in the DGGE profiles of the trap culture spores were sequenced. The middle portion of selected DGGE band was excised, and approximately 60 mg of acrylamide gel material per band was transferred to a 0.5-ml microcentrifuge tube containing 40 l of MilliQ water and frozen at 80°C for 1 h. Subsequently, the gel material was crushed with a plastic pellet mix (Treff AG), and the tubes were incubated at 37°C for 3 h. After centrifugation at 11,000 *g* for 60 s, the supernatant was transferred to a new tube, and 1 l of it was used as the template for subsequent PCR-DGGE analysis to check band position and purity. This procedure was repeated until a single sharp band was detected. After that, PCR was performed with the same primer pair used in the DGGE analysis without the GC clamp, and the product was prepared for sequencing analysis.

Cloning of AMF rDNA

In order to obtain clones for different variants of the ribosomal genes present (ribotypes) in one species (spore), amplicons were obtained from DNA extracted from individual spores after PCR amplification with the primer pair NS1 and ITS4 (White et al 1990) as described previously. PCR products were purified with the High Pure PCR product purification kit (Boehringer, Mannheim, Germany). Purified PCR products were then cloned into the pGEM-T easy vector, with *Escherichia coli* strain JM109 used for transformation, according to the procedure given by the manufacturer (Promega Benelux, Leiden, The Netherlands). The clones obtained were cultured and, after plasmid extraction by the Wizard Plus SV miniprep DNA purification system (Promega, Benelux), used as templates for PCR (see below).

Clone selection with DGGE and sequence analysis

Plasmids containing an insert obtained from different *Gigaspora* isolates were used as the templates for reactions with the forward primer NS7-GC in combination with the reverse primer F1Ra as described above. DGGE screening was performed as described above, and the PCR products obtained from the original isolates were used as reference to select clones that corresponded to each of the ribotypes detected in each isolate examined. Prior to sequencing, DNA templates were purified with

Qiaquick purification columns. Sequencing reactions were performed with the Perkin Elmer Biosystems Big Dye terminator sequence reaction kit (Perkin Elmer, Foster City, Calif.) and run on a Perkin Elmer 3700 capillary sequencer at the National Institute for Public Health and the Environment (Bilthoven, The Netherlands).

Sequence alignments

Sequences recovered from the GenBank/EMBL database or generated in this work were first aligned with Clustal-X (Thompson et al 1997), and then the alignment was improved by manual inspection. Phylogenetic analysis was conducted with the parsimony method in PAUP* version 4.0 Beta 10 (Swofford 2003).

Nucleotide sequence accession numbers

The sequences and alignment generated in this study were deposited in the EMBL database under accession numbers AJ539236 to AJ539305 and alignment number ALIGN_000606.

RESULTS

DGGE profiles targeting the V3-V4 region of the 18S rRNA gene

All *Gigaspora* strains tested migrated to approximately the same position in the gel, with the *G. margarita*, *G. decipiens*, and *Gigaspora* sp. TW-1 strains showing a tight doublet and all other species showing only a single band (results not shown).

The level of inter- and intraspecies heterogeneity within the V3-V4 region was not sufficient to discriminate among the different *Gigaspora* species tested. Nevertheless, two groups were distinct: the first group was formed by *G. margarita*, *G. decipiens*, and *Gigaspora* sp. strain TW-1 (double band), and the second group was composed of the other strains (single band).

DGGE profiles targeting the V9 region of the 18S rRNA gene

PCR-DGGE analysis of the V9 region of the 18S rRNA gene could differentiate all *Gigaspora* species based on the type materials used, including those (*G. candida* and *G. ramisporophora*) declared to be invalid by morphological analysis (Fig. 2). Almost all isolates yielded multiple bands, indicating the presence of intraspore variation between ribotypes in this region in the strains examined. To test the consistency of the PCR-DGGE profile for a given isolate, we examined several single- and multiple-spore DNA isolations per isolate. No between-spore variations could be

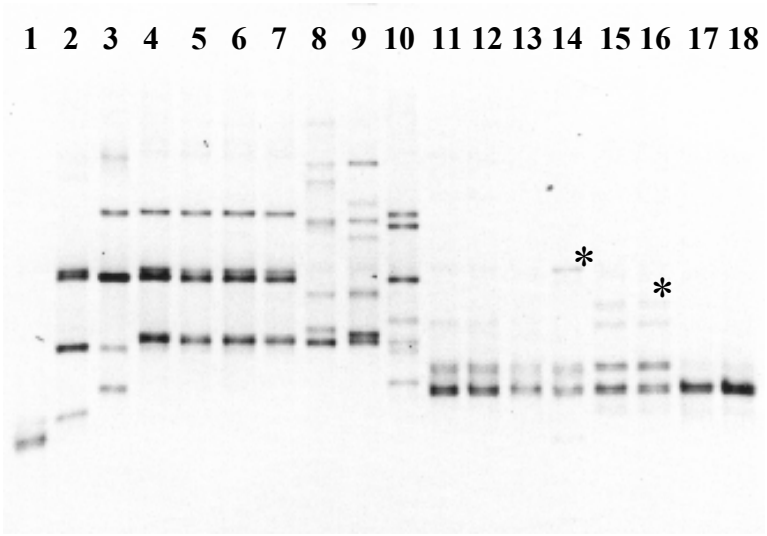

FIG. 2. PCR-DGGE analysis of 18S rRNA gene fragments amplified from *Gigaspora* species and run for 15 h at 95 V for analysis of the V9 region. lanes: 1, *G. gigantea* UFLA872; 2, *G. gigantea* MN453A-7; 3, *G. gigantea* VA105C; 4, *G. rosea* BEG9; 5, *G. rosea* FL105; 6, *G. rosea* IES19; 7, *G. albida* INVAM927; 8, *G. albida* BR607A; 9, *G. candida* BEG17; 10, *G. ramisporophora* CNPAB22; 11, *G. margarita* CNPAB1; 12, *G. margarita* CNPAB16; 13, *G. margarita* IES32; 14, *G. margarita* WV205A; 15, *G. margarita* BEG34 France; 16, *G. margarita* BEG34 Italy; 17, *G. decipiens* AU102; 18, *G. decipiens* W3516. Asterisks show *G. margarita* strain-specific bands.

detected for any of the 48 isolates tested with the exception of *G. albida* CL151 and *G. margarita* UFLA36. The former produced two very similar patterns (CL151a and CL151b in Fig. 3), but in the CL151b type, one of bands were absent and the intensity of the lower band was higher than that in the CL151a type. The latter accession produced two very different banding patterns (UFLA36-T1 and UFLA36-T2 in Fig. 3). These two strains may each actually represent two coisolated populations (see the Discussion). No difference was observed between different cultures of the same accession strain maintained in different laboratories (data not shown).

To determine the consistency of PCR-DGGE patterns within each species, we examined all the *Gigaspora* isolates that we could obtain (48 total). Dendrogram analysis of these banding patterns produced two major clades, the first containing the species *G. albida*, *G. candida*, *G. ramisporophora*, *G. rosea* and most of the *G. gigantea* strains and the second containing *G. decipiens*, *G. margarita*, and *Gigaspora* sp. strain TW-1 (Fig. 3). Within the first of these groups, the *G. albida* isolates formed a distinct subcluster. Of the 10 strains of *G. gigantea* analyzed, one, UFLA872, was unique and had no bands in common with any of the other 48 strains tested (Fig. 3). The banding patterns for strains of the same species were generally highly similar and clustered together (Fig. 3). Isolatespecific bands could be identified for some strains of the species *G. albida*,

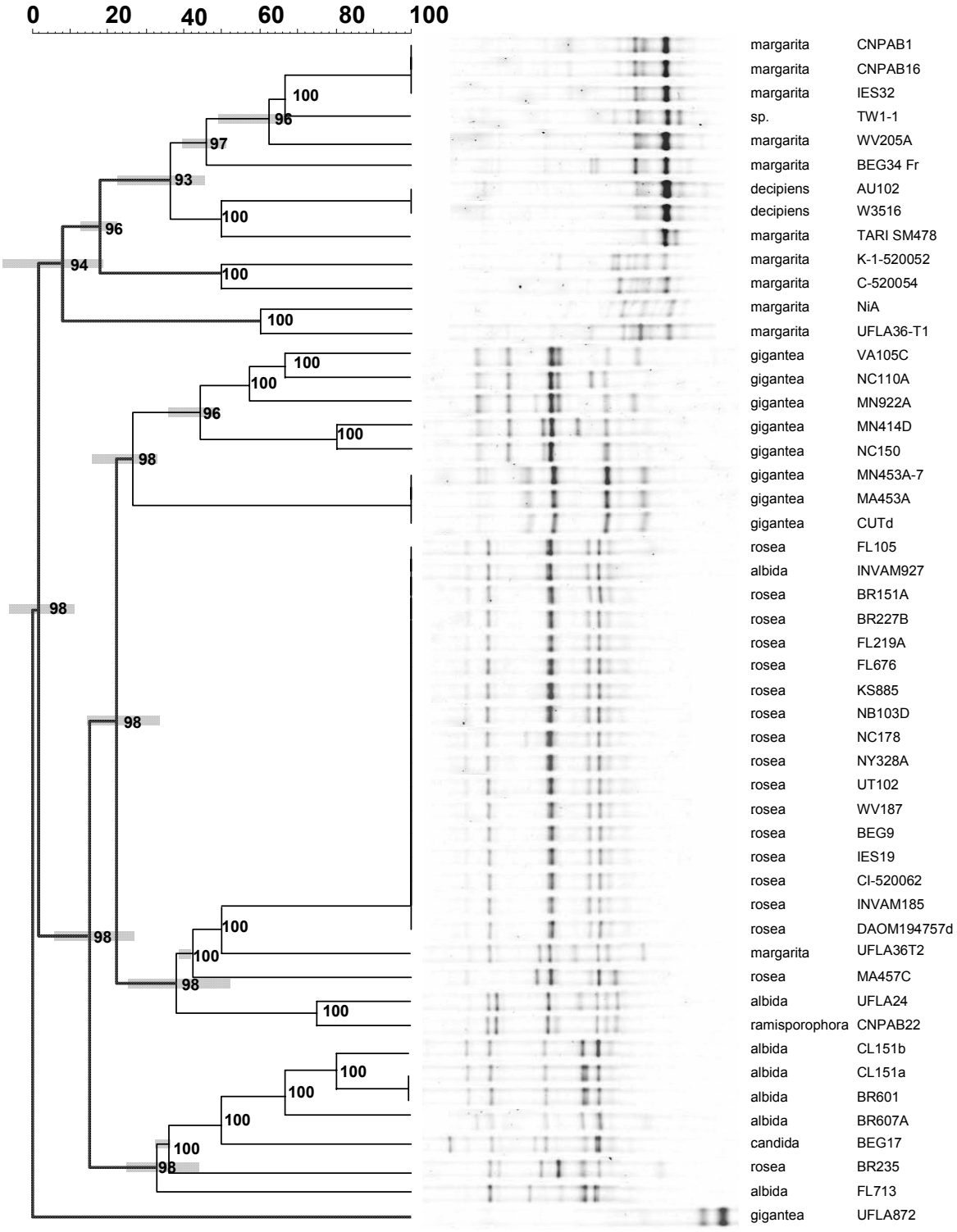

FIG. 3. Dendrogram showing the distance tree (Jaccard - UPGMA) and the PCR-DGGE banding patterns of 48 strains of *Gigaspora* and two divergent patterns found in strains *G. albida* CL151 and *G. margarita* UFLA36. Gels were run for 17 h at 95 V. Scale shows similarities of banding patterns; thicker lines indicate error flag and dashed line the separation between major clades or subclades. Numbers indicate cophenetic correlations, which are estimates of the faithfulness of each subcluster of the dendrogram.

G. gigantea, and *G. margarita* but generally not for *G. decipiens* and *G. rosea*, although accession *G. rosea* MA457C appeared to contain a number of unique ribotypes (Fig. 3).

PCR-DGGE detected potential misidentifications by revealing some patterns that did not cluster with their respective type material. For instance, within the species *G. albida*, accession UFLA24 clustered with the *G. ramisporophora* type material (CNPAB22) and accession INVAM927 produced a pattern identical to that of the large group of *G. rosea* isolates. Similarly, *G. rosea* BR235 grouped together with *G. albida* isolates; it was the blind sample sent by INVAM's curator. This result confirms the supposed misidentification of this accession based upon morphological characteristics (J. B. Morton, personal communication). Although *G. candida* BEG17 and *G. ramisporophora* CNPAB22 are both considered to represent nonvalid species names based upon morphological evaluation (Bentivenga & Morton 1995), they could be clearly distinguished from the other species examined. Interestingly, the pattern of *G. candida* BEG17 was more similar to that of the *G. albida* isolates than to those of *G. rosea*, the species to which it was previously assigned (Fig. 3). *G. ramisporophora* CNPAB22 generated a banding pattern that was more similar to those of *G. rosea* (Fig. 3) than those of *G. margarita*, the species to which *G. ramisporophora* was assigned based upon morphological characteristics. A robust molecular characterization of the species *G. candida* and *G. ramisporophora* will require the analysis of additional isolates. Unfortunately, no other well-defined isolates of *G. candida* are available at this time, to the best of our knowledge, and very few well-defined isolates of *G. ramisporophora* are available.

Sequence analysis of PCR-DGGE banding patterns

To gain further insight into the nature of the intra- and interspecies heterogeneity detected, sequence information was obtained for the region analyzed by PCR-DGGE for each of the ribotypes observed within representative isolates of each species (Table 4). Sequence analysis confirmed the identity of bands that displayed the same migratory behavior. Furthermore, sequence and phylogenetic analyses (Table 4) also confirmed a number of the relationships depicted in the dendrogram analysis of the PCR-DGGE patterns. For instance, *G. margarita* and *G. decipiens* are closely related and distinct from the group formed by *G. albida*, *G. candida*, *G. ramisporophora*, and *G. rosea* and that of *G. gigantea.* In addition, *G. gigantea* UFLA872 is quite distinct from all other accession strains and contains a DNA signature, CGCGTG, that has been reported to occur in *Scutellospora* species (Bago et al 1998). With reference to the characterization of putative invalid species, sequence analysis confirmed ribotype overlap between *G. candida* BEG17 and *G. albida* BR607A, including a shared DNA signature, TAGGTT, which is distinct from that of *G. rosea* (Bago et al 1998). The

Table 4: DNA sequences of 70 PCR-DGGE-selected *Gigaspora* clones and two DGGE bands showing alignment of 24 parsimonious informative and two uninformative positions in the V9 18S rDNA region. Bold and underlined characters show location of DNA signatures proposed by Bago et al (1998).

	Position in the alignment[d,e]
	00001111222222222222222233
	57771479366777777888889900
Species, clone code[a,b], accession code[c]	50164482456045689016792912
G. gigantea A1* UFLA872; B1* UFLA872	CCGCCCTATCGC**CGCgtG**GCCCGTAC
G. decipiens 4; *G. margarita* M5, M6*, M7, CNPAB1; T2 CNPAB16; 7*, 8, 15* BEG34; DGGE b2	CCGCCCTGTCGC**CGAgtG**GCTCGTAT
G. margarita F22, F44, CNPAB1; T3 CNPAB16	TCGCCCTGTCGC**CGAgtG**GCTCGTAT
G. margarita M9 CNPAB1; T5 CNPAB16; DGGE b1	CCGCCCTGTTGC**CGAgtG**GCTCGTAT
G. margarita 1, 21 BEG34	CCGCCCTGTCGC**CGAgtG**GTTCATAT
G. decipiens 2*; *G. margarita* 2, 10, 14 BEG34	CCGCCCTGTCGC**CGAgtG**GCCCGTAT
G. decipiens 9; *G. margarita* M18 CNPAB1; 5, 20 BEG34	CCGCCCTGTCGC**TGAgtG**GCTCGTAT
G. margarita M8* CNPAB1	CCACCCTGTCGC**TGAgtG**GCTCGTAT
G. albida 13; *G. candida* C13,C17*; *G. ramisporophora* GP18	CCGCCTTGTCGC**TAAgtG**GCTCGCTT
G. *albida* 25*	CCGCCTTGCCGC**TAAgtG**GCTCGCTT
G. *ramisporophora* GP16-11; GP22	CCGCTTTGTCGT**TAAgtG**ACTCGCTT
G. ramisporophora GP27, GP49	CTGCCTTGTCGT**TAAgtG**GCTCACTT
G. rosea genebank X58726	CCGCCTTGTCGC**TAAgtG**GCTCGCTC
G. albida 17; *G. candida* C4	CCGTCTTGTTGC**TAAgtG**GCTCGCTT
G. rosea R14, R16	CCACCTCATCGC**TAAgtG**GCTCGCTT
G. ramisporophora GP44	CCGCCTTGTCGC**TAAgtG**GCTCGCCT
G. albida genebank Z14009	CCGCCTTGTCGC**TAAgtG**GCTCGCAC
G. ramisporophora GP14	CCGCCTTGTCGC**TGAgtG**GCTCGCTT
G. albida 14; *G. candida* b4, C3, C12, C14b, C16	CCGCCTTGTCGC**TAGgtT**GCTCGTAC
G. ramisporophora 16-1, 16-2	CCGCTTTGTCGC**TATgtT**GCTCGTAC
G. ramisporophora GP33; *G. rosea* E2, R13, R15;	CCGCCTTGTCGC**TATgtT**GCTCGTAC
G. albida 19*, 31; *G. candida* C1, C14, C18, G1	CCGCCTTGTCAT**TATgtT**GCTCGTAC
G. rosea R19	CCGCCTTGCCGC**TATgtT**GCTCGTAC
G. gigantea 15 VA105C	CCGCCTTGTCGC**TAAgtT**GCTTGTAC
G. gigantea 19 VA105C	CCGCCTTGTCGC**TAAgtT**GCTCGTAC
G. gigantea 3*, 7*, 26* VA105C	CCGCCTTGTCGC**TGTgtT**GCCCGTAC
G. gigantea 10 VA105C	CCGCCTTGTCGC**TGTgtT**GCTTGTAC
G. gigantea 6 VA105C	CCGCCTTGTCGC**TTAgtT**GCTCGTAC
G. gigantea 20*, genebank Z14010*	CCGCCTTGTCGC**TGAgtT**GCTCGTAC

([a]) Different clones in the same line have 100% DNA sequences similarity with one or more clones in the same line; ([b]) Clones in the same line followed by * have less than 100% similarity with other clones in the same line. ([c]) Accession numbers not specified in the table are *G. albida* BR607A; *G. candida* BEG17; *G. decipiens* W3516; *G. ramisporophora* CNPAB22; *G. rosea* BEG9.
([d]) Position 1 starts at the 5' end of the primer NS7, according to the alignment provided at the European Bioinformatics Institute (EMBL-EBI) website (http://www.ebi.ac.uk/webin-align/webin_align_listali.html, alignment ALIGN_000606). ([e]) Grey and black shadow shows most divergent characters among the studied sequences. The GT characters in lower case are not parsimoniously informative but are shown because they are part of the DNA signature proposed by Bago et al. (3) (underlined). The line spaces in the table separate the major clades obtained by phylogenetic analysis. ([f]) Sequences obtained at the Genebank.

ribotype sequences obtained for *G. ramisporophora* were also more similar to those of *G. albida*, *G. candida*, and *G. rosea* than to those of *G. margarita* despite apparent similarity in the morphology of *G. ramisporophora* and *G. margarita* spores. Those results do not support the reclassification based on morphological analysis by which *G. candida* was reclassified as being synonymous with *G. rosea* and *G. ramisporophora* was reclassified as being synonymous with *G. margarita* (Bentivenga & Morton 1995).

Detection of Gigasporaceae species in field samples.

(i) Detection limit

To test the specificity of the *Gigasporaceae*-specific primers used, nontarget DNA, in our case DNA from *Glomus clarum* CNPAB5 spores, was used. It did not interfere in the PCR amplification even when the nontarget species was provided in 100-fold-higher numbers. Multiple *Gigaspora* species could be detected in artificial spore mixtures when a given species represented 10% or more of the total, and the relative signal intensity roughly matched the spore volume (data not shown). When *S. heterogama* and *G. margarita* spores (both targeted by the primers used) were combined at various ratios, the larger spore size (i.e., more 18S rDNA targets per spore) of the latter species skewed the range within which both species could be detected. A single *G. margarita* spore could be detected in a background of up to 100 *S. heterogama* spores, whereas the *S. heterogama* signal was no longer detected when spores of this species were outnumbered fivefold or more by *G. margarita* spores. Thus, in the analysis of bulk samples containing large numbers of spores or DNA isolated directly from root or soil material, minor populations may not be detected. The analysis of individual spores, small groups of spores, or individual root pieces may therefore offer the best strategy for detecting the full breadth of *Gigasporaceae* diversity within a sample.

(ii) Detection of Gigasporaceae species in greenhouse experiments and environmental samples

In a controlled greenhouse experiment, clover plants were inoculated with four AMF species, one species of *Gigaspora* and three of *Scutellospora*. Despite a colonization level of less than 20%, as determined by microscopic inspection, AMF-specific products could be easily detected with a nested PCR and DGGE approach. All four AMF species could be detected, although secondary bands had to be used to determine the presence of the two *Scutellospora* species, since the two species used presented a prominent DGGE band in the same position (Fig. 4).

Gigaspora spores were recovered from the trap cultures of the two Brazilian agricultural soil samples, as identified by morphological characteristics. These samples also contained spores belonging to the genera *Archaeospora*, *Acaulospora*, and *Glomus* (not targeted in our PCR-DGGE

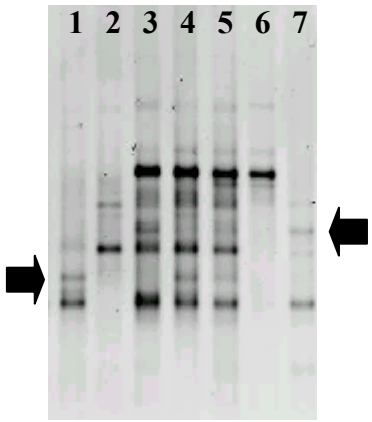

FIG. 4. Detection and identification of *Gigasporaceae* from DNA extracted from 2-month-old clover roots by PCR-DGGE analysis of the V9 region of the 18S rRNA gene. DNA templates: lane 1, *S. heterogama* CNPAB2; lane 2, *G. margarita* CNPAB16; lane 3, *Trifolium pratense* replicate 1; lane 4, *T. pratense* replicate 2; lane 5, *T. pratense* replicate 3; lane 6, *S. gregaria* CNPAB7; lane 7, *S. reticulata* CNPAB11. Note the presence of secondary bands in lanes 4 and 5 (*S. heterogama*) and 3 (*S. reticulata*) (arrows). The gel was run for 17 h at 95 V.

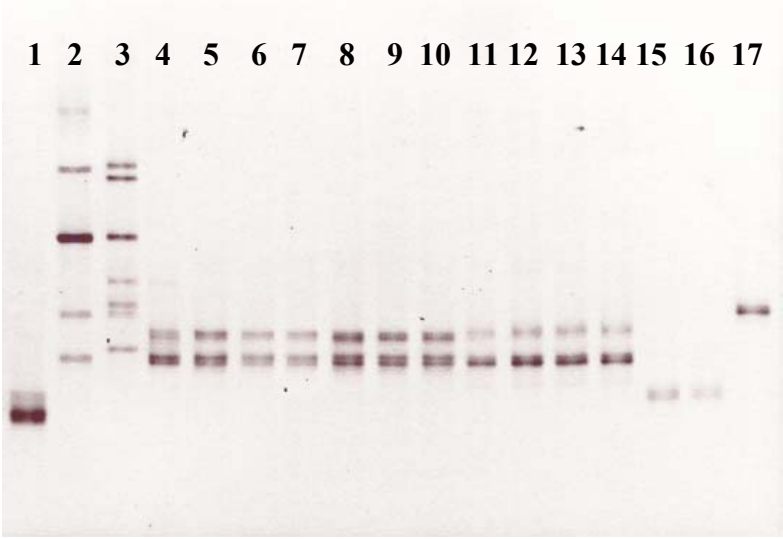

FIG. 5. Detection and identification of *Gigasporaceae* from DNA extracted from soil or single spores from trap cultures by PCR-DGGE analysis of the V9 region of the 18S rRNA gene. DNA templates: lane 1, *G. gigantea* UFLA872; lane 2, *G. gigantea* VA105C; lane 3, *G.ramisporophora* CNPAB22; lane 4, *G. margarita* CNPAB1; lanes 5 to 7, soil DNA extracted from trap culture A; lanes 8 to 10, soil DNA extracted from trap culture B; lanes 11 to 14, single-spore DNA from four different *Gigaspora* spores recovered from trap culture A; lanes 15 and 16, single-spore DNA from two different *S. heterogama* spores recovered from trap culture A; lane 17, single-spore DNA from *S. coralloidea* recovered from trap culture A. The gel was run for 15 h at 95 V.

analysis), as well as small numbers of spores of the genus *Scutellospora* (targeted by the primers used in this study; Table 3). Nested PCR-DGGE analysis with DNA extracted from these *Gigaspora* spores as well as that isolated directly from soil and roots from the trap cultures generated banding profiles similar to those observed for the type material of *G. margarita* (Fig. 5), and band identity was confirmed by sequence analysis (Table 4). In addition, the band patterns of 30 individual spores recovered from sample A were identical and produced patterns similar to that of *G. margarita* strains CNPAB1, CNPAB16, and IES32. Although some *Scutellospora* spores were present in these samples, this genus was not detected via PCR-DGGE with DNA extracted directly from the soil and roots, even though the specificity of the PCR covered this genus. Recovered *Scutellospora* spores could be used as the template for PCR-DGGE analysis and could clearly distinguish them from the *Gigaspora* species detected (Fig. 5). The relative amount of *Scutellospora* material in the soil samples (0.5 g) used to extract DNA was apparently below the detection limit of our analysis.

DISCUSSION

PCR-DGGE as a tool to characterize, identify, and detect Gigaspora species

By using PCR-DGGE targeting the V9 region of the 18S rRNA gene, we were able to generate highly reproducible profiles obtained from single-spore DNA isolations, which could be used to characterize and differentiate all *Gigaspora* species based on the type materials used. This included the discrimination of species previously thought to be invalid based upon morphological characteristics (*G. ramisporophora* and *G. candida*; see Fig. 2). While some intraspecific variation in PCR-DGGE banding patterns provided several markers that might be used to track specific isolates of a given species, species patterns were generally highly diagnostic (Fig. 3). This study provides the most complete molecular characterization available for the genus *Gigaspora*, and the specific PCR-DGGE method provided far better characterization and species identification than any other previously applied to this genus. Due to the overlap of some ribotypes across species boundaries (e.g., *G. albida*, *G. candida*, *G. ramisporophora*, and *G. rosea* or *G. decipiens* and *G. margarita*; see Fig. 3 and Table 4), it became clear that the entire pattern of 18S rDNA types needed to be used for species characterization and identification, as opposed to just a single sequence. Thus, in contrast to other molecular approaches, such as PCR followed by cloning and sequencing, in which intraspore rDNA heterogeneity impairs interpretation, the PCR-DGGE approach uses this heterogeneity to advantage for the generation of highly reproducible isolate- or species-specific patterns. Furthermore, in comparison to some other molecular methods, PCR-DGGE is easy, rapid, and inexpensive to perform.

We used two different nested PCR strategies. The first nested PCR strategy uses family-specific primers in the first PCR. With that strategy, it was possible to analyze DNA extracted from environmental samples with small amounts of material extracted directly from spores, roots, or soil without the problem of nonspecific amplification. However, despite the family-specific nature of the nested PCR strategy and the presence of both *Gigaspora* and *Scutellospora* species in the mixed spores, roots, and soils analyzed, the latter genus was not detected. However, in a control experiment, we could detect the simultaneous presence of all four of the AMF species introduced into the root system (Fig. 4). PCR-DGGE strategies will generally fail to detect some minority populations (i.e., 1% of the total target) (Brüggemann et al 2000, Muyzer & Smalla 1998), and our experiments showed that a total spore volume of 10% of the total was typically necessary to ensure the detection of a minority target species. Furthermore, the DGGE signals detected are based on the relative number of template molecules of a given species in a sample. Thus, even assuming equal amplification efficiencies, it remains difficult to translate the levels of DGGE signal detected for certain AMF species, as the ratio of 18S rDNA target to fungal biomass and the ecological importance of this ratio are not known at present. The second nested PCR strategy used universal primers that, in our case, amplified the whole 18S rRNA gene plus the internal transcribed spacer (ITS) region. That fragment was cloned, followed by PCR-DGGE selection targeting variable regions in the cloned fragment. That strategy proved to be an excellent approach to study inter- and intraspecies heterogeneity in the 18S rDNA. The principles of that strategy can be applied to study polymorphism in any other gene found in AMF. The application of a similar approach with faster-evolving genes might allow better discrimination between isolates and also might help to shed some light on evolutionary issues such as genetic drift and recombination in these ancient asexual organisms (Gandolfi et al 2003). It is interesting that the DGGE system used was highly efficient in detecting sequence variation within the V9 region of the 18S rDNA gene. Within the 344 bp (without GC clamp) of sequence targeted, 303 bp were constant, 17 bp were parsimoniously uninformative, and 24 bp were parsimoniously informative (Table 4). The V9 18S rDNA fragments analyzed by PCR-DGGE are within the optimal size range for DGGE analysis, i.e., below 500 bp. Most of the mutations found were transitions (37 of 41), which allow discrimination by DGGE, as they always cause a change in the melting temperature. However, transversions were detected in four of the parsimoniously informative positions (positions 274, 275, 279, and 299; see Table 4), and some of them did not affect the melting temperature of the amplicons; consequently, they might not be detected if they were the only mutation present in the amplicon. Despite having the ability to discriminate most single-base-pair differences within 18S rDNA V9 region fragment analyzed here, any DGGE analysis will be limited by the amount of heterogeneity, type of mutation, and fragment size of the target region (Myers et al 1987). This was exemplified by

our analyses of the V3-V4 region, which contained less variation than the V9 region and also used a longer fragment (550 bp without the GC clamp). Interestingly, the V3-V4 region could be used to discriminate between *Glomus* species (Kowalchuk et al 2002). Although the PCRDGGE banding patterns were highly reproducible within a given isolate across various DNA isolations, exact banding patterns are dependent on the electrophoresis conditions used (data not shown), and analytical consistency and the use of type strains (isolates) as markers are critical when comparing samples. The similarity between the sequences from the V9 region analyzed was high both between and within species (range, 98.6 to 100%; Table 4). Of the variable positions described, only 24 were phylogenetically informative, hampering robust phylogenetic analysis. As such, the comparison of V9 18S rDNA fingerprints (Fig. 3) provided a more reliable method of comparison than tree construction based on the sequence variations described in Table 4. With proper primer design, PCR-DGGE strategies as implemented here are also ideal for determining specific ribotypes of other AMF genera or other loci in experiments designed to address AMF reproduction and evolution (Gandolfi et al 2003, Sanders 2002), as well as similar issues with respect to intraspecies heterogeneity among rDNA copies in bacteria (Amann et al 2000, Dahllof et al 2000, Nübel et al 1996). It should be noted that our analysis of single spores does not address the homokaryotic or heterokaryotic nature of AMF nuclei (Gianinazzi-Person et al 2001, Trouvelot et al 1999), although the sensitivity of PCR should permit PCR-DGGE of single AMF nuclei.

Comparison between single- and multiple-spore DNA isolations and geographically diverse isolates

The PCR-DGGE banding patterns of all single-spore DNA isolations tested for the same isolate were identical for 46 of 48 accession strains tested (Fig. 3). Thus, at least to the level of detection afforded by the system used here, a single spore appeared to contain the full range of variation of ribotypes present in an entire spore population. The two exceptions were both isolates recovered by trap cultures. Trap cultures are the most common way to isolate AMF from field samples. However, if more than one morphologically closely related species are coisolated in the same culture, further discrimination by spore morphology is difficult. One, *G. margarita* isolate, UFLA36, produced spores with two very different DGGE patterns that clustered apart from each other; one type (UFLA36-T1) clustered in the *G. margarita – G. decipiens* cluster, and the other type (UFLA36-T2) clustered with *G. ramisporophora* and *G. rosea*. In the case of *G. albida* CL151, the two spore types were similar, differing only by the absence of one band in one spore type and the relative intensity of one of the other bands (Fig. 3). In those cultures, further purification by single-spore pot culture followed by spore identification with PCR-DGGE can be used to purify and distinguish those populations. Interestingly, some geographically distinct isolates were more similar than some isolates that were

found at the same site. Within the species *G. gigantea*, for instance, strains NC110A and NC150 are rather different single-spore cultures recovered from the same site (Bever et al 2001), whereas isolates CUT, MA453A, and MN453A-7 had identical rDNA patterns despite being isolated from disparate locations. Similarly, *G. albida* CL151 type a from the United States and BR601 from Brazil were identical (Fig. 3), while other sympatric populations showed more diversity. The presence of strain-specific bands should allow the tracking of specific AMF populations in studies dedicated to unraveling the ecological significance of such sympatric populations. The band intensities observed for different geographic isolates differed in some cases. This result suggests that the proportion of the different ribotypes may differ between isolates of the same species, but more quantitative methods, such as introduction of an internal standard in DGGE experiments (Brüggemann et al 2000), will be necessary to address this question.

PCR-DGGE characterization versus other schemes applied previously

The lack of discrete and diagnostic characters for species identification within the genus *Gigaspora* has been a major obstacle to ecological studies of this genus. The use of PCR-DGGE as applied in this study clearly offers a higher level of discrimination and reliability than previous methods used to address this issue. For instance, based on spore morphology, *G. candida* was considered synonymous with *G. rosea* and *G. ramisporophora* was considered synonymous with *G. margarita* (Bentivenga & Morton 1995). However, our results not only do not support this reclassification, they show that the so-called invalid species are actually less related to their supposed synonymous species than to other species, based on the rDNA marker. We could also distinguish between species that were grouped together on the basis of the molecular signatures proposed by Bago et al. (1998). Our detection of high degrees of heterogeneity within the V9 region of the 18S rRNA gene, which encompasses the sequence stretch examined by Bago et al. (1998), also explains the ambiguities found within the signature sequences that they defined. The mixed PCR products recovered from an isolate could be resolved by PCR-DGGE (Table 4) but produced ambiguities at heterogeneous positions upon direct sequencing as preformed by Bago et al. (1998). Our results suggest that *G. albida* INVAM927 should be reassigned as *G. rosea* (Fig. 3). *G. albida* INVAM927 was one of the strains used by Bentivenga and Morton (1995) as type material to redescribe this species. In contrast, the material we used for this isolate came from the INVAM in Florida and not from West Virginia University. Unfortunately, a comparison between the material examined in this study and the original material used by Bentivenga and Morton (1995) is no longer possible, as this accession strain has been lost from the INVAM collection (J. B. Morton, personal communication). The accession strain *G. albida* UFLA24 must also be reassigned as *G. ramisporophora* on similar grounds. One of the strains identified as *G. rosea*

obtained from INVAM was actually *G. albida*, based on information sent by the curator of the collection. This isolate was correctly identified by our PCR-DGGE analysis (Fig. 3) as strain BR235, confirming the morphological analysis. Despite being characterized as *G. gigantea*, accession UFLA872 presented a PCR-DGGE pattern and a DNA sequence that did not match those of any other isolate examined (Fig. 3). Spores of *G. gigantea* UFLA872 have the same size range as expected for *G. gigantea* and also exhibit a cytoplasm color typical of *G. gigantea.* This feature is considered a unique identifying characteristic of this species (Sejalon-Delmas 1998). Other authors have also described conflicts between morphological and molecular identifications of AMF. Bago et al. (1998) suggested the reassignment of isolate DAOM194757, morphologically identified as *G. margarita*, to the *G. rosea* group. Lanfranco et al. (2001) confirmed this result and also suggested reassignment of isolate E29 (*G. margarita* based on morphology) to the *G. rosea* group on similar grounds.

Implications of 18S rDNA heterogeneity for ecological and evolutionary studies

Heterogeneity between rRNA markers within a species or single individual is a phenomenon that has been described for a wide range of organisms (Amann et al 2000, Buckler et al 1997, Dahllof et al 2000, Gandolfi et al 2001, Kuhn et al 2001, Nübel et al 1996, Sanders 2002, Tang et al 1996). In our PCR-DGGE approach, we have not only used this heterogeneity to characterize and identify species, but have also combined this with a cloning and screening strategy to tease apart the relationships between the 18S rDNA variants detected in a single spore (species). Knowledge of this intraspecific variation is fundamental for proper assignment of operational taxonomic units for molecular sequence identification and phylogenetic analysis (Dahllof et al 2000). It is possible that cloning and sequencing will underestimate the number of species if the clones chosen for analysis happen to harbor those ribotypes held in common between the different species present in a sample. It is more likely, however, that such studies will overestimate species diversity (Dahllof et al 2000, Sanders et al 1995). Our results show that some different morphospecies share certain ribotypes but also contain species-specific variants. Given the asexual nature of AMF, the mechanisms for the establishment and maintenance of such patterns of sequence diversity remain to be discovered. Although rRNA genes have been highly useful markers for the phylogenetic study of microorganisms, including *Gigaspora* (Schüßler et al 2001), data gained from this marker alone clearly cannot resolve all phylogenetic and evolutionary issues for these organisms. The analysis of other molecular markers is an urgent issue for reconstructing phylogenetic relationships within this genus and for other AMF. Thus, although we were able to detect and utilize interesting patterns of rDNA heterogeneity within the *Gigasporaceae* for detection and identification purposes, further studies are necessary to explain these patterns and understand how they fit into the scheme of AMF life history and evolution. The ability to assess

Gigaspora diversity directly in environmental samples opens new possibilities for studying the ecology of this group under field conditions without the need for trap cultures. Recently, *G. margarita* was found in Europe, and its occurrence seems to be affected by the tillage system used (Jansa et al 2002). Some *Gigaspora* species are known to harbor an endosymbiont of a proposed new bacterial genus, candidates *Glomeribacter gigasporarum* (Bianciotto et al 2000, 2003), and the characterization of fungal and bacterial partners might clarify the evolutionary and ecological aspects of that symbiosis. PCR-DGGE targeting the V9 region of the 18S rDNA provides a fast and reliable method to identify *Gigaspora* species, to assess *Gigasporaceae* diversity in field conditions, and to characterize inter- and intraspecies rDNA heterogeneity.

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Chapter 7

Evidence for network evolution in arbuscular mycorrhizal fungi of the genus *Gigaspora*, Glomeromycota

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To be submitted.

ABSTRACT

Ancient asexual organisms are considered biological scandals because their evolutionary mechanisms to escape extinction are unknown. Arbuscular mycorrhizal fungi (AMF) are among these organisms. Recent research on AMF karyotic state have shown contrasting results. However, fungi can go through a parasexual cycle forming both homo- and heterokaryotic species. Parasexuality occurs, between genetically diverse but compatible fungi, through hyphal fusion, anastomoses, followed by nucleus exchange. Until now, only self-anastomoses has been reported in AMF, giving no support for parasexuality. Here we found evidence for reticulated evolution and recombination of rRNA genes in AMF, supporting the occurrence of parasexuality in AMF of the genus *Gigaspora*. We found more than one lineage of rRNA genes per genome, and that some lineages are polyphyletic across species boundaries, indicating hybrid species. Nevertheless, molecular clock estimates indicate these events are rare. Parasexuality could explain both the homo- and the heterokaryotic states reported for AMF, and generate genetic variability and clear deleterious mutations from their genome. These findings indicate that recombination is playing a significant role in shaping genetic diversity of this asexual group of fungi.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi (Glomeromycetes) comprise a monophyletic (Schüβler et al. 2001) group of successful plant symbionts. Their role in plant nutrition (Smith & Read 1997) and as drivers of plant diversity has often been demonstrated (van der Heijden et al. 1998). The genome organization and speciation process in this putative ancient asexual group of fungi are not fully understood (Gianninazi-Pearson et al. 2001; Sanders 2002). The fungi reproduce clonally forming large resting spores (22-1050μm diameter), which typically contain several hundreds or thousands of nuclei (Becard & Pfeffer 1993). Unfortunately, AM fungi are obligatorily biotrophic organisms, requiring symbiosis with compatible plants to complete their life cycle, a characteristic that hampers basic and applied studies. Nevertheless, the analyses of the nuclear ribosomal DNA (nrDNA) sequences obtained from single individuals (spores) have shown high polymorphism among its copies (for a review see (Sanders 2002).

Studies on nrDNA intraspecific polymorphism in AM fungi have been based on single or distantly related species. The lack of a phylogenetic framework in the study of AM fungi intraspecific

polymorphism has hampered the elucidation of evolutionary processes that generate such polymorphism. Also, recombination was recently reported to occur in AM fungi (Gandolfi et al. 2003; Pawlowska & Taylor 2004). However, these studies were again limited to the detection of recombination, providing little insight into the processes generating the observed genetic patterns. The evolutionary processes responsible for the origin and maintenance of such high nrDNA intraspecific polymorphism in AM fungi are still unknown.

Ancient asexuals are rare in nature, with apart from the AM fungi only two or three other well-known examples: the Bdelloidae (wheel animals, Ricci 1987; Birky 2004) and Darwinolidea (small Crustaceans, Butlin et al. 1998). According to theory, the early extinction of most asexual lineages can be explained by i) lack of recombination and subsequent increase of slightly deleterious mutations (Muller 1964; Kondrashov 1982) and/or ii.) low adaptability to changing environments as compared to sexual organisms; (Hamilton 1980; Bell 1982). Ancient asexuals are likely to overcome these processes, but how they do this and what it is that made them exceptional among the asexual lineages is unknown. Searching the process responsible for generating intraspecific polymorphism in the nuclear ribosomal DNA (nrDNA) of AM fungi may shed light on these intriguing questions.

AM fungi mycelium is essentially non-septate or coenocytic, where nuclei can travel via cytoplasmic streaming (Bago et al. 1998b; Bago et al. 1999). Two species are known to be halploid *Glomus intraradices* and *Scutellospora castanea* (Hijri & Sanders 2004, 2005). The spores were thought to be heterokaryotic as revealed by *in situ* hybridization assays (Trovelot et al. 1999; Kuhn et al. 2001). Recently, PCR typing of individual nuclei of *Glomus etunicatum* and *Glomus intraradices* demonstrated that AMF might be homokaryotic (Pawlowska & Taylor 2004), however, new research on this issue suggest that at least another strain of *Glomus etunicatum* is indeed heterokaryotic (Hijri & Sanders 2005). Homo and heterokaryotic states in related species could be the result of the parasexual cycle, which is the exchange of nuclei between genetically diverse but compatible fungi after fusion of somatic mycelium through anastomoses (Schardl & Craven 2003). This process results in a heterokaryotic state in the coenocytic mycelium. The heterokaryon is typically unstable, and may lead to fusion of the two genetically distinct nuclei. For instance, the anastomosis between two haploid homokaryons can generate a heterokaryon. The heterokariotic nucleus usually fuse forming a diploid, which later can loose chromosomes to return to the haploid state (Schardl & Craven 2003). The parasexual cycle might generate network (reticulated) evolution through horizontal gene transfer between genetically divergent but compatible fungi. The characteristics of AM fungi mycelium would facilitate the occurrence of parasexual cycle. However, to date anastomoses has only been observed between hyphae of the same individual (clone), and never between geographic isolates of the same morpho species (Giovannetti et al. 2003).

In this investigation, we hypothesized, that parasexual cycle is the origin of the divergent copies of nrDNA in AM fungi genome. To test this hypothesis the genus *Gigaspora* was chosen for the following reasons: i) the species of this genus are closely related based upon both morphological (Bentivenga & Morton 1995), molecular data (Bago et al. 1998a; de Souza et al. 2004), ii.) species radiations within this genus are rather recent in relation to the 600 MY old AM fungi ancestor (Redecker et al. 2000), considering, that *Gigasporaceae* are among the youngest AM fungi families, based upon fossil record (240 MYA, Phipps & Taylor 1996) and molecular clock estimates (242-252 MYA, Simon et al. 1993), iii) a high degree of nrDNA sequence polymorphism has been detected in *Gigaspora* species (Lanfranco et al. 1999; Antoniolli et al. 2000; de Souza et al. 2004), and iv) tools have recently been developed to analyze exactly the nature of nrDNA polymorphism for this genus (de Souza et al. 2004). We applied a phylogenetic approach and our data set covers all described *Gigaspora* species and one putative new species. One representative isolate of each species was chosen based upon an initial screening of 48 different *Gigaspora* strains (de Souza et al. 2004). Sequence analyses were performed for all detectable haplotypes from these selected strains for a DNA fragment spanning nearly the full 18S nrDNA until the beginning of the 28S nrDNA. In total, 37 haplotypes were analyzed across this approximately 2300 bp region. Phylogenetic and recombination analyses were performed to elucidate the mechanisms responsible for intraspecific nrDNA polymorphism.

MATERIAL AND METHODS

Fungal isolates

Eight *Gigaspora* isolates were chosen for analysis from a screening of 48 different strains (de Souza et al. 2004), representing one of each of the described species in the genus *Gigaspora* (*Gi. albida* BR 607A; *Gi. candida* BEG17; *Gi. decipiens* W3516; *Gi.* gigantea VA105C; *Gi. margarita* CNPAB1; *Gi. ramisporophora* CNPAB22; *Gi. rosea* BEG9). In addition, a *Gi. gigantea*-like accession, UFLA872, was included because of its unique 18S nrDNA sequence, which previously suggested that it might be a novel species (de Souza et al. 2004).

Selection of clones containing intraspecific polymorphism

All the laboratory procedures used, except sequencing, were as described previously (de Souza et al. 2004). We focused on 37 clones, with insert sizes of approximately 2300bp, containing the nearly complete 18S nrDNA (1744bp), the 5.8S gene and the 5' end of the 25S nrDNA, including the two

internal transcribed spacers ITS1 and ITS2. The inserts were obtained from amplification of genomic DNA of one single spore of each isolate tested. We used a single spore as representative of a species because for each of the isolates tested no spore-to-spore variation was found within different spores coming from the same fungal strains (de Souza et al. 2004). The 37 clones were selected by PCR-DGGE screening of the V9 region of the 18S nrDNA in order to obtain all detectable haplotypes within each accession studied (for details see (de Souza et al. 2004). To facilitate clone selection, we used PCR-DGGE profiles obtained from amplification of the original strains as markers. This strategy not only facilitated clone selection, but is also helped to avoid the selection of clones presenting PCR or cloning errors (Speksnijder et al. 2001). It should be noted that we initially underestimated the haplotype diversity for *Gi. Rosea* (de Souza et al. 2004). Only after full optimization of the DGGE screening method was possible to discriminate between two clones of two haplotypes that produced overlapping bands on the initial PCR-DGGE profile for the V9 region of the 18S nrDNA for this species.

DNA Sequencing

The selected clones had been partially sequenced for a, 344bp region spanning the V9 region of the 18S nrDNA, (database accession numbers AJ539236-AJ539305 (de Souza et al. 2004). This region is known to be the most variable in the SSU for *Gigasporaceae* (Simon 1996). All clones were sequenced in the most variable regions (V9 region of the 18S until the 5'end of the 25S together with the flanking regions (ITS1 and ITS2), which were the regions on which we focused our phylogenetic analysis. The remainder of the 18S nrDNA was sequenced for 26 of the clones. Sequencing reactions were obtained from the two DNA strands of each clone using the Perkin Elmer Biosystems Big Dye Terminator Sequence Reaction kit (Perkin Elmer, Foster City, Calif.) and the sequence analyses were carried out on a Perkin Elmer 3700 capillary sequencer at the RIVM (Bilthoven, The Netherlands).

Recombination analyzes

The reticulate evolution was evaluated by graphical methods and recombination by Likelihood Analysis of Recombination in DNA - LARD (Holmes et al. 1999). The Splits decomposition, a graphical method (Huson 1998), was used as a first search for evidence of reticulated evolution. This method is exploratory and it detects conflicts in the phylogenetic analyzes (discordant topologies) rather than recombination per se. LARD test was used in putative recombinant sequences. This method establishes breakpoints in the alignment that indicate the beginning of hypothesized

recombinant sites. A new phylogeny was then constructed on the different regions and the extent of the incongruence between them assessed (Holmes et al. 1999).

Phylogenetic analyzes

Sequences were aligned using Clustal-X (Thompson et al. 1997). The alignment was improved by manual inspection. Only one alignment was used, which covered the V9 region of the SSU until the 5'end of the LSU. Before performing the phylogenetic analysis we search for outgroup to infer the phylogeny. When sequences of the sister group *Scutellospora* were used as outgroup, the *Gigaspora* clones A1_UFLA872 and B1_UFLA872 were placed in the base of the *Gigaspora* clade. Thus they were chosen to be used as outgroup.

The phylogenetic analysis was conducted using Maximum Likelihood (ML) criteria, with heuristic search and tree bisection-reconnection using 10 random additional sequence replications in PAUP* version 4.0 Beta 10 (Swofford 2003). For each dataset used to construct trees the substitution model that best fit the data was selected to infer the phylogeny, using ModelTest version 3.6 (Posada & Crandall 1998). The final model used was selected after optimizing the parameters using successive searches started with improved trees and parameters. The models and the phylogenetic analyzes were calculated after removing the gapped characters from the main alignment. Maximum Parsimony (MP), and Minimum Evolution (ME) methods were also used to confirm the topology of the phylogenetic tree obtained using ML.

The data were also analyzed using Bayesian method implemented in Mr Bayes V3.0B4 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). As the ITS regions evolved faster than the rDNA genes and possessed a different base frequency richer in A and T, the data were divided in two partitions. The first containing the positions (1-387 and 478-645) of V9 18S and 5.8S rDNA regions respectively, and the second containing the positions (388-477 and 646-905) of ITS1 an ITS2 regions respectively. MrModeltest (Nylander 2004) was used to calculate the best substitution model for each data partition. The analysis was performed using 16 chains and 1,000,000 generations and sampled every 100 generations. The first 5000 trees sampled were discarded. Then a majority rule consensus tree based on 1000 best tree scores was obtained.

Relative rate tests

To test whether the different nrDNA haplotypes found in one species were evolving at constant evolutionary rates or not, we applied Tajima relative rate test (Tajima 1993) implemented in the software MEGA version 3 (Kumar et al. 2004). This test was performed with the following species: *Gi. albida*, *Gi. candida*, *Gi. gigantea*, *Gi. ramisporophora* and *Gi. rosea*. The *Gigaspora gigantea* like

A1-UFLA872 clone was used as an outgroup. This analysis is also important to properly calculate divergence times used for molecular clock assumptions (Nei and Kumar 2000).

Estimation of divergent time between sequences

The molecular clock was used to estimate the time that two closely related sequences were diverging from each other. We used only nearly full length 18S nrDNA sequences for these estimates. Two estimates of substitution rate per year, 6.67 $x10^{-11}$ and 6.42 $x10^{-11}$ were used. They had been previously used to estimate divergent time in AM fungi 18S nrDNA (Simon et al. 1993). Sequences that were not respecting the molecular clock assumption were identified by relative rate test and removed from the analysis.

RESULTS

The intraspecific variation in the nrDNA haplotypes analyzed can be indicated by the within species sequence variation in terms of similarity and transitions versus transversions mutations (Table 1). Despite of the intraspecific polymorphism in the ribotypes in the 18S nrDNA sequences indels could not be observed in this gene (Table 2), and the secondary structure of the 18S nrDNA was preserved (data not show). Differences in sequence length were obtained only in the ITS regions due to indels (Table 2). The base frequencies were slightly different for *Gi. decipiens*, *G. gigantea*-like UFLA and *Gi. margarita*, as they showed higher G and C content than the other species.

Table 1: Average sequence characteristics of the nrDNA fragment analyzed for different *Gigaspora* species. The fragment covers partially the 18S nrDNA (V9 region) and completely the ITS1, 5.8S and ITS2 sequence, and contains 813bp in length after removing the gapped sites. Designations are as follows: % - percentage of similarity; si - transitional pairs; sv - transversional pairs; R = si/sv; # - number of clones analyzed per species; and percentage of base frequencies of T – C – A – G.

Taxon	%	si	sv	R	T (U)	C	A	G	#
Gi. albida	2.9	16	3.5	4.5	30.5	17.5	31.3	20.6	6
Gi. candida	1.7	8	2.5	3.2	30.2	17.5	31.7	20.5	7
Gi. decipiens	3.6	13	7.0	1.9	30.0	17.9	30.9	21.2	2
Gi. gigantean	1.8	10	3.8	2.6	30.4	17.5	31.6	20.4	8
Gi. gigantea UFLA	2.8	10	4.0	2.5	30.1	18.3	30.6	21.0	2
Gi. margarita	4.1	11	11	1.0	30.1	18.0	30.9	21.0	2
Gi. ramisporophora	2.9	14	3.6	3.9	30.7	17.5	31.2	20.6	6
Gi. rosea	2.4	8	3.5	2.3	30.2	17.5	31.7	20.5	4
*Gigaspora/*total	3.6	16	6	2.7	30.4	17.6	31.4	20.6	37

Test for Recombination

The aligned sequences in Table 2 follow the groupings found in the phylogenetic analysis using the 37 clones analyzed. Visual inspection of this alignment already hints at the role of recombination in the evolution of these species. For instance, three haplotypes obtained from three different species, *Gi. candida* clone C13, *Gi. ramisporophora* clone GP14 and *Gi. rosea* clone R16 (indicated in the Table 2 by "R"), seem to be hybrids between haplotypes from clades A and B (designations on Table 2). The signature "CTT" at positions 297-299 in the 18S nrDNA V9 region is characteristic of sequences of Clade A, whereas the ITS2 sequences of these clones are clearly more similar to sequences from clones of Clade B, which possess a "TAC" signature in the 18S nrDNA V9 region (Table 2).

Support for recombination events was also obtained from the graphical method tested (Splits decomposition; Table 3). For single species only *Gi. gigantea* and *Gi. ramisporophora* showed evidence of reticulate evolution. In addition, reticulate evolution was also evident when all haplotypes of two or more species were analyzed together (Table 3).

To gain insight into the processes generating the reticulate evolution, we tested two of the putative hybrid sequences (R sequences, Table 2), *Gi. candida* clone C13 and *Gi. ramisporophora* clone GP14, for recombination using LARD. Putative parental sequences showing the V9 nucleotide signatures "CTT" or "TAC" were chosen (Table 2). This test was not performed for *Gi. rosea* due to a lack of sufficient information to assign both putative parental sequences. Two breakpoint positions were obtained for different sequence combinations (Table 4). From those, the breakpoint position

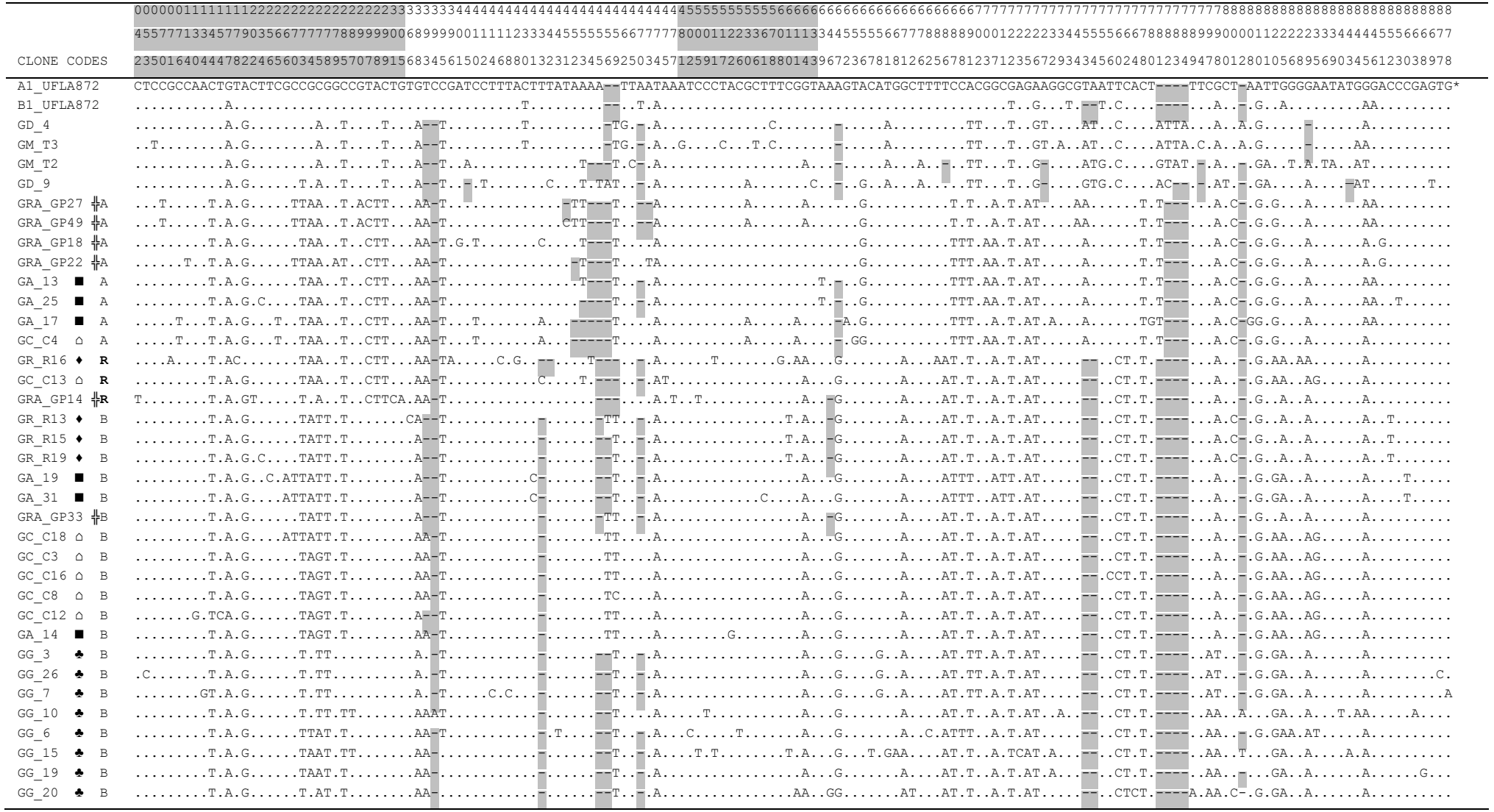
found for *Gi. candida* at the beginning of the ITS1 region (position 410) was chosen for further analysis (analysis performed using position 646 as the breakpoint resulted in similar results, data not shown). The alignment was divided in two parts (part A, positions 1-410 and part B, positions 410 to 878, according to the numbering used in table 2) and phylogenetic analysis performed on each of the sub-alignments. Trees were obtained using ML method, and the phylogenetic consistence of the three putative recombinant sequences compared (Fig. 1). In tree "A" the putative recombinant sequences grouped together with sequences having the "CTT" signature at positions 297-299 and this clade had 81% of bootstrap support. In contrast, in the tree "B", the putative hybrid sequences moved from that clade to other clades to cluster together with sequences having the "TAC" V9 signature. The putative recombinant sequences were the only sequences that moved between the clades in the partitioned sequences (Figure 1). This result provides strong evidence of recombination in the ITS region between two putative parental sequences.

Table 3. Test for reticulate evolution in *Gigaspora* species based on graphical analysis by split decomposition analysis.

Species/Cluster[a]	N	SplitsTree[b]
Gi. albida	6	NO
Gi. candida	7	NO
Gi. ramisporophora	6	YES
Gi. gigantea	8	YES
Gi. rosea	4	NO
Cluster A	8	YES
Cluster B	20	YES
Cluster C	3	NO
all	37	YES

(a) Cluster A: *Gi. albida*, *Gi. candida*, and *Gi. ramisporophora* sequences. Cluster B: *Gi. albida*, *Gi. candida*, *Gi. gigantea*, and *Gi. rosea* sequences. Cluster C: *Gi. candida* clone GC13, *Gi. ramisporophora* clone GP14, and *Gi. rosea* clone R16.
(b) Qualitative (graphical) evidence of reticulate evolution: no statistical test. Hamming distances, no gaps, only parsimonious sites.

Table 2. *Gigaspora* nrDNA sequence alignment showing variable and gapped positions in the V9 region of the 18S nrDNA, 5.8S and ITS1 and ITS2 of 37 clones. Numbered positions marked in gray represent the the 18S nrDNA and 5.8S, the ITS1 and ITS2 are not shaded.



oot note, table 2: The sequences of the clone in the alignment are ordered according to the clades obtained after phylogenetic analysis of the 37 clones. The letters "A", "B" and "R" after the clone names indicate distinct clades and putative recombinant sequences respectively. GA – *Gigaspora albida* BR607A (■), GC- *Gigaspora candida* BEG17(⌂), GD – *Gigaspora decipiens* W3516, GG – *Gigaspora gigantea* VA105C (♣), GM – *Gigaspora margarita* CNPAB01; GRA- *Gigaspora ramisporophora* CNPAB22 (╫); GR – *Gigaspora rosea* BEG9 (♦). Gapped positions in the alignment are marked in gray.

Table 4. Likelihood Analysis of Recombinant DNA (LARD) of putative hybrid nrDNA sequences of *Gigaspora candida* and *Gi. ramisporophora*.

Species	sequences (clones)[a]	Lard[b]	breakpoint
Gi. candida	C8, **C13**, and C4	0.002	410
Gi. ramisporophora	GP33, **GP14**, and GP27	0.004	646

(a) Putative hybrid sequences are marked in bold.
(b) P values are reported for no-recombination hypothesis (H0).

Phylogenetic analyses

Recombinant sequences cause incongruence in a strictly bifurcating tree. Thus, to better calculate the trees, the three recombinant haplotypes were removed from the analysis. The tree topology obtained was supported by parsimony, distance, maximum likelihood, and Bayesian approaches, with only minor changes inside the indicated clades A and B (Fig. 2 and 3). These clades indicated the non-monophyly of sequences from *Gi. albida*, *Gi. candida* and *Gi. ramisporophora* (Clade A and Clade B, fig. 2 and 3). Also *Gi. gigantea*, seems to harbor two ribotype lineages, but this evidence was not as clear as in other species. The results obtained indicate the occurrence of different ribotype lineages in those species, and that ribotype similarity, in some cases, was higher between than within species.

The polyphyletic nature of ribotypes belonging to a specific species was further tested using a topological constraint, and the likelihood of trees with and without the monophyletic constrain were compared. The following order of clones was used to calculate the constraint tree: (*Gi. gigantea*-like A1_UFLA872, B1_UFLA872, (((Gi. decipiens GD_4, GD_9), (*Gi. margarita* GM_T3, GM_T2)), ((*Gi. albida* GA_13, GA_14, GA_15, GA_17, GA_19, GA_25, GA_31), (*Gi. candida* GC_C3, GC_C4, GC_C8, GC_C12, GC_C16, GC_C18), (*Gi. gigantea* GG_3, GG_6, GG_7, GG_10, GG_15, GG_19, GG_20, GG_26), (*Gi. ramisporophora* GRA_GP18, GRA_GP22, GRA_GP27, GRA_GP33, GRA_GP49), (*Gi. rosea* GR_R13, GR_R15, GR_R19)))). The best constrained tree (- ln = 2326.79) for the monophyletic hypothesis was less likely than the best non-constrained polyphyletic tree (- ln = 2174.34). This results support the polyphyletic nature of the ribotypes in the *Gigaspora* species studied.

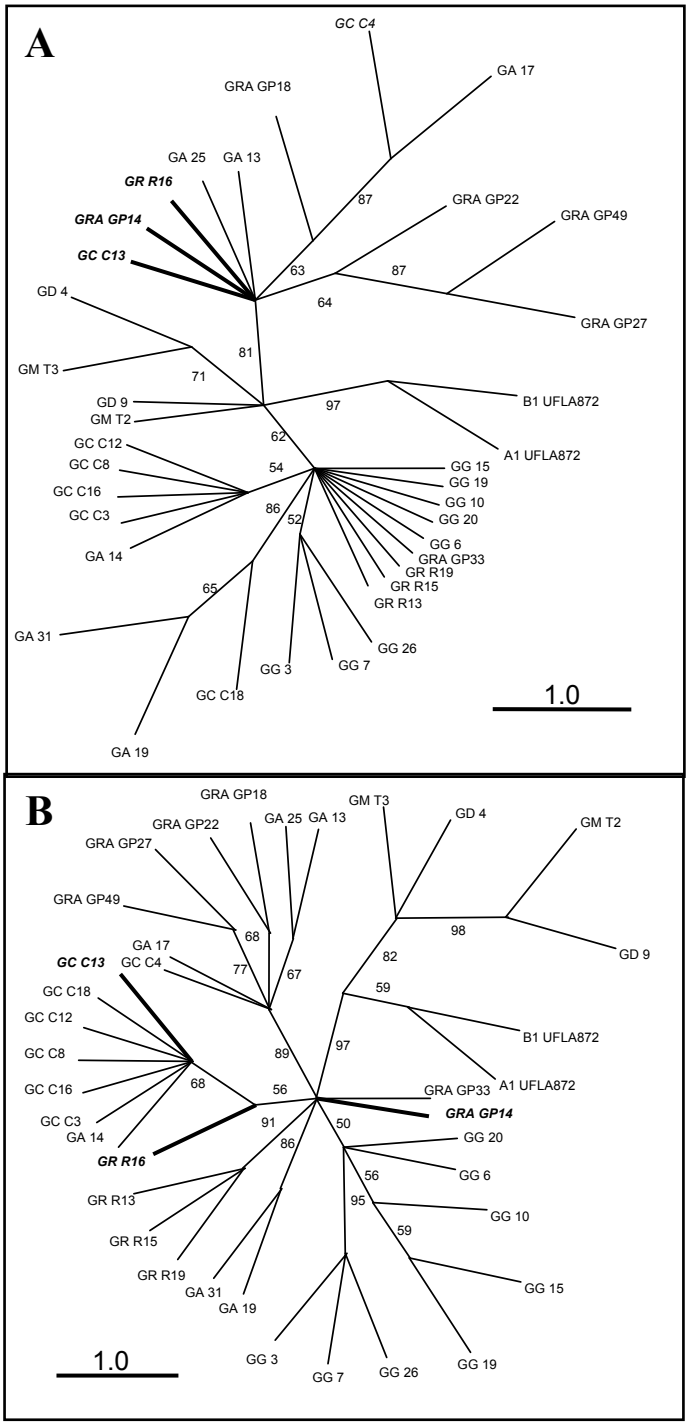


Figure 1. Phylogenetic support for recombination in the ITS region of *Gigaspora* species. Maximum Likelihood unrooted trees, showing putative recombinant sequences (thicker lines). The alignment presented at table 2 was divided in two parts: A. Positions 1 to 410 (V9-18SnrDNA); B. Positions 411 to 878 (ITS1, 5.8S and ITS2). Bootstrap values above 50% are shown (number of repetitions = 1000). For clone codes see table 2.

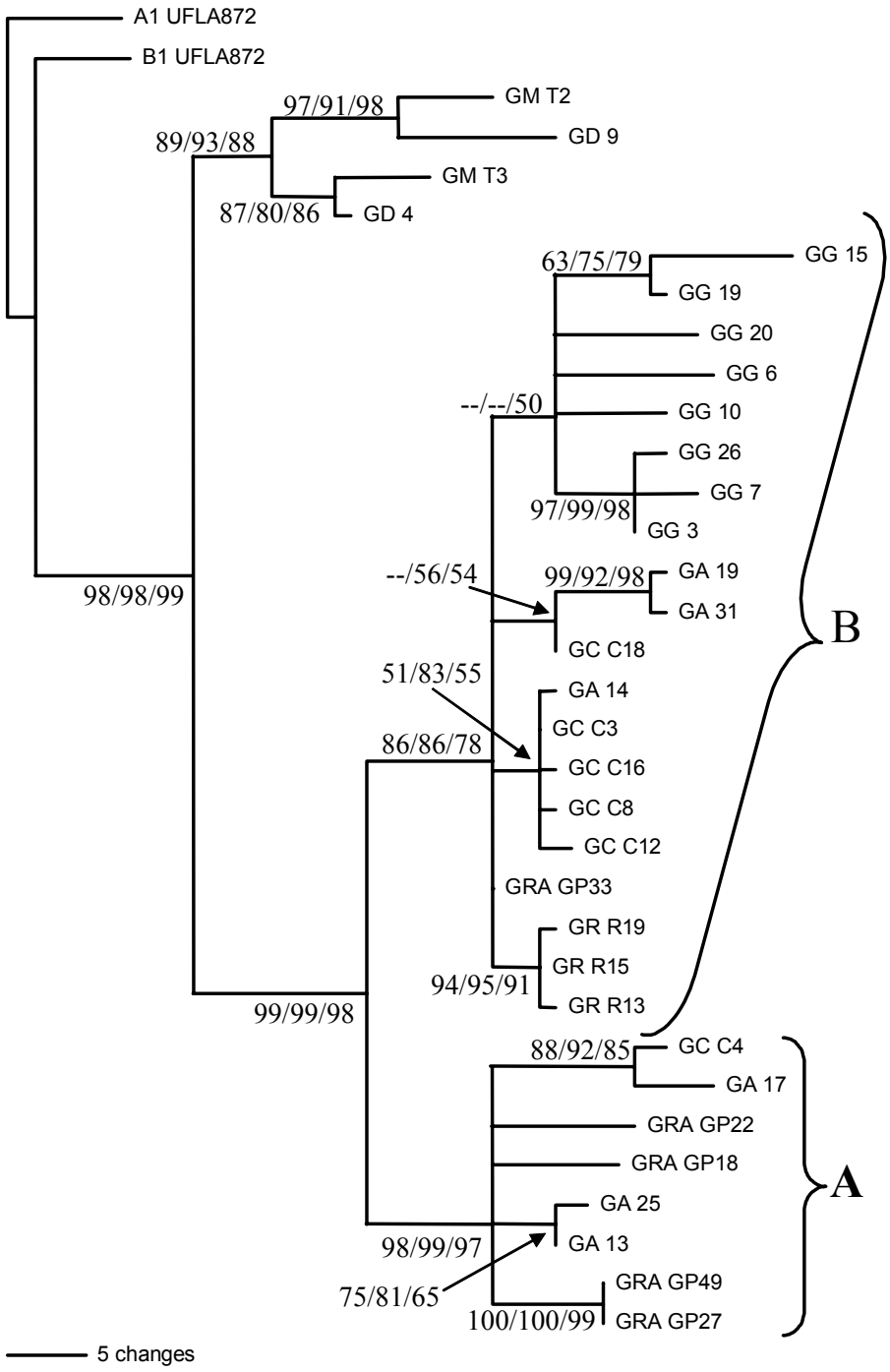


Figure 2. Phylogenetic analysis of inter and intraspecific polymorphism of nrDNA sequences revealed the within species polyphyletic nature nrDNA haplotypes. Maximum likelihood (ML) tree of 34 *Gigaspora* nrDNA sequences spanning the V9 region of the 18S nrDNA through the 5'end of the 28S. Bootstrap values of 1000 replicates for maximum parsimony, minimum evolution and ML are shown respectively. Likelihood settings for best-fit model selected by AIC test: base (0.3082 0.1840 0.2073), NST=6, Rmat (0.254 5.772 1.0058 8.63^{-12} 5.772), Rates=gamma, shape=0.703, Pinvar=0.5282. For clone codes see table 2.

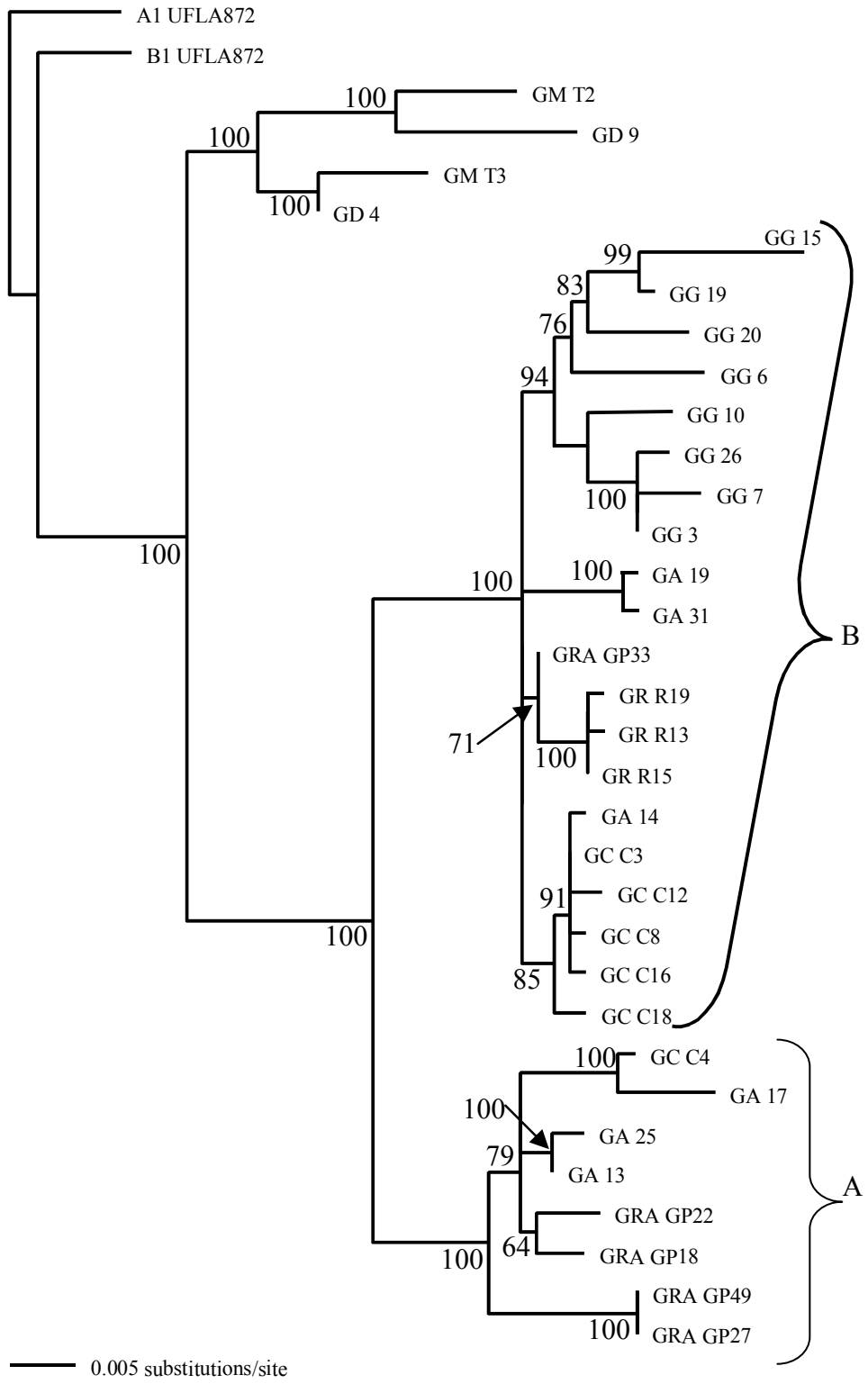


Figure 3. Bayesian phylogenetic analysis of inter and intraspecific polymorphism of nrDNA sequences revealed the within species polyphyletic nature nrDNA haplotypes. Tree constructed using 34 *Gigaspora* nrDNA sequences spanning the V9 region of the 18S nrDNA through the 5'end of the 28S. Likelihood settings for best-fit models selected by AIC for data partition 1 (positions 1-387 478-645) - nst=6 rates=invgamma and 2 (positions 388-477 646-905) - nst=2 rates=propinv. For clone codes see table 2.

The occurrence of closely related ribotypes in different morphospecies as compared to variation within a species (Fig. 2 and 3), is a characteristic found in hybrid species, and is indicative of trans-species polymorphism.

Relative rate test (RRT) and Molecular Clock estimates

Tajima's RRT confirmed that some ribotype lineages within a species are evolving at different rates. *Gi. gigantea* possessed the highest number of haplotypes for which rate constancy of substitution was rejected. Other species with sequence combinations rejected by the RRT was *Gi. albida.*

The molecular clock estimation indicates that the divergent times are high in both the following cases: split between the two haplotypes lineages (38-39 my) and divergence within a lineage (8.4 to 25 my).

DISCUSSION

Phylogenetic evidence for parasexual recombination in Gigaspora

In filamentous fungi the fusion of somatic mycelium via anastomosis allows the exchange of nuclei between genetically compatible fungi via parasexual cycle. This process results in a hybrid species. We obtained strong evidence for hybridization, exemplified by the occurrence of different ribotype lineages and trans-species polymorphism in several morphospecies (Fig. 2 and 3).

The ploidy of *Gigaspora* species is yet to be determined, however, *Gi. margarita* possess a genome 45 times larger than *Glomus intraradices* that is known to be haploid (Hijri and Sanders 2004). Yet, the techniques which we used can not address the ploidy of the fungi. This does not change or weaken the evidence obtained for parasexual recombination, because no matter the ploidy of the fungi, the occurrence of different ribotype lineages in the same species strongly supports the occurrence of parasexual cycle. This evidence will be confirmed when the location of the different ribotype lineages in the genome (chromosomes) of these species were determined. Unfortunately, there is no information about chromosome number or genome map for *Gigaspora* species. Despite of the lack of information, (Trovelot et al. 1999) carried out fluorescent *in situ* hibridzation in spore interphasic nuclei using 25S nrDNA probes. They found 3 to 5 and 3 to 7 different hybridization spots per nucleus in *Gi. rosea* and *Gl. intraradices* respectively. This result suggests the occurrence of more than one rDNA in tandem repeated arrays per nucleus, which might suggest their occurrence in different chromosomes.

The frequency of occurrence of parasexual recombination in *Gigaspora* cannot be determined by our analysis, but the following information suggests that parasexual cycle occur in low frequency in *Gigaspora*. Anastomosis is common in *Glomus* species, but it occur in very low frequency in *Gigasporaceae* (Giovannetti et al. 1999) (de la Providencia et al. 2005). Besides, no anastomoses has been observed to occur between different isolates of a morpho species (Giovanetti et al. 2003). In addition, molecular clock estimation indicates that the divergent times are high in both the following cases: split between the two haplotypes lineages (38-39 my) and divergence within a lineage (8.4 to 25 my). Thus, although parasexual cycle must have profound consequences for *Gigaspora* evolution, it probably occurs at low frequency.

A parasexual process where the dikaryon undergoes nuclear fusion followed by chromosome losses could lead to the formation of species complex, as observed in *Gi. albida*, *Gi. candida*, *Gi. ramisporophora.* In addition, *Gi. gigantea* and *Gi. rosea* certainly are linked with this complex, although all sequences clustered together in the clade B (Fig. 2 and 3). Interestingly, *Gi. gigantea* seems to have an additional ribotype lineage, not yet found in any other species. Another species complex is formed by *Gi. decipiens* and *Gi. margarita*. The coenocytic nature of the AM fungi mycelium might facilitate the process of radiation after parasexual recombination, because nuclei located in different regions of a continuous somatic mycelium could undergo different chromosome losses, and suffer different selective pressure.

The other possible mechanism that can generate allelic sequence divergence in asexual lineages is through the Meselson effect, caused by freely random accumulation of neutral or nearly neutral mutations in asexual organism (Welch & Meselson 2000); (Birky 2004). This mechanism has been conclusively demonstrated for the ancient asexual class Bdelloidea (Rotifera) (Welch et al. 2004). This process can explain the divergence between the haplotypes belonging to the same lineage occurring in different morphospecies. Indeed the high divergence and different mutation rates in haplotypes of the same lineage suggest that this mechanism is operating in AM fungi. However, this cannot explain the recombinant sequences and the polyphyletic nature of haplotypes within species. Through purely random accumulation of mutations the generation of very divergent nrDNA copies (hard polytomy) might be expected, as is evident for the Bdelloidea (Rotifera) (Birky 2004).

Recombination

In addition to the evidence of parasexual cycle, we also obtained evidence for recombination as exemplified by the recombinant sequences found (Table 2), confirmed by LARD and the break point phylogenetic analysis (Table 4 and Fig. 2). The mechanism responsible for such recombination in these putative asexuals is still unknown. The mechanisms of recombination and parasexual cycle must

be able to clean the AM fungi genome from deleterious mutations and generate variability necessary for adaptation. These recombination processes could be an alternative to overcome the need of a true sexual cycle.

Advantages of nrDNA intraspecific polymorphism for phylogenetic and evolutionary studies of AM fungi

AM fungi phylogeny as predicted by using single nrDNA sequences to represent a species has been very informative for inferring evolutionary relationships from the genus level to above (Schüssler et al 2001). However, at the species level, the use of single nrDNA sequences for identification and phylogenetic inference of AM fungi is seriously hampered by the high levels of sequence similarity. Here we demonstrated that the characterization of the nrDNA intraspecific variation in closely related species is not only useful for AM fungi species identification (de Souza et al. 2004), it also provide glimpses into the evolutionary history of organisms possessing such variation.

If parasexual recombination is shaping the AM fungi evolution as suggested by our data, the phylogeny based on intraspecies polymorphism of the rDNA genes will provide a strong means to infer relationshipness of closely related species in this group. In contrast, the analysis of single copy genes might not provide evidence of parasexual recombination because only one copy may remain in the genome if after parasexual fusion occurs chromosome lost.

The evidence presented in this paper, demonstrated that through the analysis of intraspecific nrDNA polymorphism, occurring in closely related species, additional insight into the evolution of these organisms could be obtained, beyond simply predicting phylogenetic relationships. nrDNA intraspecific polymorphism is a characteristic present in different organisms (see references in de Souza et al. 2004) and the analysis of closely related species as demonstrated here, might be useful to understand the evolutionary path of other organisms too.

We found phylogenetic evidences to support the occurrence of network evolution via parasexual recombination in AM fungi of the genus *Gigaspora*.

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Chapter 8

Summary and General Discussion

de Souza FA

SUMMARY AND GENERAL DISCUSSION

This thesis has focused on biological, ecological and evolutionary aspects of Arbuscular Mycorrhizal Fungi (AMF), and in particular the family *Gigasporaceae.* AMF form the most common underground symbiosis with vascular plants, and this association is considered important for the functioning of plant-soil ecosystems. Moreover, their economic significance to sustainable agriculture, land reclamation and efficient use of non-renewable resources such as phosphate has been widely accepted. However, major bottlenecks in AM research are the lack of knowledge on ecology, and in particular on life history strategies (LHS) among the different AM fungal families, and the lack of knowledge on evolutionary process, in particular the maintenance of genetic variability in this putative ancient asexual group of fungi.

Based on 18S nuclear ribosomal sequences (nrDNA), AMF form a monophyletic group, phylum *Glomeromycota*, with approximately 200 morphologically described species. *Gigasporaceae* (*Gigaspora* and *Scutellospora*) represents 20% of the total number of described species and is one of the most common worldwide. An example of the economical value of *Gigasporaceae* can be demonstrated by the positive response of coffee plants in Brazil and India to the inoculation with isolates of *Gigaspora margarita.* However, little is known about the ecology of these fungi in agricultural or natural conditions. Such knowledge would improve the chances of successful and durable inoculation responses.

In recent years, the use of DNA-based techniques has facilitated microbial ecology studies opening possibilities of studying organisms that cannot (yet) be cultured, and to infer phylogenetic relationships between organisms and taxa. However, the apparent complexity of AMF genomic organization has complicated the application of such techniques to their study. The only markers used so far to characterize and identify AMF in natural assemblages are the nuclear ribosomal DNA (nrDNA). Although, nrDNA sequences are typically identical within an organism, AMF and some other organisms contain a considerable intragenomic polymorphism in the rDNA copies, but the mechanisms generating such polymorphic copies are unclear.

This thesis had two major aims. The first was to study the life history strategies of members of the *Gigasporaceae*. The second was to assess the extent, nature and implications of inter and intra-genomic polymorphism of nuclear ribosomal (nrDNA) sequences, and to test the use of this marker to differentiate species and infer phylogeny and evolutionary processes at a fine scale (species and sub-species level).

To achieve the first aim, the life cycle of the *Gigasporaceae* was explored in search of LHS patterns, which are considered to obey a set of rules related to organism size, rates of growth and

development, and reproduction. To facilitate the study of traits involved in fitness and to compare different AMF, a set of experiments (Chapters 2 and 3) was carried out using a monoxenic culture (MC) system (microcosm), which consisted of Ri-T DNA transformed carrot root explants inoculated with contaminant free AMF spores in a defined medium. This microcosm system facilitates laboratory investigation of this biotrophic obligatory group of fungi. *Scutellopora reticulata* CNPAB11 was used as model species to elucidate aspects of *Gigasporaceae* life cycle (**Chapter 2** and **3**). Since no *Scutellospora* species had been cultivated in MC before, the successful cultivation of *S. reticulata* would allow comparison with other species previously studied in this system (*Gigaspora* and *Glomus*, see **Chapter 4**). The monoxenic culture (MC) proved to be an excellent microcosm system to study biological aspects of AMF such as mycelium architecture, anastomosis formation, spore development, and facilitated quantification of mycelial structures. *S. reticulata* seems to be a good model species to study the life cycle of *Gigasporaceae*, because its behavior was similar to *Gigaspora* species studied previously (see discussion in **Chapter 2** and **4**) and its mycelium and spore characteristics (size and color) allowed observation of colony development (**Chapter 2**, **3**, and **5**). In addition, anastomosis was recorded in *S. reticulata* mycelium, a phenomenon not previously reported in *Gigasporaceae*. However the type of anastomosis reported, connecting secondary or lower order hyphae, and the low frequency observed does not permit the formation of extensive anastomosis networks, such as the ones reported for *Glomus*. Another process observed was a hyphal healing mechanism that was operating in the major hyphae of the fungus. Hyphal bridges were formed to reconnect broken hyphae or circumvent obstructed areas within a hypha. Despite the healing mechanism, however, damaged mycelium negatively affected spore formation. In addition, numerous auxiliary cells (AC) were produced during culture development. The biological function of AC in the life cycle of *Gigasporaceae* is not known. The results obtained suggested a possible role of the AC in carbon storage, as an energy reserve for spore and/or mycelial production and repair.

1. What are the roles of Auxiliary Cells in the life cycle of *Gigasporaceae*?

Time-course sequential development and quantification of the extraradical mycelium, AC and spores of *S. reticulata*, was used to examine the role of AC in spore formation (**Chapter 3**). It was found that the extraradical mycelium development followed classical lag-exponential-plateau phases, with an additional late decline phase of 7% in the number of auxiliary cells. Spore production started 12 weeks after root colonization, when a critical extraradical mycelium (ERM) biomass (1360 ± 625 cm of ERM

length and 501± 96 AC) was reached, and roots were no longer growing in the experiment. Sporulation continued until 8 months. Moreover, 30% of the total spore production occurred during the phase of late decline in AC numbers. At that phase, almost eight month after starting the culture, the medium resources should have been incorporated into root and fungal biomasses or consumed (respiration), and resources stored by AC must have been the only ones available for spore formation at that phase. These results supported the hypothesis that reallocation of resources stored in the AC contribute to spore formation (**Chapter 3**).

2. Are members of the *Gigasporaceae* K-strategists in relation to other common AMF species?

An important characteristic revealed during the MC of *S. reticulata* and *Gigaspora rosea* is that these species increase the colonization and extraradical mycelium biomass after root had ceased growth (see **Chapter 3** and **4**), for *S. reticulata* the roots reached a plateau after 7 wk and did not increase any further until the end of the experiment (week 36). Just after the root reached the plateau phase the mycelium growth started its exponential growing phase, showing a huge increase in length, AC number and spores (**Chapter 3**). At that time, much of the resources in the medium were already consumed by the root culture, indicating a capacity of *Gigasporaceae* to live and reproduce with a small portion of the resources available. This scenario is similar to the conditions expected for competitive species or referring the K-strategist concept (**Chapter 4**).

Gigasporaceae spores are thought to be the main, if not the solely, type of propagule (see **Chapter 4**). In *S. reticulata*, spores were mainly formed in the extraradical mycelium (**Chapter 2** and **3**). Based on the results, it was hypothesized that in disturbed soils, such as tilled agricultural fields, *Gigasporaceae* species would be negatively selected. Other factors that might negatively affect *Gigasporaceae* fitness in tillage soils include their long vegetative period before sporulation and the lengthy process of spore expansion and development.

Another important observation was the maintenance of arbuscules by *S. reticulata* in apparently non-active growing roots harvested at the plateau phase, a characteristic never reported for *Glomus* species, but apparently common for *Gigasporaceae* (see **Chapter 4**). The comparison of life cycles of different *Gigasporaceae* and some *Glomeraceae* species cultivated monoxenically (**Chapter 4**) provided strong evidence for disparate LHS strategies between these families. The available data for comparative analyses of *Gigasporaceae* and *Glomeraceae* LHS were obtained from experiments focused on growth kinetics and development characteristics such as the timing of the first daughter

spore produced, the rate of sporulation, and the duration of the reproductive phase. The data obtained for *Gigasporaceae* and *Glomeraceae* species cultivated in MC, strongly suggests that they concentrate their reproductive efforts at different periods of time. These results are in agreement with those obtained with reference to Malthusian fitness (MF). In this case, MF compared the instantaneous change of spore production over time in relation to the starting inoculum, and it gives an idea of the reproductive effort of the organism. Those results suggested that coexistence between different AMF species in the same piece of root is facilitated by disparate LHS of different AMF species (**Chapter 4**).

The *Gigasporaceae* isolates studied in MC exhibited several characteristic traits (investment in somatic growth rather than in reproduction, development of large spore size and few offspring) of organisms adapted to live in stable ecosystems, where inter- and intra-species competition for resources is high, conditions favoring somatic growth is favored above reproduction. *Glomus* species that differentiate single spores in the soil seems to follow the opposite trend, i.e. they seemed to be adapted for growth in disturbed ecosystems that are rich in available resources, which favor reproduction over somatic growth (**Chapter 4**). The life history traits reported for *Gigasporaceae* support with the hypothesis (**Chapter 2**) that those species are negatively selected in agricultural fields cultivated that are submitted to frequent plowing that disrupts the mycorrhizal mycelium. In addition, a hypothesis to explain the coexistence of different AMF species in the same niche (piece of root) was formulated based on maintenance of disparate LHS between *Gigasporaceae* and *Glomus* species studied (**Chapter 4**).

To achieve the second aim of this thesis a PCR-Denaturing Gradient Gel Electrophoresis (DGGE) approach was combined with phylogenetic analysis of individual rDNA copies to decipher the nature of rDNA intragenomic polymorphism found in AMF, and to test the use of this polymorphism to differentiate closely related species. However, species determination in AMF are traditionally based on morphological characteristics of spore and its wall components, and developmental patterns during spore ontogeny are still considered to be the best way to infer phylogeny based on morphological analysis in AMF. Thus, this approach was first compared with rDNA-based phylogeny to determine the strengths and weaknesses of each approach. To this end, *Scutellospora* species were chosen, because this genus is well characterized using morphological analysis. Thus, *S. reticulata* was further characterized using a combination of morphological and molecular methods (**Chapter 5**).

3. Can nrDNA intragenomic polymorphism be used to differentiate species of AMF?

The molecular phylogeny of AMF based on 18S rDNA has been proven useful in the discrimination from genus and sub-genus level and above but it fails to discriminate between species, because nrDNA is too conserved, as confirmed by this thesis (**Chapter 5**). In *Scutellospora,* the 18S nrDNA sequence produced three shallow clades supported by strong bootstrap values. These clades have poor resolution to discriminate between species. On the other hand, they are indicative of sub-groups within *Scutellospora.* The three clades obtained by molecular phylogeny clustered species that are closely related based on developmental patterns of the spores, but within each clade more than one developmental group was present. If the molecular phylogeny is correct then the developmental analysis might cluster species with different ancestry based on convergence of morphological characters. This might be possible if constraints related to germination events take place during species radiation. In such a scenario, nrDNA data might more accurately discriminate at the sub-genus level than developmental patterns because nrDNA are probably under low selective pressure during speciation in asexual organisms. In addition, PCR-DGGE, targeting the 18S nrDNA V9 region, could be used to differentiate *S. reticulata* from 16 other *Scutellospora* species based upon nrDNA sequence variation and intragenomic polymorphism. However, *S. gregaria* and *S. coralloidea*, which are closely related species based on morphological analysis, could not be differentiated using V9 18S rDNA region. Nevertheless, the technique has great potential to discriminate AMF, although more variable regions such as the ITS might be necessary to discriminate very closely related species.

To get further insight into intragenonic nrDNA polymorphism, inter- and intragenomic variation of 18S nrRNA genes of the genus *Gigaspora* was explored to assess the use of this marker for discrimination of *Gigaspora* isolates and of *Gigasporaceae* populations from environmental samples. I concentrate the analysis in the genus *Gigaspora* (**Chapter 6**) for the following reasons: 1.) The species of this genus are closely related based upon both morphological and molecular data, 2.) Species radiations within this genus are rather recent in relation to the 600 million year old AMF ancestor, 3.) A high degree of rDNA sequence polymorphism has been detected in *Gigaspora* species, 4.) There are several strains available covering all described species and coming from different geographic origins. Forty eight *Gigaspora* isolates, including all known described species and geographically distinct isolates of several species, were screened by PCR-DGGE analysis of DNA extracted from single spores (**Chapter 6**). PCR-DGGE analysis of the 18S rDNA V9 region revealed

multiple bands for most of the species analyzed indicating intragenonic polymorphism of nrDNA copies in that region.

4. Do different geographic isolates of an Arbuscular Mycorrhizal Fungi morphospecies differ in their nrDNA copies? If yes, how can species boundaries be defined?

PCR-DGGE banding patterns could be used for reliable identification of all recognized species within *Gigaspora*, to provide insight into some putative misidentifications, and to differentiate some geographic isolates of *G. albida*, *G. gigantea* and *G. margarita*, but not for *G. rosea*. Two major clusters were apparent upon comparison of PCR-DGGE ribotype patterns, one containing *G. albida*, *G. candida*, *G. ramisporophora* and *G. rosea* and the other containing *G. decipiens* and *G. margarita*. Dissection of DGGE patterns by cloning, DGGE screening and sequencing, confirmed these groupings and revealed that some ribotypes were shared across species boundaries. The PCR-DGGE patterns obtained from different spores of the same isolate were usually indistinguishable. Therefore, a single spore appeared to typify the genetic variation present in the entire spore population of a given isolate. The usefulness of this approach to identification of *Gigasporaceae* in field samples was also demonstrated using a *Gigasporaceae*-specific nested PCR approach. An analysis of Brazilian agriculture soils revealed a dominance of *G. margarita* within this family.

rDNA polymorphism seems to be a good marker to differentiate morphospecies, however, species boundaries must be determined by the vegetative compatibility test (VCT), because a morphospecies might represent disparate species as observed for the *Gigaspora gigantea* strains studied. In this case, the VCT based on the wound-healing mechanism has great potential to determine species boundaries in *Gigasporaceae* (**Chapter 4**).

5. What are the phylogenetic and evolutionary implications of nrDNA variation in Arbuscular Mycorrhizal Fungi?

The partial sequence analysis of V9 18S rDNA fragment used in the PCR-DGGE analysis indicated a species complex containing *Gi. albida*, *Gi. candida*, *Gi. ramisporophora* and *Gi. rosea* (**Chapter 6**). To gain further insight into the intragenomic polymorphism found in *Gigaspora* species and its phylogenetic and evolutionary implications, a detailed phylogenetic and recombination analysis of nrDNA variability in *Gigaspora* was performed, using eight selected species out of the 48 accessions tested previously (**Chapter 7**). One isolate of each species was used plus one putative new *Gigaspora* species. PCR-amplified DNA fragments of approximately 2300bp, covering 18S, 5.8S and the ITS1 and ITS2 regions, were obtained from DNA extracted from single spores and cloned in *E. coli*. Clones containing different haplotypes were selected using PCR-DGGE targeting the 18S rDNA V9 region and sequenced. Phylogenetic analysis revealed the existence of reticulated evolution, occurrence of multiple haplotypes lineages within a single species, and polyphyletic haplotype lineages within the species *G. albida*, *G. candida*, *G. ramisporophora*. In addition, evidence for recombination was found in one haplotype lineage in *Gi. candida*, *Gi. rosea* and *Gi. ramisporophora*. The polyphyletic nature of nrDNA copies in some *Gigaspora* species provides evidence of trans-species polymorphism, which is characterized by higher similarity between nrDNA copies across species boundaries than within the same genome. This evidence strongly suggests the occurrence of parasexual recombination in AMF of the genus *Gigaspora*. These findings indicate that recombination played a significant role in shaping genetic diversity in these putatively asexual fungi.

PCR-DGGE targeting the 18S rDNA V9 region revealed the possibility of discrimination of closely related species on the basis of intragemonic polymorphism of the nrDNA copies. This is a great advantage of PCR-DGGE analysis of AMF over other molecular techniques applied to date, because PCR-DGGE takes advantage of the intraspecific polymorphism of the nrDNA copies to discriminate close related species. Furthermore, although, anastomosis between different species or even between different strains of the same morpho-species had never been observed in AMF, the interpretation of nrDNA intragenomic polymorphism of closely related *Gigaspora* species provided strong evidence for network evolution via parasexual recombination in AMF.

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Abstract

Research described in this thesis focused on biological, ecological and evolutionary aspects of Arbuscular Mycorrhizal Fungi (AMF), and in particular of the family *Gigasporaceae* (*Gigaspora* and *Scutellospora*, genera). This study had two major objectives. The first objective was to obtain better knowledge about the life history strategies (LHS) of *Gigasporaceae,* and the second objective was to study the intragenomic polymorphism of the ribosomal rDNA copies in order to understand its origin and to test the use of such polymorphism to discriminate between closely related species. The LHS patterns observed for the *Gigasporaceae* species studied suggests that they behave like K-strategists, i.e. organisms adapted to live at stable and predictable environments, as compared to *Glomeraceae* species that form single spores in the soil. Based on LHS patterns of *Gigasporaceae* an hypotheses that those species will be negatively selected in disturbed ecosystems have been formulated. Although intraspecific rDNA polymorphism in *Gigasporaceae* has been previously reported, this study, for the first time, systematically describes this heterogeneity and analyses this phenomenon with respect to *Gigasporaceae* phylogeny and evolution. It has been demonstrated that the intragenomic polymorphism of the rDNA copies can be used to differentiate species and in the case of *Gigaspora* even isolates. The phylogenetic analysis of inter and intragenomic rDNA polymorphism of closely related *Gigaspora* species strongly suggested that reticulated evolution, characterized by occasional hybridization or horizontal gene transfer between two species, played a significant role in evolution of this asexual group of fungi, via parasexual recombination. In addition, a molecular method was developed to assess *Gigasporaceae* diversity in environmental samples.

Samenvatting

Het onderzoek, dat in dit proefschrift is beschreven, was gericht op de biologische, ecologische en evolutionaire aspecten van arbusculaire mycorriza schimmels, AMF, en in het bijzonder schimmels die behoren tot de familie van de *Gigasporaceae* (de soorten *Gigaspora* and *Scutellospora*). De voornaamste twee doeleinden van deze studie waren: 1) om een meer kennis te verwerven van de 'Life History Strategies', LHS, van *Gigasporaceae* en 2) om een beter begrip te krijgen van de oorsprong van het intragenomische polymorfisme van het ribosomale DNA, om daarmee een methode te ontwikkelen om nauw verwante soorten te kunnen onderscheiden.

Het LHS patroon van de onderzochte leden van de familie van de *Gigasporaceae* duidt erop dat zij zich gedragen als typische K-strategen, oftewel organismen die zijn aangepast aan stabiele en voorspelbare milieu's, in tegenstelling tot de andere belangrijke mycorrhiza familie van de Glomeraceae soorten. Gebaseerd op LHS patronen van *Gigasporaceae* is de hypothese geformuleerd, dat die soorten negatief geselecteerd zullen worden in verstoorde ecosystemen. Ofschoon intragenomisch polymorfisme al eerder voor *Gigasporaceae* is gerapporteerd, is in dit onderzoek voor de eerste keer op systematische wijze deze heterogeniteit beschreven en is dit verschijnsel geanalyseerd in relatie tot de fylogenie en evolutie van *Gigasporaceae*. Aangetoond is dat het intrgenomische polymorfisme van rDNA gebruikt kan worden om verschillende soorten en zelfs isolaten van *Gigasporaceae* te onderscheiden.De fylogenetische analyse van het inter en intragenomisch polymorfisme van nauw verwante *Gigaspora* soorten wijst er sterk op dat 'reticulated'evolutie, gekenmerkt door hybridisatie of horisontale gen overdracht tussen twee soorten, een doorslaggevende rol heeft gespeeld in de evolutie van deze groep van aseksuele schimmels via paraseksuele recombinatie. Voorts werd een methode ontwikkeld om de diversiteit van *Gigasporaceae* in de bodem te kunnen bepalen.

Resumo

A pesquisa descrita nesta tese focou aspectos biológicos, ecológicos e evolutivos de Fungos Micorrízicos Arbusculares (FMA) e em particular a família *Gigasporaceae* (gêneros *Gigaspora* e *Scutellospora*). Este estudo teve dois objetivos principais. O primeiro objetivo foi avançar o conhecimento sobre histórico de estratégia de vida (HEV) em *Gigasporaceae*, e o segundo objetivo foi estudar o polimorfismo intra-genômico do ribossomo rDNA visando elucidar sua origem e testar o uso deste polimorfismo para discriminar entre espécies próximas. Os padrões de HEV observados para as espécies de *Gigasporaceae* estudadas sugerem que elas se comportam como estrategistas em K, i.e. organismos adaptados a viverem em ambientes estáveis e previsíveis, quando comparado com espécies da família *Glomeraceae* que formam esporos isolados no solo. Baseado no padrão de HVE de Gigasporaceae foi formulada uma hipótese de que estas espécies serão selecionadas negativamente em ecossistemas perturbados. Embora, a ocorrência de polimorfismo intra-específico no rDNA tenha sido demonstrado anteriormente, pela primeira vez, está heterogeneidade foi sistematicamente descrita e este fenômeno analisado em relação à filogenia e evolução da *Gigasporaceae.* Foi demonstrado que o polimorfismo intra-específico das copias do rDNA pode ser utilizado para diferenciar espécies e no caso de *Gigaspora* até isolados geográficos de uma mesma espécie. A análise filogenética do polimorfismo do rDNA intra e entre genomas de espécies de *Gigaspora* sugere claramente que a ocorrência de evolução reticular, caracterizada por hibridizações ocasionais e transferência horizontal de genes entre espécies, tiveram um papel significativo na evolução deste grupo de fungos assexuados, via recombinação parasexual. Além disso, um método molecular para acessar a diversidade de *Gigasporaceae* em amostras ambientais foi desenvolvido.

Curriculum Vitae

Francisco Adriano de Souza was born on April 1st (no fooling), 1964 in Santos (São Paulo State), Brazil. Santos is known by its harbor and the soccer team of the legendary Pelé, "Santos Football Club", which is still considered one of Brazil's best soccer teams. Francisco spent his childhood on the island of Santo Amaro, in Gurarujá city located near Santos, where he divided his time between school and the sea. In 1982, he got a technician degree in Chemistry at the Lyceum CARMO in Santos. Chemistry was his passion, but the experience of living and working close to one of the most polluted cities in the world (Cubatão) deflated the idea of continuing his studies in Chemistry. Then he spent some years just surfing waves, but a knee problem jeopardized his life as a sportsman. Then in August 1985, he started studies in Agronomy at the Rural Federal University of Rio de Janeiro (UFRRJ), in Seropédica, Rio de Janeiro State. There he was awarded a CNPq fellowship to work with soil fertility under the supervision of Drs. Luiz Freire and Nelson Moura-Brasil in the department of soil science of the UFRRJ. There he met Dr. Ricardo Berbara, who led him into the mycorrhizal world. He got his degree in Agronomy in January 1991. That same year he started his MSc in Agronomy – Soil Science where he defended the thesis entitled: "Effects of pre-crops on the inoculum potential of arbuscular mycorrhizal fungi and production of cassava (Manihot esculeta)" supervised by Dr. José Guilherme Marinho Guerra. In January 1994, he started his first permanent position obtained by public examination as researcher at PESAGRO, the Agriculture Research Organization of Rio de Janeiro State. In March of the same year, he changed jobs to work in the rubber tree program of the Institute Agronômico de Campinas (IAC), Campinas, São Paulo State, one of the most renowned agriculture research institutes in Brazil. However, his fascination for the mycorrhizal symbiosis made him change jobs once more, in December of the same year, for a position at Embrapa Agrobiologia, where he works to this day. In 1999 he submitted a grant project to the Brazilian Council for Scientific and Technological Development (CNPq) to allow him a four-year grant to study abroad. This grant proposal was approved, and he was selected to work with the Prof. Dr. Sally Smith (Adelaide University, Australia), Prof. Dr. Peter Young (York University, UK) or Prof. Dr. J.D. van Elsas (PRI-The Netherlands). In December 1999 he started his PhD program in the Netherlands at Plant Research International (PRI). The following year it became apparent that the focus of his research was a better match with research activities within the Netherlands Institute of Ecology (NIOO-KNAW). Thus, in November 2000, he made a change of research institute, to join the NIOO department of Plant Microorganism Interactions (PMI) under the supervision of promoters Prof. J.A. (Hans) van Veen and Prof. George A. Kowalchuk in collaboration with Dr. Eric Smit of the RIVM. The resulting research led to the production of this thesis.