Restriction Analysis of PCR-Amplified Internal Transcribed Spacers of Ribosomal DNA as a Tool for Species Identification in Different Genera of the Order Glomales

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A technique combining PCR and restriction fragment length polymorphism analysis was used to generate specific DNA fragment patterns from spore extracts of arbuscular mycorrhizal fungi. With the universal primers ITS1 and ITS4, DNA fragments were amplified from species of *Scutellospora* and *Gigaspora* that were approximately 500 bp long. The apparent lengths of the corresponding fragments from *Glomus* spp. varied between 580 and 600 bp. Within the genus *Glomus*, the restriction enzymes *Mbo*I, *Hinf*I, and *Taq*I were useful for distinguishing species. Depending on the restriction enzyme used, groups of species with common fragment patterns could be found. Five tropical and subtropical isolates identified as *Glomus manihotis* and *G. clarum* could not be distinguished by their restriction patterns, corresponding to the morphological similarity of the spores. The variation of internal transcribed spacer sequences among the *Gigaspora* species under study was low. Fragment patterns of *Scutellospora* spp. showed their phylogenetic relationship with *Gigaspora* and revealed only a slightly higher degree of variation.

Arbuscular mycorrhiza is the most widespread type of mycorrhiza, and its relevance for the mineral nutrition of a vast variety of plants is well known. Approximately 130 species of the presumed mycosymbionts have been described (31) on the basis of morphological features of the spores that were standardized by Walker (29). These characteristics may be difficult to discern and are subject to alterations during spore ontogeny or by parasitism. The hyphal connections of the spores, normally needed for genus determination, can be lost. Overviews of difficulties and perspectives of morphological analysis were presented by Walker (30) and Morton (16).

The Glomales form a group of fungi that traditionally is placed in the Zygomycetes. Their diversification coincides with the evolution of land plants (23). Two suborders have been erected, Glomineae and Gigasporineae (17).

Efforts have been made to identify taxa by means of antibodies (10), isozyme patterns (25), or lipid profiles (1). A specific DNA probe for *Scutellospora castanea* was developed (35). DNA-based methods are not affected by character changes during ontogenesis or organ differentiation. Genes for rRNA are among the most promising target DNA sequences because in many organisms they are found in multiple copies per genome and parts of them are highly conserved. This is a feature that allows the generation of PCR primers with broad applicability. Among other highly variable parts of these genes (3, 24, 26), the internal transcribed spacers (ITS) have been used for identification or phylogenetic analyses of a wide range of fungi (e.g., see references 5, 8, and 11) and fungus-like organisms (14). In the Glomales, fragment patterns generated

by restriction analysis of the amplified ITS are reproducible among different spores of the same species (18, 21).

However, few restriction analyses of ITS from isolates of defined species of arbuscular mycorrhizal fungi (AMF) have been performed, so that their diversity on the species, genus, or family level is still unclear. Moreover, after reports that ITS sequences show a relatively high level of heterogeneity even within single spores (12, 21) it has been uncertain whether ITS can be used for standard identification of the Glomales.

Therefore, in the present study a range of isolates from the genera *Glomus*, *Entrophospora*, *Scutellospora*, and *Gigaspora* were analyzed by determining the restriction patterns of their ITS. These patterns are discussed in relation to the morphological diversity of the spores.

MATERIALS AND METHODS

The fungi studied were produced in pot cultures with various host plants (Table 1). Two cultures of the same isolate of *Glomus caledonium* (BEG20) were used: one was directly obtained from the Banque Européenne de Glomales (BEG), and the other, a gift from C. Azcón-Aguilar, Granada, Spain, has been cultivated in Marburg, Germany, for several years.

Spores were extracted from the pot culture substrate by wet sieving and decanting (9). Under a dissecting microscope, spores were sorted twice with a micropipette (Varipette, 0.5 to 10 μ l; Eppendorf, Hamburg, Germany) and transferred each time into a petri dish with sterile tap water. They were placed in an Eppendorf tube with a minimum amount of water. In the case of small spores that were difficult to select individually, 30% (wt/vol) sucrose was layered into the petri dish so that the spores could be collected from the water-sucrose interface. Voucher specimens were mounted on microscope slides in polyvinyl alcohol lacto-glycerol and retained in the personal herbarium of C. Walker.

Spore extracts were produced for PCR by crushing spores with a pipette tip in 2 μ l of 0.25 M NaOH, incubating them in a boiling water bath for 1 min, adding 1 μ l of 0.5 M Tris HCl (pH 8.0) and 2 μ l of 0.25 M HCl, boiling them again for 2 min (18). Extracts were directly used for PCRs, which were performed by using each nucleotide at 50 μ M, primers ITS1 and ITS4 at 0.2 μ M each (32), 0.1 U of *Taq* polymerase (Amersham/USB, Cleveland, Ohio) per μ l, and the reaction buffer supplied by the manufacturer. The concentration of MgCl₂ was adjusted to 1.5 mM. Alternatively, 0.05 U of *Taq* polymerase from Appligene (Illkirch, France) per μ l was used. A TC1 thermal cycler (Perkin Elmer, Norwalk, Conn.)

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TABLE 1. Fungal isolates analyzed in this study

AMF	Code(s)	Voucher no.	Source(s) ^a	Country of origin
<i>Entrophospora colombiana</i>	#47, C-18-3	W2859	CIAT, E. Sieverding	Colombia
<i>Gigaspora albida</i>	BR205-1	W2382	CIAT/INVAM	Brazil
<i>Gigaspora candida</i>	BEG17	W2418	BEG	Taiwan
<i>Gigaspora decipiens</i>	BEG45	W1939	BEG	Australia
<i>Gigaspora rosea</i>	FL105-5	W2379	CIAT, INVAM	United States
<i>Glomus caledonium</i>	BEG20	W2426	BEG	United Kingdom
<i>Glomus clarum</i>	CL883A	W2860	INVAM	Colombia
<i>Glomus clarum</i>	BR147B-4	W2861	INVAM	Brazil
<i>Glomus clarum</i>	#842	W2862	CIAT, N. C. Schenck	United States
<i>Glomus geosporum</i>	BEG11		BEG	United Kingdom
<i>Glomus intraradices</i>	H3		H. von Alten	Germany
<i>Glomus manihotis</i>	LMNH980	W2863	H. Baltruschat	Colombia
<i>Glomus manihotis</i>	FL879-3	W2864	INVAM	United States
<i>Glomus mosseae</i>	BEG12		C. Azcón-Aguilar	United Kingdom
<i>Glomus</i> sp.	S328		D. M. Sylvia	United States
<i>Scutellospora cerradensis</i>		W1743	EMBRAPA	Brazil
<i>Scutellospora heterogama</i>	BR154C-2	W2383	CIAT, INVAM	Brazil
<i>Scutellospora pellucida</i>	#1332, C-139-3A	W2385	CIAT, E. Sieverding	Colombia

^a Abbreviations: CIAT, Centro Internacional de Agricultura Tropical (Cali, Colombia); INVAM, International Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (Morgantown, W.Va.); EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária.

was programmed as follows. After a hot start at 60°C, an initial denaturation of 3 min at 95°C was followed by five cycles of 30 s at 95°C, 30 s at 52°C, and 1.5 min at 72°C. Thereafter, 25 to 30 cycles with annealing at 51°C were performed. In the case of *S. pellucida*, PCR was performed with purified DNA (27).

Aliquots of the reaction mixture were examined by electrophoresis on 1.5% agarose gels. DNA fragments from successful reactions were concentrated and purified by precipitation with ice-cold ethanol in the presence of 0.3 M sodium acetate and 60 mg of glycogen (molecular biology grade; Boehringer GmbH, Mannheim, Germany) per ml.

The purified DNA was digested with the restriction enzymes *Mbo*I (Sigma, Deisenhofen, Germany), *Hin*II, and *Taq*I (Promega, Madison, Wis.) and electrophoresed on 4% agarose gels (3:1 NuSieve-SeaKem; FMC Corp., Rockland, Maine). As a molecular weight marker, a 100-bp ladder (Gibco BRL, Eggenstein, Germany) was used. Gels were stained in 5 mg of ethidium bromide per ml and photographed or scanned under UV illumination.

RESULTS

The apparent PCR product length was approximately 500 bp for *Gigaspora* and *Scutellospora* and ranged between 550 and 600 bp for *Glomus* species. *Entrophospora colombiana* had a

DNA fragment of 610 bp. In most cases, it was possible to amplify from a single spore. The approximate restriction fragment lengths of the glomalean species were calculated by using the molecular weight marker and are presented in Tables 2 and 3. The precision and reproducibility of the measurements were approximately 10 bp for fragments of up to 350 bp.

Figure 1 shows the length variation of the uncut fragments of several *Glomus* species and restriction patterns generated with the enzyme *Mbo*I. All five species possess clearly distinct patterns.

The constancy of the characters under study throughout fungal propagation had been established by performing analyses of different generations of a single spore culture of *G. intraradices* (18). Two cultures of the same isolate of *G. caledonium* that had been cultivated separately for several years at different locations were examined. The banding patterns produced with *Mbo*I, *Taq*I, and *Hin*II from these isolates remained constant over time (data not shown).

The enzymes *Hin*II and *Taq*I also distinguished many of the

TABLE 2. Apparent lengths of restriction fragments of PCR products from several AMF^a

AMF	Fragment length(s) (bp)			
	<i>Mbo</i> I	<i>Hin</i> II	<i>Taq</i> I	Uncut fragment
<i>Glomus manihotis</i> - <i>Glomus clarum</i>	315, (240), 115, 90 ^b	(340), 230, (200), 130, 115, 90	350, 180, 70	580
<i>Glomus intraradices</i> H3	400, 90 ^b	330, 120	350, 180, 80	580
<i>Glomus</i> sp. strain S328	400, 330, 230, 130 ^b	330, 120	320, 240, 180, 90, 70	580
<i>Glomus caledonium</i>	210, 170, 140, 100 ^b	380, 230	200, 95, 80, 70	590
<i>Glomus geosporum</i>	290, 160, 85	380, 220	(300), 200, 100, 90, 80	590
<i>Glomus mosseae</i>	190, 160, 130, 95	340, 190	220, 140, 80, 70	590
<i>Entrophospora colombiana</i>	180, 150, 120, 90	220, 195, 130	170, 120, 100	610
<i>Glomus mosseae</i> ^c	182, 152, 115, 91, 22, 17	345, 182, 44, 8	206, 126, 76, 65, 59, 47	579
<i>Glomus intraradices</i> DAOM197198 ^d	407, 79, 26, 17, 7	327, 118, 106, 8	329, 171, 59	559

^a PCR was performed with primers ITS1 and ITS4. Numbers in parentheses represent weak bands. For comparison, fragment lengths derived from previously reported nucleotide sequences were added (13, 21).

^b From reference 18.

^c From reference 21.

^d From reference 13.

TABLE 3. Apparent lengths of restriction fragments of PCR products from several Gigasporaceae^a

AMF	Fragment length(s) (bp)			Uncut fragment
	<i>Mbo</i> I	<i>Hin</i> FI	<i>Taq</i> I	
<i>Gigaspora albida</i>	200, 175, 100	310, 195	360, 175, 140	500
<i>Gigaspora rosea</i>	200, 175, 100	310, 195	360, 175, 140	500
<i>Gigaspora candida</i>	200, 175, 100	310, 195	360, 175, 140	500
<i>Gigaspora decipiens</i>	380, 200, 175, 100	310, 195	360, 140	500
<i>Scutellospora heterogama</i>	190, 100	300, 195	360, 140	500
<i>Scutellospora pelliculosa</i>	205, 170, 110	200, 120	ND ^b	510
<i>Scutellospora ceradensis</i>	200, 180, 100	300, 195	360, 140	500
<i>Scutellospora castanea</i>	210, 173, 105, 23	311, 200	364, 147	511

^a PCR was performed with primers ITS1 and ITS4. For comparison, fragment lengths derived from the nucleotide sequence of *S. castanea* (6, 7) are included.

^b ND, not determined.

Glomus spp. under study but revealed groups of species with banding patterns in common.

G. intraradices, *G. clarum*-*G. manihotis*, and *Glomus* sp. strain S328 share at least one restriction pattern with each other (Table 2). *G. caledonium* and *G. geosporum* could be differentiated with *Mbo*I but not with *Hin*FI or *Taq*I.

To analyze the variation of ITS restriction patterns within species and between closely related species, five isolates of *G. clarum* and *G. manihotis* from tropical and subtropical areas were screened for variability of these features. Their possible conspecificity has been discussed, since they can be distinguished by isozyme analysis, the differences apparently being biogeographically founded (19). With all three of the enzymes tested, these isolates showed considerable similarity in their banding patterns (Fig. 2 and Table 2), indicating low intraspe-

FIG. 1. Restriction fragment patterns of ITS fragments from *Glomus* spp. PCR products were digested with the enzyme *Mbo*I as indicated and run on a 4% agarose gel (3:1 NuSieve-SeaKem). Lane M, molecular size marker (100-bp ladder).

FIG. 2. Restriction fragment patterns of ITS fragments from several isolates of *G. clarum*-*G. manihotis* and other *Glomus* spp. PCR products were digested with the enzyme *Hin*FI and run on a 4% agarose gel (3:1 NuSieve-SeaKem). Lane M, molecular size marker (100-bp ladder).

cific variation. This represents a necessary precondition for recognition of isolates of the same species from different origins. Figure 2 presents the identical *Hin*FI banding patterns of the isolates mentioned. Only the intensity of some faint bands found in all five isolates varied, which is without diagnostic relevance. For the enzymes *Mbo*I and *Taq*I, all patterns were identical (Table 2).

Contrary to the findings of Sanders et al. (21), no restriction site for the enzyme *Alu*I was found in a wide range of Glomales tested, except for *Glomus* sp. strain S328. Analysis of the ITS sequence of *G. mosseae* BEG12 (GenBank accession no. X84232) reported by Sanders et al. (21) confirmed that it lacks an *Alu*I site.

The restriction fragment patterns of *E. colombiana* are shown in Fig. 3. The *Mbo*I pattern of this fungus was almost identical to that produced by *G. mosseae*. This was confirmed by analyzing the sequence of Sanders et al. (21) with regard to its *Mbo*I sites. With *Hin*FI and *Taq*I, no bands corresponding between the two organisms were found.

All of the species of *Gigaspora* tested possess similar restriction patterns (Fig. 4 and Table 3). The banding patterns of *Gigaspora albida*, *G. rosea*, and *G. candida* were identical. Some of the restriction patterns seem to indicate the presence of two sets of sequences (*G. rosea* *Taq*I digest in Fig. 4) because the overall length of the fragments is much larger than the original uncut PCR product. These results were reproducible with the same PCR product from a single spore that showed nonambiguous results with other restriction enzymes. Therefore, they are due to sequence heterogeneity within the spores (21). The 175-bp band (Fig. 4, *G. rosea* *Taq*I digest) can be easily explained by an additional restriction site on a subpopulation of the ITS sequences that divided the 360-bp fragment partially into two 175-bp fragments. *G. decipiens* differs from *G. albida*, *G. rosea*, and *G. candida* by the partial loss of an *Mbo*I restriction site that divides the 380-bp band into two bands of 200 and 175 bp (Fig. 4). Therefore, the few differences

FIG. 3. Restriction fragment patterns of ITS fragment from *E. colombiana*. PCR products were digested with the enzyme *Mbo*I, *Hinf*I, or *Taq*I and run on a 4% agarose gel (3:1 NuSieve-SeaKem). Lane M, molecular size marker (100-bp ladder).

between *Gigaspora* sp. restriction patterns are only present in subpopulations of amplified ITS sequences.

The close phylogenetic relationship between *Gigaspora* and *Scutellospora* correlates with several fragment patterns shared by the species examined (Table 3). With the exception of *S. pellucida*, all samples from the suborder Gigasporineae show the same *Hinf*I pattern (Fig. 5). This only aberration can be explained by a sequence mutation that led to an additional *Hinf*I site. Therefore, the 310-bp fragment of most of the Gigasporaceae is cut into a 120-bp fragment and 200-bp fragment in *S. pellucida*.

S. heterogama shows *Hinf*I and *Taq*I patterns identical to those of the other members of the Gigasporaceae examined, but its *Mbo*I pattern was distinguishable. *S. cerradensis* combines restriction characteristics of some other *Gigaspora* and

FIG. 4. Restriction fragment patterns of ITS fragments from several *Gigaspora* spp. PCR products were digested with the enzyme *Mbo*I, *Hinf*I, or *Taq*I and run on a 4% agarose gel (3:1 NuSieve-SeaKem). Lane M, molecular size marker (100-bp ladder).

FIG. 5. Restriction fragment patterns of ITS fragments from several *Scutellospora* spp. PCR products were digested with the enzyme *Hinf*I or *Mbo*I and run on a 4% agarose gel (3:1 NuSieve-SeaKem). Lane M, molecular size marker (100-bp ladder).

Scutellospora spp. but does not show a unique pattern with any of the enzymes tested.

DISCUSSION

Previous sequence analysis of ITS presented little variation among different isolates of *G. mosseae*, *G. fasciculatum*, and *G. dimorphicum* (15). Most of this variation was due to insertions-deletions of short runs of a single base and was not useful for distinguishing morphologically defined species. In this study, we prove for other *Glomus* spp. that restriction analyses of the ITS can indeed distinguish taxonomic entities near the level of currently defined species. However, as with isozyme studies (20), several enzymes should be used to characterize the isolates in question.

Our results suggest that the ITS sequence divergence within the genus *Glomus* is higher than within the whole family Gigasporaceae. Some investigators (19, 30) have discussed a possible division of *Glomus* because there is evidence that, as currently defined, it is not a monophyletic group. Restriction analysis and sequence data have the potential to contribute considerably to future revisions of the concept of this genus.

G. caledonium and *G. geosporum*, although quite distinct morphologically, share two restriction fragment patterns (Table 2) and also show similar isozyme patterns and sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles (4). The restriction patterns derived from the complete ITS sequence of a second isolate of *G. intraradices* from North America (13) were highly similar to the German isolate characterized in this study (Table 2). The Gigasporaceae are united by a whole range of restriction patterns. However, to draw conclusions about phylogenetic relationship, the whole sequences should be considered.

Within the Gigasporaceae, the fragment patterns were not as variable as in *Glomus* and do not allow fast and convenient identification, even to the genus level. It is well established that morphological characteristics used to distinguish *Gigaspora* spp. from each other are scarce, and therefore low genetic variation might be expected within this genus. Recently, Bentivenga and Morton (2) synonymized *G. rosea* and *G. candida* but maintained the species *G. albida* and *G. rosea*. Our data show that none of the three can be distinguished from the others by ITS restriction analysis with the restriction enzymes tested.

Although species of *Scutellospora* were chosen that were easy to distinguish morphologically and can be expected to represent a wide range of species, analyses with several restriction enzymes were required even to separate these isolates from *Gigaspora*. Fragment patterns derived from the nucleotide sequence of *S. castanea* (6, 7) are very similar to those of *S. cerradensis* (Table 3).

As the Acaulosporaceae were not the focus of our study, only *E. colombiana* from this group was analyzed. This species shows an *Mbo*I pattern almost undistinguishable from that of *G. mosseae*. As neither the *Hinf*I nor the *Taq*I pattern supports this, analyses of the total sequence are required to define if this is a homologous characteristic. No similarity could be detected between the restriction patterns of *E. colombiana* (this study) and *Acaulospora laevis* (21).

It is well established that ITS restriction patterns are useful for identification of taxonomic entities near the species level in organisms other than the Glomales (8, 11, 14, 34). However, it was shown that ITS sequences are heterogeneous within spores of *G. mosseae* (15, 21) and *Gigaspora* (this study). Recent findings (12) have even indicated that highly divergent sets of ITS sequences that can be differentially amplified by PCR exist within spores. The question arises as to whether the occurrence of different ITS in a single organism precludes species identification by examination of such sequences. Some of these sets may not be amplified with the primers ITS1 and ITS4 because the binding site is altered, as already shown for the primer VANS1 (22). Discordant phylogenetic groupings obtained by using ITS (15, 28) might be due to multiple sets of ITS. Therefore, care should be taken when using ITS to construct Glomales phylogenetic trees.

The intention of this study was mainly to test the suitability of our method for diagnostic purposes. In this context, it is not necessary to characterize all of the sets of ITS sequences present in the genome if a reproducible and well-defined protocol is used to select a certain set of ITS from a sequence pool. Although it was not the main intention of this study to analyze phylogenetic relationships in the Glomales, our restriction patterns confirm the current morphological classification.

Interpretation of the restriction fragment patterns is facilitated by the fact that, unlike banding patterns obtained by random amplification of polymorphic DNA (33), the bands of ITS digests follow certain inherent rules. In most cases, the total length of the banding pattern equals the approximate size of the uncut PCR product and the staining intensity of the bands decreases proportionally with their length. Exceptions to the latter indicate two fragments of the same apparent size or polymorphisms caused by minor ITS types.

Restriction fragment analysis of the ITS proved to be a valuable tool for characterization of many glomalean fungi on or above the species level. Our method is technically relatively simple to perform and thus can be of practical value. The connection of few morphological characters and highly variable ITS sequences in the current genus *Glomus* indicates that this method will be more useful in that genus than in the Gigasporaceae. Further analyses are necessary to clarify the usefulness of the technique in *Acaulospora*. When a larger library of fragment patterns is available, the described methods may allow assignment of species names reliably from a minute amount of fungal material. Once the ITS sequences can be amplified from extracts of colonized roots with AMF-specific PCR primers, the problem of characterizing active fungal populations of field sites on the species level can be addressed.

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