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Phylogenetic analysis of Glomeromycota by partial LSU rDNA sequences

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Abstract We analyzed the large subunit ribosomal RNA (rRNA) gene [LSU ribosomal DNA (rDNA)] as a phylogenetic marker for arbuscular mycorrhizal (AM) fungal taxonomy. Partial LSU rDNA sequences were obtained from ten AM fungal isolates, comprising seven species, with two new primers designed for Glomeromycota LSU rDNA. The sequences, together with 58 sequences available from the databases, represented 31 AM fungal species. Neighbor joining and parsimony analyses were performed with the aim of evaluating the potential of the LSU rDNA for phylogenetic resolution. The resulting trees indicated that *Archaeosporaceae* are a basal group in Glomeromycota, *Acaulosporaceae* and *Gigasporaceae* belong to the same clade, while *Glomeraceae* are polyphyletic. The results support data obtained with the small subunit (SSU) rRNA gene, demonstrating that the LSU rRNA gene is a useful molecular marker for clarifying taxonomic and phylogenetic relationships in Glomeromycota.

Keywords Glomeromycota · LSU rDNA · Phylogeny · Arbuscular mycorrhiza · Taxonomy

Introduction

Arbuscular mycorrhizal (AM) fungi, with about 157 described species (Kirk et al. 2001), establish the most widespread symbiotic association (mycorrhiza) between plants and fungi in nature; it has been suggested that 80% of all vascular plants belong to families that characteristically form mycorrhizae (Trappe 1987).

Morton and Benny (1990) grouped these fungi, characterized by arbuscule formation and at that time placed in *Endogonales* (*Zygomycota*), in the order *Glomales*, containing three families and six genera, mainly defined by spore formation. Simon et al. (1993), in pioneering work using ribosomal DNA (rDNA) sequences for molecular phylogeny of arbuscular mycorrhiza fungi, observed some discrepancies in the classification of the group as far as morphological analysis was concerned. All species included in *Glomales* were supposed to form arbuscular mycorrhizae, but Schüβler et al. (1994) suggested, on the basis of morphological similarities with *Glomus*, that *Geosiphon pyriformis*, not known to form arbuscular mycorrhizae but known to form an endosymbiosis with *Nostoc* (cyanobacteria), might be a species belonging to the group. Later, Gehrig et al. (1996) found evidence through small subunit (SSU) rDNA sequencing that *G. pyriformis* could be an ancestral species in *Glomales*. From this, a new classification using DNA sequences was initiated. For example, Redecker et al. (2000a) transferred *Sclerocystis coremioides* (type species) to *Glomus*, making *Sclerocystis* a taxonomic synonym for *Glomus*. Two new families (*Archaeosporaceae* and *Paraglomeraceae*) were proposed by Morton and Redecker (2001) and *Glomales*, together with *Geosiphon,* was moved to a new phylum (Glomeromycota), with four orders (Schüβler et al. 2001b). Recently, two families (*Pacisporaceae* and *Diversisporaceae*) were described as including species transferred from *Glomus* (Walker et al. 2004; Walker and Schüβler 2004; Oehl and Sieverding, 2004).

In spite of the advances reached in phylogenetic interpretation of the Glomeromycota, there are still some unsolved problems, such as the two large groups (*Glomus* group A and *Glomus* group B) in the order Glomerales (Schüβler et al. 2001b) and the *Diversisporaceae*, whose species are not yet completely defined (Walker and Schüβler 2004).

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The current classification of higher taxa in the Glomeromycota is mainly based on SSU rDNA sequences (Schüβler et al. 2001b). It is important to clarify the informative value and the resolution power that other genes could have at different taxonomic levels. The aim of this work was to evaluate the potential of a large subunit (LSU) rDNA region for phylogenetic resolution to solve problems that are not completely understood, as well as to confirm or suggest new evolutionary tendencies in Glomeromycota. In total we analysed 68 partial LSU ribosomal RNA (rRNA) gene sequences from 31 AM fungal species, 58 available from the databases and ten obtained in this work with two new specific primers.

Materials and methods

AM fungi

Ten isolates, propagated in pot cultures and belonging to the genera *Gigaspora, Glomus*, and *Scutellospora*, were used for DNA extraction and sequencing (in bold in Table 1).

DNA extraction

The DNA of spores was extracted as described in Lanfranco et al. (2001), with some modifications. Ten to 50 spores were washed in distilled water, sonicated three to four times, crushed in 50–100 μl of 1× REDTaq polymerase chain reaction (PCR) buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, and 0.01% jelly) (Sigma-Aldrich, Milan, Italy), centrifuged at 1,000 rpm for 2 min, and the supernatant was incubated at 95°C for 13 min. After extraction, the DNA was stored at −20°C.

Design of PCR primers, amplification, and sequencing

The AM fungal PCR primers were designed on the basis of LSU rDNA sequences from different AM fungi previously published in GenBank (the sequences aligned are located in the position 180–800 of the *Saccharomyces cerevisae* LSU rDNA).

The two new primers, 28G1 (5'-CATGGAGGGTGAG AATCCCG-3') and 28G2 (5'-CCATTACGTCAACATC CTTAACG-3'), were designed to amplify approximately 600 bp of Glomeromycota LSU rDNA, and they were tested for specificity by two methods: first, by finding the homologous DNA sequences by means of the GenBank basic local alignment search tool (BLAST) tool; and second, by amplifying the target DNA with regular PCR, and visualizing the amplicon on 1.5% agarose gel with ethidium bromide.

PCR reactions were carried out in a volume of 50 μl, containing 200 μM each deoxyribonucleotide triphosphates, 1 μM of each primer 28G1–28G2, and 2 units of REDTaq DNA polymerase (Sigma-Aldrich, Milan, Italy); cycling parameters were 45 s at 94°C, 1 min at 55°C, 1 min at 72°C for 40 cycles, and a final elongation of 7 min at 72°C followed the last cycle. The amplified products were purified with a QIAquick kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's instruction, and sequenced (accession numbers are listed in Table 1). Sequencing was provided by GeneLab (Rome, Italy).

Sequence alignment and phylogenetic analysis

Querying the National Center for Biotechnology Information databases with the BLASTn program, we verified that the ten sequences obtained from the seven species were affiliated to the Glomeromycota before using them for phylogenetic analysis. In all, 68 AM fungal sequences (each one with more than 500 bp), ten obtained in our laboratory and 58 from the GenBank (Table 1), were aligned with the program ClustalX (Thompson et al. 1997) and edited with the GeneDoc program (Nicholas et al. 1997) to obtain a ~600 bp final alignment deposited at the European Molecular Biology Laboratory database under accession number ALIGN_000914.

For phylogenetic analyses and tree construction, maximum parsimony (MP) and neighbor joining (NJ) analyses with 1,000 and 10,000 bootstrap replications, respectively, were performed using the Phylogenetic Analysis Using Parsimony program version 4 (Swofford 2002). The NJ analysis was performed using the Kimura parameters (Kimura 1980). Sequences from *Mortierella polycephala* (AF113464), *Boletus edulis* (AF336240), and *Neurospora crassa* (AF286411) were used as the out-group.

Results

PCR amplification of AM fungal DNA

BLASTn analysis of the new 28G1 and 28G2 primers indicated that the 28G2 primer matched with sequences of Glomeromycota, while 28G1 was also homologous with the LSU rDNA of several other fungi. The combination of the above primers is appropriate for the amplification of Glomeromycota LSU rDNA sequences. The amplification product obtained by PCR with the primer pairs 28G1–28G2 from spore DNA of the ten isolates (two *Glomus*, four *Gigaspora*, and one *Scutellospora* species, Table 1) was about 600 bp. These new primers were also tested on DNA extracted from mycorrhizal roots, and they successfully amplified different AM fungi sequences without recognition of plant DNA (data not shown).

Table 1 Isolates from Glomeromycota used for the phylogenetic analyses

N°	Species	Isolate code	Origin	Source or contributors[a]	Accession N°
1	*Acaulospora lacunosa*	BEG 78	USA	BEG	AJ510230
2	*Ac. laevis*	BEG 13	New Zealand	BEG	AJ510229
3	*Ac. longula*	BEG 08	UK	BEG	AJ510228
4	*Ac. mellea* 1	NA	Kenya	NA	AY900514
5	*Ac. mellea* 2	NA	Kenya	NA	AY900512
6	*Ac. paulinae* 1	NA	NA	NA	AY639328
7	*Ac. paulinae* 2	NA	NA	NA	AY639263
8	*Archaeospora gerdemannii* 1	NC169-3	USA	INVAM	AJ510234
9	*Ar. gerdemannii* 2	AU215-6	Tasmania	INVAM	AJ510233
10	*Ar. gerdemannii* 3	NC169-3	USA	INVAM	AJ271712
11	***Gigaspora albida***	BR 601	Brazil	INVAM	AJ852007
12	***Gi. gigantea* 1**	MN 414D	USA	INVAM	AJ852009
13	***Gi. gigantea* 2**	NC 150	USA	INVAM	AJ852010
14	*Gi. gigantea* 3	NA	Kenya	NA	AY900506
15	*Gi. gigantea* 4	NA	Kenya	NA	AY900504
16	***Gi. margarita* 1**	K-1-520052	Japan	MAFF	AJ852013
17	***Gi. margarita* 2**	BEG 34	New Zealand	Torino	AJ852011
18	***Gi. margarita* 3**	C-520054	Japan	MAFF	AJ852014
19	*Gi. margarita* 4	Gigmar58	NA	NA	AF396783
20	*Gi. margarita* 5	Gigmar60	NA	NA	AF396782
21	*Gi. rosea* 1	BEG 9	USA	BEG	Y12075
22	***Gi. rosea* 2**	UT 102	USA	INVAM	AJ852015
23	*Glomus caledonium* 1	SC_658	NA	NA	AF396799
24	*G. caledonium* 2	BEG 86	Denmark	BEG	AJ510239
25	*G. caledonium* 3	BEG 86	Denmark	BEG	AJ628059
26	*G. caledonium* 4	RMC_658	NA	NA	AF396789
27	*G. claroideum* 1	BEG 31	Finland	BEG	AJ271929
28	*G. claroideum* 2	BEG14	Denmark	BEG	AF235007
29	*G. claroideum* 3	NA	NA	NA	AY639287
30	*G. claroideum* 4	NA	NA	NA	AY639185
31	*G. cf. claroideum* 1	NA	NA	NA	AY639346
32	*G. cf. claroideum* 2	NA	NA	NA	AY639338
33	*G. clarum* 1	LPA64	NA	NA	AJ510243
34	***G. clarum* 2**	UFPE 08	Brazil	Recife	AJ852005
35	*G. clarum* 3	LPA16	NA	NA	AJ510242
36	*G. cf. clarum*	NA	NA	NA	AY639330
37	*G. constrictum*	BEG 130	Spain	BEG	AF145741
38	*G. coronatum* 1	BEG 28	Italy	BEG	AF145739
39	*G. coronatum* 2	BEG 49	Spain	BEG	AF145740
40	*G. deserticola*	BEG73	Spain	BEG	AJ746249
41	*G. cf. eburneum* 1	NA	NA	NA	AY639306
42	*G. cf. eburneum* 2	NA	NA	NA	AY639225
43	***G. etunicatum***	UFPE 06	Brazil	Recife	AJ852006
44	*G. fragilistratum*	BEG 05	Denmark	BEG	AF145747
45	*G. geosporum*	BEG 11	UK	BEG	AJ510241
46	*G. intraradices* 1	SI 141	NA	NA	AF396797
47	*G. intraradices* 2	DAOM181602	NA	NA	AY842577
48	*G. intraradices* 3	DAOM181602	NA	NA	AY842575
49	*G. mosseae* 1	BEG 25	UK	BEG	AF145735
50	*G. mosseae* 2	BEG 85	Denmark	BEG	AF145736
51	*G. mosseae* 3	BEG 12	UK	BEG	Y07656
52	*G. mosseae* 4	NA	NA	NA	AY639273
53	*G. versiforme* 1	BEG 47	USA	BEG	AY842574
54	*G. versiforme* 2	BEG 47	USA	BEG	AY842573

Table 1 (continued)

N°	Species	Isolate code	Origin	Source or contributors[a]	Accession N°
55	*Paraglomus occultum*	CL700C-2	Colombia	INVAM	AJ271713
56	*Scutellospora calospora*	BEG 32	UK	BEG	AJ510231
57	*S. castanea*	BEG 01	France	BEG	Y12076
58	*S. gregaria*	LPA48	NA	NA	AJ510232
59	***S. heterogama* 1**	UFPE 19	Brazil	Recife	AJ852016
60	*S. heterogama* 2	NA	Kenya	NA	AY900503
61	*S. heterogama* 3	NA	Kenya	NA	AY900500
62	*S. nigra* 1	NA	Kenya	NA	AY900498
63	*S. nigra* 2	NA	Kenya	NA	AY900494
64	*S. pellucida* 1	Scutpell90	NA	NA	AF396784
65	*S. pellucida* 2	NA	NA	NA	AY639313
66	*S. pellucida* 3	NA	NA	NA	AY639309
67	*S. verrucosa* 1	NA	Kenya	NA	AY900508
68	*S. verrucosa* 2	NA	Kenya	NA	AY900507

NA information not available

[a]*BEG* European Bank of *Glomales*, Dijon, France; *INVAM* International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, Morgantown, West Virginia; *MAFF* Ministry of Agriculture, Forest and Fisheries, Genebank Project of the National Institute of Agrobiological Sciences, Ibaraki, Japan; *Torino* Dipartimento di Biologia Vegetale dell'università di Torino, Italy; *Recife* Departamento de Micologia, CCB, Universidade Federal de Pernambuco, Recife, PE, Brasil

Phylogenetic analyses

The tree topology obtained either by MP or NJ analysis was congruent. The NJ phylogenetic tree is shown in Fig. 1.

The phylogenetic analyses, obtained with partial sequences from LSU rDNA, grouped *N. crassa* and *B. edulis* with bootstrap values of 91% (NJ). In both MP and NJ analysis, *Paraglomus occultum* was not grouped with other species.

The families *Acaulosporaceae*, *Gigasporaceae*, and *Diversisporaceae* were grouped together, forming a cluster supported with bootstrap values of 87% for NJ and 75% for MP analysis. The species of *Acaulosporaceae* and those belonging to *Gigasporaceae* and *Diversisporaceae* were grouped with bootstrap values higher than 92%, respectively. In the NJ analysis, the *Scutellospora* species were grouped with bootstrap values of 86%, but the *Gigaspora* species were not solved.

In *Glomus*, two groups, here designated *Glomus* group A (GlGr A) and *Glomus* group B (GlGr B), were observed. The GlGr B showed high bootstrap values (100%) and the GlGr A was supported by 96 and 92% bootstrap values (NJ and MP analysis, respectively). Moreover, the latter group can be divided into two subgroups (GlGr Aa and GlGr Ab); both of them were supported by bootstrap values of more than 97%.

Discussion

Our phylogenetic analyses on the partial LSU rDNA sequences generated trees that were congruent with those based on SSU rDNA that were obtained by Schwarzott et al. (2001). In NJ analysis, the *Archaeosporaceae* appear as a basal group in Glomeromycota, whereas in MP analysis, as well as in Redecker et al. (2000b) and Schwarzott et al. (2001), there is not a clear definition of the most ancestral group (*Archaeosporaceae* or *Paraglomeraceae*). However, in our analyses, both *Archaeosporaceae* and *Paraglomeraceae* were only represented by a single species because no other LSU rDNA sequences were available in the Databases.

The results of our work confirm data regarding the proximity between *Acaulosporaceae* and *Gigasporaceae* (Schwarzott et al. 2001; Schüβler et al. 2001b). This grouping of the two families was also shown by Simon et al. (1993) and Redecker et al. (2000b). However, Helgason et al. (2003), using sequences from actin and elongation factor 1-alpha for phylogenetic analyses, observed that *Acaulospora laevis* was closer to *Glomus caledonium* than to *Gigaspora margarita* and *Scutellospora dipurpurescens*. Based on morphological data, Morton (1990) indicated that *Acaulosporaceae* might be closer to *Glomeraceae*, as was supported by Gianinazzi-Pearson et al. (1994), who found β (1 → 3) glucans only in the walls of species belonging to these two families.

In *Gigasporaceae*, ontogenesis data (Franke and Morton 1994; Bentivenga and Morton 1995; Morton 1995 and fatty acid profiles (Bentivenga and Morton 1996) indicated that *Gigaspora* might be basal to *Scutellospora*, as established before by morphological data (Morton 1990). Conversely, the trees obtained by Simon et al. (1993) and Redecker et al. (2000b) from molecular data have indicated that *Scutellospora* appeared earlier in relation to *Gigaspora* in the evolutionary process. Kramadibrata et al. (2000) reported that *Scutellospora projecturata* might belong to a

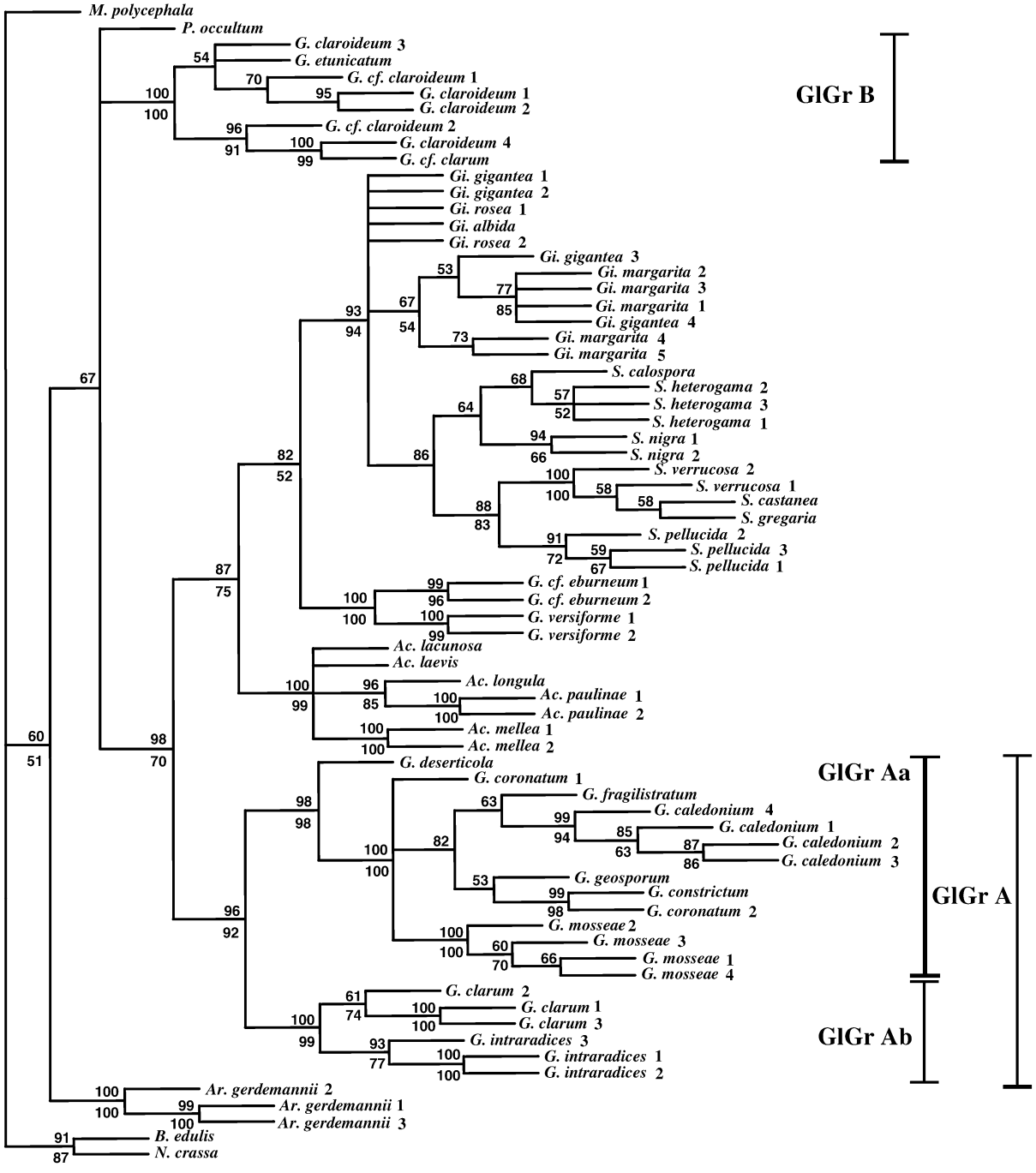

Fig. 1 Phylogenetic tree obtained from partial LSU rDNA sequences. *Numbers above branches* indicate the bootstrap values (above 50%) of the neighbor joining analysis; *numbers below branches* indicate the bootstrap values of the maximum parsimony analysis. (CI, Consistency Index =0.45; RI, Retention Index =0.80)

group closer to *Gigaspora*, and different from that of the species of *Scutellospora* sequenced until then. Currently, *Gigaspora* has been considered as an advanced genus in *Gigasporaceae* (Redecker 2002). In our MP analysis, the position of some *Scutellospora* species was unresolved with respect to other *Scutellospora* and *Gigaspora* species, as already observed by Schwarzott et al. (2001) and Schüβler et al. (2001a), whereas in our NJ analysis, all *Scutellospora* species were grouped together. The *Gigaspora* species were present in just one clade in most phylogenetic work; conversely, the evolutionary pathways in *Scutellopora* remain unclear.

Schwarzott et al. (2001) grouped some species of *Glomus* (*Glomus etunicatum*-like isolate W2423, *Diversispore spurcum* (syn. *G. spurcum* and *Glomus versiforme*) with *Acaulosporaceae* and *Gigasporaceae*, forming the order

Diversisporales recently revised by Walker and Schüβler 2006. Another isolate of *G. etunicatum* (isolate UT 316), was grouped in GlGr B (Schwarzott et al. 2001). Our data indicate that *G. versiforme* and *Glomus cf. eburneum* group in *Diversisporales*, but the isolate of *G. etunicatum*, UFPE 06, falls in GlGr B together with *Glomus claroideum*. The sequence of SSU rDNA obtained from the isolate UFPE 06 (data not shown) confirmed these results. Jansa et al. (2003) analyzed phylogenetically partial sequences for LSU rDNA, and observed that *G. claroideum* and *G. etunicatum* grouped together in the tree. Further important data regarding *G. claroideum* and *G. etunicatum* are that the fatty acid C20:0 ISO was observed only in these species (Graham et al. 1995).

Our analyses confirm the *Glomus* groups obtained by Schwarzott et al. (2001), and we have retained their terminology for these groups. Similar results with LSU sequences have been obtained by Rodriguez et al. (2001) for the phylum Glomeromycota in general, and by Kjoller and Rosendahl (2001) and Rosendahl and Stukenbrock (2004) for some *Glomus* species.

In general, our data, together with sequences already deposited in GenBank, indicate that the use of LSU rRNA sequences is suitable to investigate Glomeromycota phylogeny. However, it is necessary that other species of Glomeromycota have their genes sequenced, i.e., the rDNA or other phylogenetic-informative genes (Helgason et al. 2003), to establish inter-species relations and to determine the evolutionary tendencies in this group of fungi.

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