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Phylogenetic Analysis of a Dataset of Fungal 5.8S rDNA Sequences Shows That Highly Divergent Copies of Internal Transcribed Spacers Reported from *Scutellospora castanea* Are of Ascomycete Origin

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Redecker, Dirk, Hijri, Mohamed, Dulieu, Hubert, and Sanders, Ian R. 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. *Fungal Genetics and Biology* 28, 238–244. Using a dataset comprising 5.8S rDNA sequences from a wide range of fungi, we show that some sequences reported recently from the arbuscular mycorrhizal (AM) fungus *Scutellospora castanea* most likely originate from Ascomycetes. Other ITS and 5.8S sequences which were previously reported are confirmed as being clearly of mycorrhizal origin and are variable within one isolate of *S. castanea.* However, these results mean that previous conclusions which were drawn regarding the heterokaryotic status of AM fungal spores remain unproven. We provide an enlarged 5.8S rDNA dataset that can be used to check ITS sequences for conflicts with well-established phylogenies of the organisms that they were obtained from.

***Index Descriptors:* *Scutellospora castanea;* Glomales; arbuscular mycorrhiza; AM fungi; internal transcribed spacers; ribosomal DNA; phylogeny; internuclear polymorphism; Ascomycete.**

Arbuscular mycorrhiza (AM)[1] is an extremely widespread symbiosis between plant roots and zygomycetous fungi from the order Glomales. The vast majority of land plants take advantage of this association. It is of great agricultural and ecological importance, exerting strong influences on the species diversity of plant ecosystems (Smith and Read, 1997; van der Heijden *et al.,* 1998). Molecular and fossil data indicate that AM fungi are ancient and that AM-like fungi were present in the roots of the first land plants (Simon *et al.,* 1993; Remy *et al.,* 1994) approximately 500 million years ago and they may, therefore, have played a key role in the colonization of land by plants.

The only fungal structures of the Glomales which can be distinguished as individuals are their large (40 to 800 μm) spores that can contain several thousand nuclei (Bécard and Pfeffer, 1993). Several authors reported that a considerable degree of variation can be detected among the copies of ribosomal DNA within single spores of the Glomales (Sanders *et al.,* 1995; Lloyd MacGilp *et al.,* 1996; Redecker *et al.,* 1997). The occurrence of varying copies of rDNA within a genome alone is not unusual, as it was also shown for plants (Buckler *et al.,* 1997). A recent study

[1] Abbreviations used: AM, arbuscular mycorrhiza; ITS, internal transcribed spacer.

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claimed that extremely divergent internal transcribed spacer (ITS) sequences are located on different nuclei within single spores of the AM fungus *Scutellospora castanea* (Glomales; Zygomycetes) (Hijri *et al.,* 1999). A second publication concerning the same isolate of the fungus reported that 18S ribosomal subunit genes are so variable that they phylogenetically group into different genera of the Glomales (Hosny *et al.,* 1999). Such high heterokaryotic diversity in these coenocytic organisms would have far-reaching consequences for population genetics and evolutionary biology of these fungi (Sanders, 1999). The ITS1 and ITS2 regions are too variable to allow alignments among organisms that are not very closely related and, therefore, are not sufficiently useful to produce robust results in phylogenetic analyses. Embedded in between the ITS1 and 2 regions is the more conserved gene for the 5.8S rRNA. Using phylogenetic analyses of a comprehensive dataset of these sequences from all known subgroups of the Glomales, we show that some of the rDNA sequences Hijri *et al.* (1999) and Hosny *et al.* (1999) presented as variants of ITS from the AM fungus *S. castanea* appear to be of Ascomycete origin. Therefore the evolutionary and phylogenetic conclusions drawn from these data can no longer be substantiated. As ITS have become popular in studies of the Glomales, our 5.8S dataset will also constitute a necessary tool to elucidate the origin of ITS amplified from AM fungal spores and colonized roots.

TABLE 1

Isolates of Arbuscular Mycorrhizal Fungi Used in This Study and Sequence Accession Numbers

Species	Isolate	Source	Accession no.
Acaulospora laevis	AU211-3	INVAM	AJ242499
Acaulospora morrowiae	BR226-1	INVAM	AJ242500
Acaulospora denticulata	CL139-3	INVAM	AJ239115
Acaulospora mellea	BR983-4	INVAM	AJ239116
Entrophospora colombiana	C18-3	CIAT	AJ239117
Gigaspora albida	BR205-1	CIAT	AJ239118
Gigaspora decipiens	AU102-3	CIAT	AJ239119
Scutellospora heterogama	BR154	CIAT	AJ245961
Scutellospora pellucida	C-139-3A	CIAT	AJ239121
Glomus geosporum	BEG11	BEG	AJ239122
Glomus clarum	CL883A	INVAM	AJ243275/AJ239123
Glomus etunicatum	UT316	INVAM	AJ239125
Glomus spec.	S329	Sylvia	AJ239124
Acaulospora trappei	AU219	INVAM	AJ243419
Acaulospora gerdemannii/ Glomus leptotichum	NC176	INVAM	AJ012109
Glomus occultum	HA771	INVAM	AJ012113
Glomus brasilianum	WV219	INVAM	AJ012112

Note. INVAM, International Collection of Vesicular–Arbuscular and Arbuscular Mycorrhizal Fungi, Morgantown, WV; CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; BEG, Banque Europeenne de Glomales; Sylvia, Dr. D. M. Sylvia, University of Florida, Gainesville.

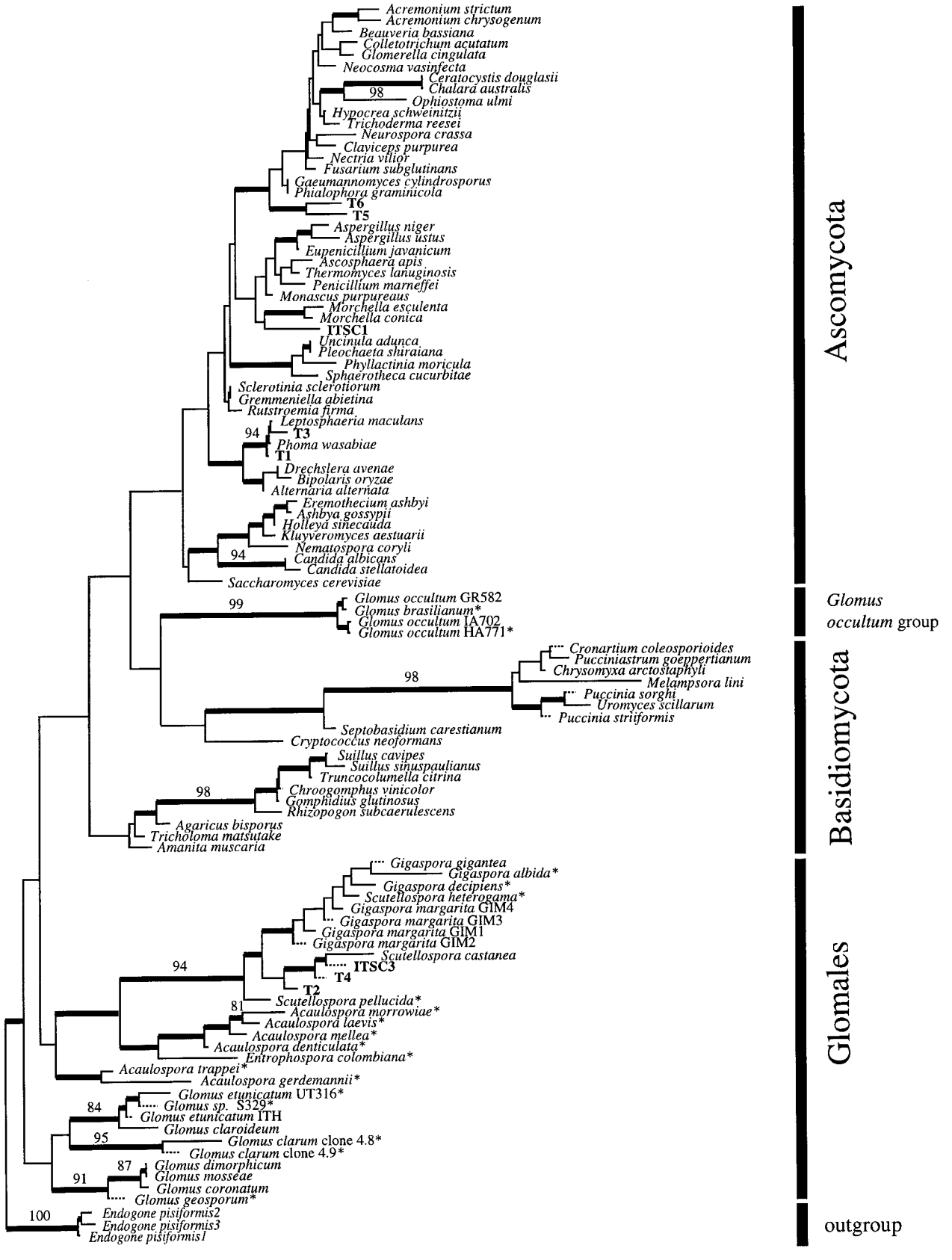
MATERIALS AND METHODS

Sequences of the ITS and 5.8S rDNA were obtained by D.R. from spores of the AM fungi given in Table 1 and were used to supplement the data in the GenBank and EMBL databases for phylogenetic analyses: The sequences were amplified by PCR followed by cloning and sequencing. PCR was conducted on genomic DNA from single AMF spores using the primers ITS1F (Bruns and Gardes, 1993) and ITS4 (White *et al.,* 1990) as previously described (Redecker *et al.,* 1997). PCR products were cloned into pCR 2.1 with the Invitrogen TA Cloning Kit (Invitrogen, San Diego, CA) and then sequenced. A PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer, Foster City, CA) was used. Electrophoresis and data collection were carried out on an ABI Model 377 DNA sequencer (Perkin–Elmer). DNA Sequencing Analysis (version 2.01) and Sequence Navigator (version 1.01) were used for processing the raw data. DNA sequences were submitted to the EMBL database under the accession numbers given in Table 1.

The 5.8S subunit gene embedded in between these and other glomalean ITS sequences was included in a dataset originally compiled by Cullings and Vogler (1998). 5.8S sequences of plants and animals from this dataset were omitted to facilitate display of trees. Another reason was that some taxa from those groups show extremely long branches that could interfere with phylogenetic analyses within the fungi. Sequences were aligned by hand and no characters were excluded from phylogenetic analysis. Thus, the 5.8S dataset we used consisted of sequences of 46 Ascomycetes, 18 Basidiomycetes, and 31 glomalean fungi. Trees were rooted with three variant sequences from a strain of the zygomycete *Endogone pisiformis.*

For analysis of 18S rDNA sequences, an alignment based on a dataset of Berbee and Taylor (1999) that comprised 60 species from fungi, choanoflagellates, and animals was used. One thousand five hundred thirty-three characters that could be aligned with confidence were used for the analyses.

Phylogenetic analyses were conducted with PAUP* 4.0b2 (Swofford, 1999). Neighbor joining (Kimura two-


Acremonium strictum
Acremonium chrysogenum
Beauveria bassiana
Colletotrichum acutatum
Glomerella cingulata
Neocosma vasinfecta
Ceratocystis douglasii
98
Chalara australis
Ophiostoma ulmi
Hypocrea schweinitzii
Trichoderma reesei
Neurospora crassa
Claviceps purpurea
Nectria vilior
Fusarium subglutinans
Gaeumannomyces cylindrosporus
Phialophora graminicola
T6
T5
Aspergillus niger
Aspergillus ustus
Eupenicillium javanicum
Ascosphaera apis
Thermomyces lanuginosis
Penicillium marneffei
Monascus purpureaus
Morchella esculenta
Morchella conica
ITSC1
Uncinula adunca
Pleochaeta shiraiana
Phyllactinia moricula
Sphaerotheca cucurbitae
Sclerotinia sclerotiorum
Gremmeniella abietina
Rutstroemia firma
Leptosphaeria maculans
94
T3
Phoma wasabiae
T1
Drechslera avenae
Bipolaris oryzae
Alternaria alternata
Eremothecium ashbyi
Ashbya gossypii
Holleya sinecauda
Kluyveromyces aestuarii
Nematospora coryli
94
Candida albicans
Candida stellatoidea
Saccharomyces cerevisiae
99
Glomus occultum GR582
Glomus brasilianum*
Glomus occultum IA702
Glomus occultum HA771*
Cronartium coleosporioides
Pucciniastrum goeppertianum
Chrysomyxa arctostaphyli
98
Melampsora lini
Puccinia sorghi
Uromyces scillarum
Puccinia striformis
Septobasidium carestianum
Cryptococcus neoformans
Suillus cavipes
Suillus sinuspaulianus
Truncocolumella citrina
98
Chroogomphus vinicolor
Gomphidius glutinosus
Rhizopogon subcaerulescens
Agaricus bisporus
Tricholoma matsutake
Amanita muscaria
Gigaspora gigantea
Gigaspora albida*
Gigaspora decipiens*
Scutellospora heterogama*
Gigaspora margarita GIM4
Gigaspora margarita GIM3
Gigaspora margarita GIM1
Gigaspora margarita GIM2
Scutellospora castanea
94
ITSC3
T4
T2
Scutellospora pellucida*
81
Acaulospora morrowiae*
Acaulospora laevis*
Acaulospora mellea*
Acaulospora denticulata*
Entrophospora colombiana*
Acaulospora trappei*
Acaulospora gerdemannii*
Glomus etunicatum UT316*
84
Glomus sp. S329*
Glomus etunicatum ITH
Glomus claroideum
95
Glomus clarum clone 4.8*
Glomus clarum clone 4.9*
87
Glomus dimorphicum
91
Glomus mosseae
Glomus coronatum
Glomus geosporum*
100
Endogone pisiformis2
Endogone pisiformis3
Endogone pisiformis1
0.01 changes
Ascomycota
Glomus occultum group
Basidiomycota
Glomales
outgroup

parameter method, $\gamma = 0.5$) was the preferred method with the 5.8S dataset because parsimony analysis produced large numbers of equally parsimonious trees. To conduct a heuristic search under the parsimony criterion, the number of trees in memory was restricted to 5000. Alternative tree topologies of distance trees were assessed by the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) as implemented in PAUP*. Manually edited tree files that forced certain taxa into the branch containing the Gigasporaceae were loaded as "backbone constraints." The resulting constrained neighbor joining trees were compared to the unconstrained original by the Kishino–Hasegawa test under the maximum likelihood criterion.

RESULTS AND DISCUSSION

Analysis of newly acquired sequences confirmed that single glomalean spores of several species showed a considerable degree of variation with respect to their ITS and 5.8S rDNA as shown by other authors (Sanders *et al.,* 1995; Lloyd MacGilp *et al.,* 1996). For instance, 34 variable positions from a total of 510 were found between two cloned sequences (clones 4.8 and 4.9) from a single spore of *Glomus clarum.* The ITS themselves cannot be aligned across the Glomales but only within certain groups of species that correspond to the phylogenetic lineages supported by the 5.8S data. Phylogenetic analysis of the 5.8S subunit that is embedded between the ITS always put clones from the same spore and sequences from the same species on one major branch of the tree (Fig. 1). Cullings and Vogler (1998) already stated that the phylogenetic signal in the relatively short (170 bp) 5.8S subunit is limited but sufficient to assign the sequences with acceptable bootstrap values to already defined taxonomic groups. One such group comprises the Gigasporaceae, which are supported by the high bootstrap value of 94%. *S. castanea* belongs to this group. Divergent ITS from single spores of *Gigaspora margarita* (Lanfranco *et al.,* 1999) also clustered closely together on this clade.

The grouping of taxa within the Glomales, though only in the major groupings supported by bootstrap, is in concordance with results from better supported 18S rDNA studies, including the position of *Acaulospora gerdemannii* and *Acaulospora trappei* as deeply divergent glomalean lineages (Redecker *et al.,* 1999). A notable exception is the *Glomus occultum* group, which is a very deeply branching lineage according to 18S rDNA analyses (Redecker *et al.,* 2000). While the 18S phylogenetic analysis shows clearly that this group belongs to the Glomales (Redecker *et al.,* 2000), in the 5.8S tree, the group does not cluster with the Glomales but groups with a deep branch of Basidiomycetes (Uredinales, Septobasidiales, and Tremellales). This grouping is clearly due to long branch effects (Carmean and Crespie, 1995), because the *G. occultum* group consistently associates with the longest branch present in the dataset: the basal Basidiomycetes or, if present, plants or animals (not shown). Nevertheless, all included sequences of the *G. occultum* group, independently determined in different laboratories, form a common cluster. We sequenced a clone containing both the ITS and the 3′ end of the 18S rDNA from *Glomus brasilianum,* which clearly shows that the ITS are of glomalean origin. This example illustrates the limitations of the phylogenetic signal in the 5.8S subunit, which does not allow the establishment of phylogenetic relationships between very deeply divergent clades and analyses and should be used strictly as a diagnostic tool to assign ITS sequences to clades already present.

Three of the sequences (T2, T4, and ITSSc3) published by Hijri *et al.* (1999) and Hosny *et al.* (1999) group closely to the 5.8S rDNA sequence previously reported for *S. castanea.* As these sequences are deeply nested within the well-supported Gigasporaceae clade, there is no reason to assume that these sequences do not originate from *S. castanea.* The sequences also indicate that there is indeed sequence heterogeneity in the ITS from this isolate, but not to the extent of variation previously reported. However, all other sequences that were reported to be ITS variants of this AM fungus (T1, T3, T5, T6, and ITSc1) do not belong to that clade. T1 and T3 are in a group within the Ascomycetes with *Phoma wasabiae* and *Leptosphaeria maculans* as closest relatives. The 5.8S rDNA sequences of

FIG. 1. Five of the ITS sequences reported to originate from *S. castanea* by Hijri *et al.* (1999) and Hosny *et al.* (1999) group with the Ascomycetes and three group within the Glomales. Phylogenetic tree of 5.8S rDNA sequences obtained by distance analysis with the neighbor joining method. *Endogone pisiformis* was used as outgroup. Branches supported by bootstrap values from 1000 replications higher than 50% are shown in bold, bootstrap values over 80% are shown. *Sequences that were not previously published. Names of sequences reported by Hijri *et al.* (1999) and Hosny *et al.* (1999) are shown in boldface. A similar tree topology was obtained by parsimony analysis.

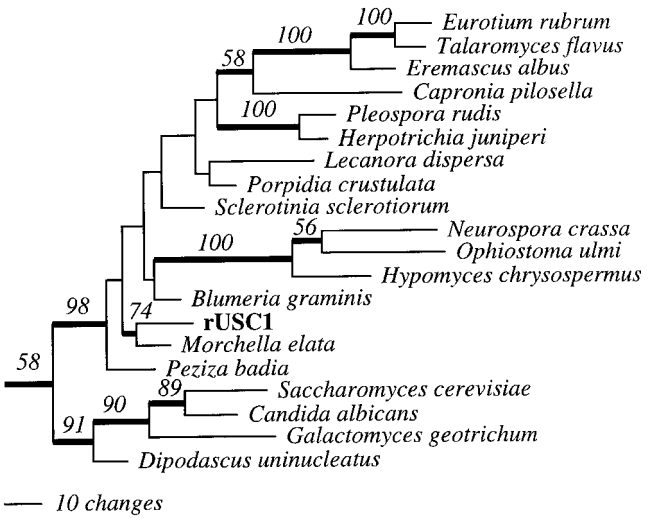


FIG. 2. Phylogenetic analysis of the 18S subunit (rUSc1) confirms the placement of ITSc1 in the Ascomycetes. Depicted is the Euascomycete/Hemiascomycete branch of a phylogenetic tree obtained from parsimony analysis of 18S rDNA sequences that yielded 12 equally parsimonious trees. Bootstrap values over 50% from 200 replications are shown on the branches.

Phoma, Leptosphaeria, and T1 are completely identical. The similarities between the highly variable ITS sequences of T1/T3 and *Phoma/Leptosphaeria* are strong (Fig. 3). T5 and T6 group in another part of the Ascomycetes with weak bootstrap support.

The closest match in the dataset for ITSc1 is *Morchella.* ITSc1 and ITSc3 were sequenced from clones that also contained the respective 18S ribosomal small subunit sequences (rUSc1 and rUSc3). Because it is nearly 10 times as long as the 5.8S rDNA gene, the small subunit usually is superior for phylogenetic analysis. The placement of rUSc1 close to *Morchella* (Fig. 2) confirmed with good bootstrap support (74%) the analysis of the 5.8S rDNA from ITSc1.

The phylogenetic placements of T1/T3, T5/T6, and ITSc1 in the 5.8S rDNA tree were assessed by the Kishino–Hasegawa test. When these sequences were forced into the Gigasporaceae two of the trees were significantly worse than the unconstrained tree at $P < 0.05$ (Table 2).

The phylogenetic analysis by Hijri *et al.* (1999) included no sequences outside the Glomales as an outgroup. Given this insufficient taxon sampling, no other result was possible than the grouping of the putative *S. castanea* sequences on a common branch, i.e., within the Glomales. Our results clearly show that the root of that tree has to be put between the branches containing *Glomus/Scutellospora/* T2/T4 and ITSc1/T1/T3/T5/T6. A similar mistake occurred in the trees presented by Hosny *et al.* (1999). What remains unclear is the true origin of these sequences. There are three possibilities: (1) An Ascomycete contaminant from the surface of the spores was present. (2) Ascomycete fungi are living inside a high proportion of *S.*

```
Scutellospora pellucida      TTGAAGGTCAGTTAAAA-----TAAATCGTACATCT-TTGATGTTACGGATCTGGGTTATCCGA--
Scutellospora heterogama     TTGAGGGTCAGTTAAAAAAAAATAA-TCGTACATCTTTTGATGTGGCGGATCTGGGTTATCCGA--
T2                           TTGAGGGTCAGTTAAAAAA---TA--TCGTACATCTCT-GATGTTACGGATCTGGGTTATCCAATT
T4                           TTGAGGGTCAGTTAAAAAAA--TA--TCGTACATCTCT-GATGTTACGGATCTGGGTTATCCGATT
ITSC3                        TTGAGGGTCAGTTAAAAAAAA-TA--TCGTACATCTCT-GATGTTACGGATCTGGGTTATCCGATT

Leptosphaeria maculans       TCGAGCGTCATTTGTACCC-T-CAA-GC-TT-TGCTT--GGTGTT--GGGTGAATGTTCTCTG-T-
Phoma wasabiae               TCGAGCGTCATTTGTACCC-T-CAA-GC-TT-TGCTT--GGTGTT--GGGTGAATGTTCTCTG-T-
T1                           TCGAGCGTCATTTGTACCT-TTCAA-GC-T-CTGCTT--GGTGTT--GGGTG--T-TTGTCC--TT
T3                           TCGAGCGTCATTTGTACCT-T-CAA-GC-C-CTGCTT--GGTGTT--GGGTG--T-TTGTCC--TT


Scutellospora pellucida      -TTCTT------AAGGTCGG-TTA-CCT-AA--AA-TTAAATTTTATGTAATGTG---TGAAGCG
Scutellospora heterogama     --TCTTATT-----AATCGG-TTA-CCT-AA--AATTTAAGATGGATATATAATG---TGAAGTG
T2                           TTTTTTTTTTTTTAGAATTGG-TTA-CCT-AAA--AATTAAATT--ATGTAA--TG---TGAA-CG
T4                           TTTCTTTT----AGAATTGG-TTA-CCT-AAA--AATTAAAA---TTGTAA--TG---TGAAGGG
ITSC3                        TTTCTTT-----AGAATTGG-TTA-TCT-AAA--AATTACA---TTTGTAA--TG---TGAAGCG

Leptosphaeria maculans       --GCTTGCAC--AGA-TTGGACTCGCCTGAAAACAATTGGCAGCCGGCATATTGGCC-TGGAGCG
Phoma wasabiae               --GCTTGCGC--AGA-CTGGACTCGCCTGAAAACAATTGGCAGCCGGCATATTGGCC-TGGAGCG
T1                           --GCTCTAGT--GGC--GGGATTCGCCTTAAAGTAATTGGCAGCCAGTGTTTTGGTTTTGAAGCG
T3                           --GCTCTAGT--GGC--GGGATTCGCCTTAAAGTAATTGGCAGCCAGTGTTTTGGTTTTGAAGCG
```

FIG. 3. ITS sequences T1 and T3 show strong homology to *Phoma wasabiae* and *Leptosphaeria maculans* and not to *Scutellospora.* A representative detail of an ITS sequence alignment is shown, with bases from the 5.8S rDNA in boldface. Note that ITS cannot be aligned across the two groups.

TABLE 2
Results of the Kishino–Hasegawa Test

Tree	−ln L	Diff −ln L	sd (diff)	*T*	*P*[a]
Unconstrained	2059.40455 (best)				
ITSC1 into Gigasporaceae	2087.49583	28.09128	13.78448	2.0379	0.0431**
T1/T3 into Gigasporaceae	2108.42709	49.02254	13.86107	3.5367	0.0005**
T5/T6 into Gigasporaceae	2090.55836	31.15381	16.91400	1.8419	0.0672

Note. Constraint trees in which taxa were forced into the Gigasporaceae branch were compared to the unconstrained tree. L, likelihood; sd, standard deviation.

[a] Probability of getting a more extreme *T* value under the null hypothesis of no difference between the two trees (two-tailed test).

** Significant at $P < 0.05$.

castanea spores. (3) These sequences really form part of the glomalean genome. AM fungi can be cultivated only together with a plant host, usually in open pot cultures. Therefore, the presence of contaminating microorganisms is not unexpected and contamination remains the most parsimonious explanation for our findings. The other two possibilities are considerably less likely at present and warrant further investigation. The fact that different copies of rDNA occur within single spores of AMF is undisputed. This variation begs the question of whether those variant rDNA sequences are found within single nuclei or among the different nuclei in single spores of the Glomales, but as yet this remains an exciting, but open question.

As completely sterile AM spores can hardly ever be obtained (Walley and Germida, 1996), there is always a high probability of amplifying non-glomalean DNA by PCR with universal primers as our own experience has shown. In this situation we suggest that, where possible, sequences obtained from glomalean spores by universal or nonspecific primers should be subjected to phylogenetic analyses to assess their origin. However, many regions of the genome other than rDNA will not allow such analysis. A good example of this was given by Clapp *et al.* (1999), who did not assign short sequences from the 18S subunit from spores and colonized roots to the Glomales unless substantiated by phylogenetic analyses. The 5.8S dataset we present should be a helpful tool to check ITS sequences from AM fungi. The alignment will be available in the World Wide Web (http://plantbio.berkeley.edu/~bruns/ftp/glomales58S.nex).

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