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Evolution

Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae

(*Gigaspora*/Glomales/phylogeny/*Scutellospora*)

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ABSTRACT **Arbuscular mycorrhizal (AM) fungi (Order Glomales, Class Zygomycetes) are a diverse group of soil fungi that form mutualistic associations with the roots of most species of higher plants. Despite intensive study over the past 25 years, the phylogenetic relationships among AM fungi, and thus many details of evolution of the symbiosis, remain unclear. Cladistic analysis was performed on fatty acid methyl ester (FAME) profiles of 15 species in *Gigaspora* and *Scutellospora* (family Gigasporaceae) by using a restricted maximum likelihood approach of continuous character data. Results were compared to a parsimony analysis of spore morphological characters of the same species. Only one tree was generated from each character set. Morphological and developmental data suggest that species with the simplest spore types are ancestral whereas those with complicated inner wall structures are derived. Spores of those species having a complex wall structure pass through stages of development identical to the mature stages of simpler spores, suggesting a pattern of classical Haeckelian recapitulation in evolution of spore characters. Analysis of FAME profiles supported this hypothesis when *Glomus leptotichum* was used as the outgroup. However, when *Glomus etunicatum* was chosen as the outgroup, the polarity of the entire tree was reversed. Our results suggest that FAME profiles contain useful information and provide independent criteria for generating phylogenetic hypotheses in AM fungi. The maximum likelihood approach to analyzing FAME profiles also may prove useful for many other groups of organisms in which profiles are empirically shown to be stable and heritable.**

Arbuscular mycorrhizal (AM) fungi (Order Glomales, Class Zygomycetes) are ubiquitous, asexually reproducing soil fungi that colonize the roots of most species of vascular plants. These obligately symbiotic fungi obtain inorganic nutrients, most notably phosphorus, from the soil, which are translocated to the plant in return for organic carbon. These fungi contribute to the nutrient status of plants in both native and agronomic ecosystems and often improve plant survival and productivity (1). An intriguing aspect of the symbiosis is its ancient origin. Fossil evidence indicates that AM fungi were colonizing terrestrial plants in the early Devonian (≈400 million years ago) (2). Molecular evidence based on DNA sequence data confirms the fossil record (3). Thus, the symbiotic partners have had several hundred million years to coevolve.

Currently, about 150 species of AM fungi have been formally described based on morphological characters of spores (4). However, characters at other levels of organization (e.g., biochemical, molecular) can also be important determinants of fungal diversity (5). The extent to which patterns resulting from these processes are coupled (or not) with morphology must be understood to establish species concepts that encompass these levels of diversity. Empirical evidence is needed at all taxonomic levels to more thoroughly circumscribe taxon boundaries and reconstruct phylogenetic relationships.

Fatty acid methyl ester (FAME) profiles have been used as biochemical characters to study many different groups of organisms, such as bacteria (6) and yeasts (7). While fatty acids of many filamentous fungi have been characterized (8, 9), few studies have addressed taxonomic issues. Those addressing taxonomy (10, 11) have done so from a phyletic (or intuitive) approach as opposed to using more rigorous cladistic methods. In the case of AM fungi, initial suggestions as to the taxonomic relevance of FAME profiles have been made without empirical evidence of the true value of these characters (12, 13). Recent work has shown that FAME profiles are sufficiently stable and heritable in AM fungi to be taxonomically informative in comparative studies (14) and that the rank of resolution of these characters is likely to range from the species to the family level (14, 15). The objectives of the present work were to determine the composition of FAME profiles from spores of AM fungi in the genera *Gigaspora* and *Scutellospora* (family Gigasporaceae) and to compare the profiles using cladistic (phylogenetic) approaches. These analyses then were compared to cladistic analyses based on morphological characters.

MATERIALS AND METHODS

All fungi used in these studies were from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, West Virginia University). Multiple isolates of each species were chosen to determine intraspecific variability in FAME profiles. Isolates of the following species in *Gigaspora* and *Scutellospora* were analyzed (INVAM numbers in parentheses): *Gigaspora albida* Schenck & Smith (BR201, BR205, BR214, FL927); *Gigaspora decipiens* Hall & Abbott (AU102, AU104); *Gigaspora gigantea* (Nicolson & Gerdemann) Gerdemann & Trappe (HA150C, HA953, MA453, MN922, NC110A, NC111B, NC120A, VA103C, VA105C, VA106A); *Gigaspora margarita* Becker & Hall (BR444, WV205A); *Gigaspora rosea* Nicolson & Schenck (BR151A, BR155, FL105, FL219A, FL676, KS885, NC114B, NC121A, NY328A, UT102); *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders (AU212, AU222, CL370, NC111A, NC114A, NY348); *Scutellospora dipurpurascens* Morton & Koske (MX921, MX923, WV930, WV989); *Scutellospora erythropa* (Koske & Walker) Walker & Sanders (HA150B, MA438B, MA453); *Scutellospora fulgida* Koske & Walker (VA101B, VA105A); *Scutellospora heterogama* (Nicolson & Gerdemann) Walker & Sanders (BR154C, FL312B, FL654, IL203, NC141, SN722, VZ103, WV108, WV858); *Scutellospora pellucida* (Nicolson & Schenck) Walker & Sanders (BR208, FL966, WV104, WV205B, WV872, WV873, WV935); *Scutellospora persica* (Koske & Walker) Walker & Sanders (HA101, MA461A, VA102C); *Scutellospora reticulata*

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Abbreviations: AM, arbuscular mycorrhizal; FAME, fatty acid methyl ester.

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(Koske, Miller & Walker) Walker & Sanders (BR100; CL756); *S.* species (an undescribed *Scutellospora* similar to *S. heterogama*, except lacking ornamentation) (BR101, BR211); *Scutellospora verrucosa* (Koske & Walker) Walker & Sanders (HA151A, VA103A, VA105B).

All isolates were grown in pot-cultures with *Sorghum sudanense* (Piper) Stapf as the host. The growth medium consisted of a steam-pasteurized soil (Lily series) mixed 1:2 (vol/vol) with quartzite sand (mean particle size, 0.9 mm). Further details on culturing conditions are given in ref. 16.

Spores of the fungi were extracted from pot cultures by wet-sieving and decanting followed by sucrose gradient centrifugation (17). Spores then were pipetted individually from remaining debris and then rinsed with copious amounts of tap water and with at least three final rinses of distilled water. All spores were examined again to ensure a homogenous population of one morphotype. Approximately 10–20 mg (dry weight) of mature spores (as determined by microscopic examination) of each isolate were placed in a sterilized test tube and dried at 80°C overnight.

Cellular fatty acids (both free and bound) in each sample were saponified, methylated, and extracted according to previously described methods (14). Fatty acid methyl esters were separated via gas-liquid chromatography and the molecules were named by using the Microbial Identification System (version 3.70) from the MIDI Corporation (Newark, DE), also as described in ref. 14. Only FAMEs that comprised at least 0.25% of the total profile in at least three different fungal isolates were used in the cladistic analysis.

Cladistic analysis of FAME profiles was performed with the program CONTML from PHYLIP version 3.5c (18) by choosing the following options: continuous character data, global rearrangements, and 100 jumbles (randomization) of the input order. This program estimates phylogenies from continuous character data by the restricted maximum likelihood approach (19, 20). *Glomus leptotichum* Schenck & Smith (isolates NC171A, NC171B, WV109, WV954) was selected as the outgroup because it is hypothesized to be an ancestral species in the genus *Glomus* and thus may be closely derived from an ancestor at the Glomaceae–Gigasporaceae split (21). The cladistic analysis was first performed on only one isolate of each species. Inclusion of alternate isolates of the species did not substantially change tree topology, so data for all isolates within a species were combined and the means for each species was used in the final analysis. To determine the effect of the outgroup taxon, the analysis was again repeated with *Glomus etunicatum* Becker & Gerdemann (putatively a derived *Glomus* species; ref. 21) as the outgroup.

Cladistic analysis of the morphological characters (Table 1) was performed by using the PENNY algorithm of PHYLIP version 3.5c, with the Wagner parsimony option (no assumption of ancestral states). PENNY uses a branch and bound approach to find the most parsimonous tree(s). *Gl. leptotichum* again was chosen as the outgroup.

RESULTS

Eighteen FAMEs comprised at least 0.25% of the total profile in at least three fungal isolates (Table 2). Among these molecules, some appeared to be relatively uninformative (such as 14:0), whereas others appeared to provide a high degree of taxonomic information (such as 18:1 ω9c and Feature 7). Maximum likelihood analysis resulted in only one tree (Fig. 1). With *Gl. leptotichum* as the outgroup, *Gigaspora* spp. were found to be the most ancestral. *S. persica*, *S. verrucosa*, and *S. fulgida* were intermediate, and the remaining *Scutellospora* spp. were the most derived in Gigasporaceae. One species, *S. erythropa*, was separated from the others by a substantial distance (24.2 relative distance units). When the same FAME data were analyzed with *Gl. etunicatum* as the outgroup, polarity of the entire tree was reversed with *S. erythropa* being the most ancestral, and *Gigaspora* spp. the most derived group (not shown).

Parsimony analysis of the morphological characters was confounded by the inability of the limited number of chosen characters to differentiate between several species with similar spore morphologies. When species with identical character sets were eliminated, the analysis produced only one most parsimonious tree. Manual addition of the eliminated species resulted in a tree with several unresolved terminal clades (Fig. 2). This cladogram indicates that *Gigaspora* spp. (which have no flexible inner walls) are most ancestral and that successively more derived clades were formed by *Scutellospora* spp. with one, two, or three flexible inner walls, respectively. The characteristic of the innermost flexible wall layer being "amorphous" (22) appears to be a derived character as well. Changing the outgroup from *Gl. leptotichum* to *Gl. etunicatum* did not alter the topology of the resulting cladogram, as it did using FAME characters.

DISCUSSION

To our knowledge, this is the first report of FAME profiles being used in a cladistic analysis for any organism. Traditional

Table 1. Morphological characters and character states used in the parsimony analysis

Species	Arbuscules	Intraradical vesicles	Extraradical auxiliary cells	Bulbous sporogenous cell	One flexible inner wall	Two flexible inner walls	Three flexible inner walls	Innermost wall layer "amorphous"
Gl. leptotichum	1	1	0	0	0	0	0	0
G. albida	1	0	1	1	0	0	0	0
G. decipiens	1	0	1	1	0	0	0	0
G. gigantea	1	0	1	1	0	0	0	0
G. margarita	1	0	1	1	0	0	0	0
G. rosea	1	0	1	1	0	0	0	0
S. calospora	1	0	1	1	1	1	0	1
S. dipurpurascens	1	0	1	1	1	1	0	1
S. erythropa	1	0	1	1	1	1	1	1
S. fulgida	1	0	1	1	1	0	0	0
S. heterogama	1	0	1	1	1	1	0	0
S. pellucida	1	0	1	1	1	1	0	1
S. persica	1	0	1	1	1	0	0	0
S. reticulata	1	0	1	1	1	0	0	0
Scutellospora sp.	1	0	1	1	1	1	0	0
S. verrucosa	1	0	1	1	1	0	0	0

Character states are as follows: 0 = absent; 1 = present.

types of cladistic analyses that are based on discrete characters (e.g., parsimony analysis) could not be used because FAME profile data are continuous quantitative characters. The restricted maximum likelihood method proved to be sufficiently robust to adequately resolve species within the family Gigasporaceae. FAME analysis may prove useful for other organisms that have limited or equivocal morphological data sets, and may provide character sets in addition to DNA sequence data.

Comparison of Figs. 1 and 2 revealed strong congruence between the networks derived from the FAME data and the morphological data, when *Gl. leptotichum* was set as the outgroup. One notable exception to this is the position of *S. reticulata*, which is morphologically similar to *S. persica*. Even though these characters (both morphological and FAME profiles) occur at different hierarchical levels of organization (5), evolutionary pressures may have channeled these different character types similarly. This similarity would not be unexpected if both FAME and morphological characters evolved from drift alone. The fact that the morphological and FAME characters examined occur in the same portion of the fungal thallus (the spores) may also account for some similarities between the FAME and morphological analyses. Nonetheless, this congruence is quite remarkable since DNA sequence data suggest that members of the family Gigasporaceae arose ≈250 million years ago (3), and so these characters have had substantial time to diverge.

The morphological data suggest that terminal addition of separate flexible inner walls represents a derived state. During spore development, these walls are laid down sequentially (23–25). When spore development in the entire family is considered, the later stages of development in those species with either no (*Gigaspora* spp.) or only one inner wall (e.g., *S. fulgida*) are present as early developmental stages in those fungi with more complicated wall structures (e.g., *S. erythropa*). Thus, it appears that wall structure and development in spores of Gigasporaceae represent an example of the theory that ontogeny recapitulates phylogeny, first popularized by Ernst Haeckel (see ref. 26). The strong congruence of the trees derived from FAME and morphological data rooted with *Gl. leptotichum* lend support to the above hypothesis of Haeckelian recapitulation of spore morphological characters, since it is unlikely that the noted congruence is a coincidence.

An unexpected observation in this study was a reversal in polarity of the tree when *Gl. etunicatum* was used as the outgroup (not shown). We hypothesize this is due to convergent evolution of FAME profiles of *Gl. etunicatum* and several morphologically complex *Scutellospora* spp., suggesting that in addition to drift, selection pressures have influenced evolution of these characters. Evidence supporting this hypothesis includes the morphological cladogram that did not vary when *Gl. etunicatum* was used as the outgroup. It is difficult to imagine these complex *Scutellospora* spp. with two and three inner walls as being directly descended from a simple-walled *Glomus*, and then losing these walls during the evolution of *Gigaspora*. Nonetheless, this observation underscores the importance of choosing an appropriate outgroup when performing cladistic analysis on FAME profiles. Further research is needed to conclusively determine which taxon is most appropriate as an outgroup for the Gigasporaceae.

Maximum likelihood analysis is based on the assumption that characters evolve independently from one another. This assumption is undoubtedly violated in the case of FAME profiles. First, the type of data (percentage of total profile) necessitates that as one fatty acid increases, at least one other must decrease its contribution to the total profile. Secondly, fatty acid biosynthesis involves the building of longer chain molecules from smaller ones. Having many (eighteen) molecules in the analysis minimized the covariance of individual molecules. FAME molecules still provided a satisfactory test

Table 2. Fatty acid profiles (expressed as percentage of the total profile) of spores of the fungi used in the maximum likelihood analysis

Fungus*	14:0	16:1ω7*c*	16:1ω5*c*	16:0	18:3ω6*c*	Feature 6†	18:1ω9*c*	Feature 7‡	18:1ω5*c*	18:0	Unknown 18.593§	20:4ω6, 9,12,15*c*	Unknown 19.470§	Unknown 19.480§	18:0 3-OH	Unknown 19.709§	20:0*iso*	20:1ω9*c*
Gl. etunicatum (6, 24)	0.00	0.00	37.06	31.06	0.80	0.71	1.73	8.44	0.95	0.00	1.17	0.00	0.00	0.00	0.69	0.62	14.87	0.00
Gl. leptotichum (4, 4)	0.00	4.93	0.00	37.25	0.06	1.55	46.04	3.76	0.00	1.18	1.77	0.05	0.17	0.00	0.33	0.86	0.00	1.24
G. albida (4, 9)	0.00	0.11	1.49	30.31	0.35	0.36	42.07	6.08	0.27	0.60	0.39	0.97	1.06	0.64	0.90	0.55	0.02	13.34
G. decipiens (2, 2)	0.00	0.26	5.70	30.60	0.66	0.92	40.82	6.11	1.12	1.36	0.87	2.45	1.10	0.00	0.00	0.00	0.00	7.15
G. gigantea (11, 20)	0.01	0.34	1.12	26.72	0.82	0.89	48.30	3.58	0.25	0.14	0.47	1.72	1.48	0.78	0.36	0.15	0.00	12.75
G. margarita (2, 4)	0.00	0.38	5.72	33.03	0.47	0.67	40.13	6.05	0.89	0.61	0.31	1.45	1.40	0.00	0.00	0.21	0.00	8.63
G. rosea (10, 35)	0.00	0.13	2.49	31.33	0.41	0.78	45.64	3.84	0.09	0.41	0.74	0.97	0.65	0.62	0.14	0.56	0.00	11.01
S. calospora (6, 7)	0.00	0.05	49.32	29.92	0.42	0.61	4.09	13.90	0.03	0.15	0.00	0.95	0.19	0.00	0.00	0.00	0.00	0.13
S. dipurpurascens (4, 5)	0.00	0.19	53.63	23.86	0.69	0.72	3.01	15.48	0.35	0.08	0.00	1.33	0.09	0.00	0.20	0.00	0.00	0.15
S. erythropa (3, 3)	0.19	0.08	72.13	12.61	0.25	0.65	1.39	10.30	0.57	0.09	0.27	0.55	0.41	0.00	0.00	0.20	0.00	0.10
S. fulgida (2, 3)	0.05	0.16	23.17	29.14	0.97	0.99	7.07	34.24	0.83	0.15	0.52	0.99	0.39	0.00	0.50	0.39	0.08	0.33
S. heterogama (9, 21)	0.04	0.72	47.34	25.50	0.38	0.84	7.94	13.28	1.24	0.09	0.26	0.18	0.29	0.59	0.09	0.09	0.20	1.14
S. pellucida (7, 11)	0.00	0.02	55.22	20.93	0.56	0.86	2.16	19.19	0.46	0.00	0.00	0.64	0.07	0.00	0.15	0.00	0.00	0.00
S. persica (3, 5)	0.00	0.62	19.37	15.09	1.34	1.49	36.46	19.66	0.17	0.05	0.00	1.00	0.83	0.00	0.50	0.00	0.13	0.82
S. reticulata (2, 2)	0.22	0.07	47.88	39.59	0.93	0.46	1.65	6.78	0.51	0.77	0.00	1.56	0.00	0.00	0.00	0.00	0.00	0.08
S. species (2, 3)	0.00	0.95	40.40	41.76	0.00	0.43	3.85	9.55	0.91	1.29	0.00	0.18	0.68	0.00	0.00	0.00	0.00	0.00
S. verrucosa (3, 4)	0.00	0.56	18.50	22.16	1.42	1.83	35.02	17.09	0.52	0.00	0.00	1.23	0.34	0.00	0.00	0.00	0.44	0.85

*Numbers in parentheses represent the number of isolates analyzed, followed by the total number of extractions (number of extractions per isolate varied).
†Feature 6 is an unresolved mixture of 18:2 ω6, 9*c*/18:0 *anteiso*.
‡Feature 7 is an unresolved mixture of 18:1 ω7*c*/ω9*t*/ω12*t*.
§Unknown fatty acids are described by their equivalent carbon length.

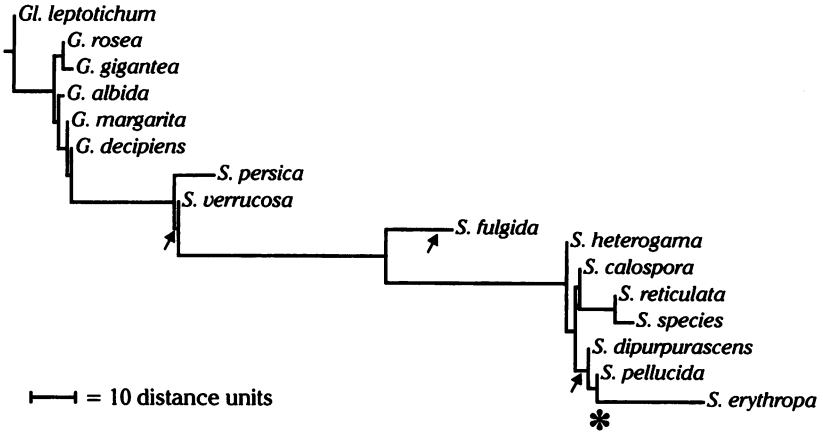

FIG. 1. Single tree resulting from restricted maximum likelihood analysis of FAME profiles with *Gl. leptotichum* set as the outgroup. Distance units are the expected accumulated variance (not time). The existence of each horizontal branch (except those marked with an arrow) is supported at $P = 0.05$ (based on 95% confidence intervals of branch lengths). Asterisk indicates the node where *Gl. etunicatum* roots the tree.

of phylogenetic relationships, despite violating the assumption of independent evolution.

In summary, FAME profiles provide unique characters that, when analyzed with maximum likelihood algorithms, can be useful in cladistic analysis. Data provided here support cladistic hypotheses generated from morphological data. The rank of resolution of FAME profiles for AM fungi appears to range between the species and family levels, since convergent evolution of FAME profiles appears to have taken place between members of different families. The usefulness of FAME profiles in phylogenetic reconstruction of other types of organisms will depend on the stability and heritability of profiles in those organisms. In addition, the rank of resolution of these characters will need to be determined empirically for each group of study organisms.

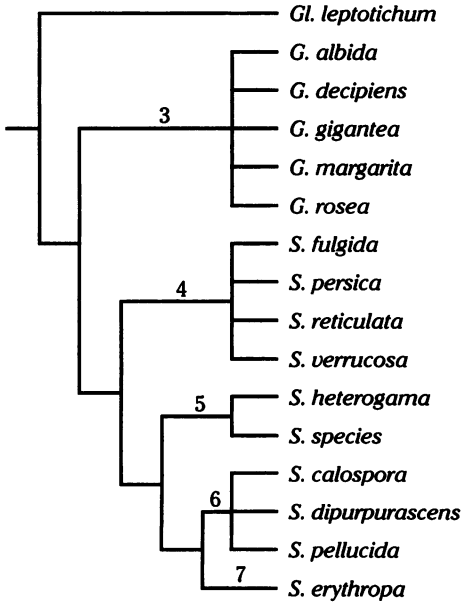

FIG. 2. Most parsimonious tree (length = 7) resulting from Wagner parsimony analysis of the morphological data. Note that several terminal clades are unresolved. Species with identical morphological data sets were eliminated from the actual analysis and then added back manually. Numerals indicate the number of steps from the root.

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