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Phylogenetic systematics of the *Gigasporales*

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Abstract — The classification, phylogeny, and evolutionary pathways of the *Gigasporales* are re-evaluated based on concomitant morphological and molecular phylogenetic analyses. Only *Cetraspora* was not supported in the morphology-based tree, while *Quatunica* formed a monophyletic group with its sister genus *Dentiscutata*. Only a few taxa were not completely supported in the SSU rDNA phylogenetic analyses, namely *Dentiscutata* and *Fuscutata* (*Dentiscutataceae*) and *Racocetra* and *Cetraspora* (*Racocetraceae*). However, all trees generated by the LSU, SSU (rDNA), and β-tubulin genes supported the existence of the families with strong support for all genera represented in the LSU rDNA and β-tubulin analyses. In conclusion, the current classification of the *Gigasporales* has a strong morphological and molecular congruency.

Key words — *Glomeromycetes*, gigasporoid, scutellosporoid

Introduction

The arbuscular mycorrhizal (AM) fungi form a symbiotic association with plants, being the most important mutualism between plants and fungi in the nature. These fungi are grouped in the phylum *Glomeromycota*, in which the order *Gigasporales* (Oehl et al. 2011a) with 54 currently described species (Table 1) forms a clade of outstanding interest and particularity for taxonomic, phylogenetic, and ecological reasons. Fungi in this order were grouped according to their spore formation mode on sporogenous cells and phylogenetic analyses of the rDNA and β-tubulin genes (Oehl et al. 2011a). Besides, members of *Gigasporales* do not form vesicles in the colonized roots but rather form auxiliary cells in mycorrhizospheric soils. Recently, Dotzler et al. (2006) reported ~400 million year-old fossil scutellosporoid spores with very structures similar to those in *Gigasporales* (bulbous bases, germinal walls, and

Table 1. Species in *Gigasporales**

Dentiscutataceae F.A. Souza et al.
- *Dentiscutata biornata* (Spain et al.) Sieverd. et al.
- *D. cerradensis* (Spain & J. Miranda) Sieverd. et al.
- *D. colliculosa* B.T. Goto & Oehl
- *D. hawaiiensis* (Koske & Gemma) Sieverd. et al.
- *D. heterogama* (T.H. Nicolson & Gerd.) Sieverd. et al.
- *D. nigerita* Khade
- *D. nigra* (J.F. Redhead) Sieverd. et al.
- *D. reticulata* (Koske et al.) Sieverd. et al.
- *D. scutata* (C. Walker & Dieder.) Sieverd. et al.
- *Fuscutata aurea* Oehl et al.
- *F. heterogama* Oehl et al.
- *F. rubra* (Stürmer & J.B. Morton) Oehl et al.
- *F. savannicola* (R.A. Herrera & Ferrer) Oehl et al.
- *F. trirubiginopa* (X.L. Pan & G. Yun Zhang) Oehl et al.
- *Quatunica erythropus* (Koske & C. Walker) F.A. Souza et al.

Gigasporaceae J.B. Morton & Benny
- *Gigaspora albida* N.C. Schenck & G.S. Sm.
- *G. alboaurantiaca* W.N. Chou
- *G. candida* Bhattacharjee et al.
- *G. decipiens* I.R. Hall & L.K Abbott
- *G. gigantea* (T.H. Nicolson & Gerd.) Gerd. & Trappe
- *G. margarita* W.N. Becker & I.R. Hall
- *G. ramisporophora* Spain et al.
- *G. rosea* T.H. Nicolson & N.C. Schenck

Intraornatosporaceae B.T. Goto & Oehl
- *Intraornatospora intraornata* (B.T. Goto & Oehl) B.T. Goto et al.
- *Paradentiscutata bahiana* Oehl et al.
- *P. maritima* B.T. Goto et al.

Racocetraceae Oehl et al.
- *Cetraspora armeniaca* (Błaszk.) Oehl et al.
- *C. gilmorei* (Trappe & Gerd.) Oehl et al.
- *C. helvetica* Oehl et al.
- *C. nodosa* (Błaszk.) Oehl et al.
- *C. pellucida* (T.H. Nicolson & N.C. Schenck) Oehl et al.
- *C. striata* (Cuenca & R.A. Herrera) Oehl et al.
- *Racocetra alborosea* (Ferrer & R.A. Herrera) Oehl et al.
- *R. beninensis* Oehl et al.
- *R. castanea* (C.Walker) Oehl et al.
- *R. coralloidea* (Trappe et al.) Oehl et al.
- *R. fulgida* (Koske & C. Walker) Oehl et al.
- *R. gregaria* (N.C. Schenck & T.H. Nicolson) Oehl et al.
- *R. minuta* (Ferrer & R.A. Herrera) Oehl et al.
- *R. persica* (Koske & C. Walker) Oehl et al.
- *R. tropicana* Oehl et al.
- *R. undulata* T.C. Lin & C.H. Yen
- *R. verrucosa* (Koske & C. Walker) Oehl et al.
- *R. weresubiae* (Koske & C. Walker) Oehl et al.

Scutellosporaceae Sieverd. et al.
- *Orbispora pernambucana* (Oehl et al.) Oehl et al.
- *O. projecturata* (Kramad. & C. Walker) Oehl et al.
- *Scutellospora arenicola* Koske & Halvorson
- *S. aurigloba* (I.R. Hall) C. Walker & F.E. Sanders
- *S. calospora* (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders
- *S. crenulata* R.A. Herrera et al.
- *S. dipapillosa* (C. Walker & Koske) C. Walker & F.E. Sanders
- *S. dipurpurescens* J.B. Morton & Koske
- *S. spinosissima* C. Walker & Cuenca
- *S. tricalypta* (R.A. Herrera & Ferrer) C. Walker & F.E. Sanders

*The nomenclature and its authorities are based on Goto et al. (2009, 2010, 2011, 2012), Khade (2010), Lin & Yen (2011), Mello et al. (2012), Oehl et al. (2008, 2010, 2011b,d), Silva et al. (2008), and Tchabi et al. (2009).

Placement of *S. spinossisima* is tentative because its germ shield morphology and phylogeny are not yet sufficiently resolved and need re-analysis.

germination shields). This discovery indicates diversification of *Gigasporales* earlier than suggested by other researchers (Phipps & Taylor 1996, Redecker et al. 2000).

Until 2008 little attention was given to *Gigasporales* classification, although some problems regarding the monophyly and position of *Gigaspora* and *Scutellospora* had been discussed earlier (Kramadibrata et al. 2000, Souza et al. 2005, Silva et al. 2006). Studying the morphological phylogeny of AM fungi, Morton (1990) reported *Gigaspora* as basal to the former *Scutellospora*, and his results were supported by ontogenetic studies (Bentivenga & Morton 1995, Morton 1995, Franke & Morton 1994) and fatty acid profiles (Bentivenga &

Morton 1996). On the other hand, molecular phylogenetic analyses increasingly demonstrated that *Gigaspora* might be monophyletic with *Scutellospora* basal and polyphyletic (Simon et al. 1993, Souza et al. 2005, Redecker & Raab 2006).

Based on concomitant morphological and molecular phylogenetic analyses, Oehl et al. (2008) confirmed the former *Scutellospora* as polyphyletic and proposed four families (*Dentiscutataceae*, *Gigasporaceae*, *Racocetraceae, Scutellosporaceae*) and seven genera (*Cetraspora*, *Dentiscutata*, *Fuscutata*, *Gigaspora*, *Quatunica*, *Racocetra, Scutellospora*) within the sporogenous cell forming AM fungi, i.e. the *Gigasporales*. Countering this revision of *Scutellospora*, Morton & Msiska (2010) reported that such segregation destabilized the taxonomy of the group, proposing that beside the former *Gigaspora* and *Scutellospora*, only one genus (*Racocetra*) described by Oehl et al. (2008) was valid. However, Krüger et al. (2012) provided support for some more genera described by these authors, and a several recent studies with a broader database (e.g. Goto et al. 2010, 2011, 2012, Oehl et al. 2010, 2011b) sustain the revision by Oehl et al. (2008).

Currently, *Gigasporales* comprises five families (*Dentiscutataceae*, *Gigasporaceae*, *Intraornatosporaceae*, *Racocetraceae, Scutellosporaceae*) and ten genera (*Cetraspora*, *Dentiscutata*, *Fuscutata*, *Gigaspora*, *Intraornatospora*, *Orbispora*, *Paradentiscutata*, *Quatunica*, *Racocetra, Scutellospora*) (Oehl et al. 2008, 2011b, Goto et al. 2012). We wish here to evaluate the classification, phylogeny, and evolutionary pathways in *Gigasporales*.

Materials & methods

Morphological phylogenetic analyses

As in Morton & Msiska (2010), we selected just 27 species from *Gigasporales* and 23 characters to construct a matrix for the morphological phylogenetic analysis. In general, characters and the plesiomorphic and apomorphic character states were defined and coded as in Morton & Msiska (2010). We did not include the *Intraornatosporaceae* or *Intraornatospora*, *Orbispora,* and *Paradentiscutata* in our morphological analysis, because these taxa were described after the Morton & Msiska (2010) study.

The phylogenetic analysis and tree construction were performed using Phylogenetic Analysis Using Parsimony (PAUP 4.0b10) (Swofford 2003). The matrix-generated data were calculated by maximum parsimony (MP). *Acaulospora mellea* and *A. laevis* served as outgroups.

Evolutionary phylogenetic terms used in this study (from Wiley et al. 1991) include (1) homoplastic = a character shared by two taxa that does not meet the criteria of homology (i.e., the character does not derive from a common ancestor), (2) plesiomorphic = ancestral character state and apomorphic = derived character state, (3) synapomorphy = a derived character state shared by a group of species with evidence of a common ancestor, (4) symplesiomorphy = an ancestral character state shared by a group of species but useless for phylogenetic analyses since clearly representing common ancestry for all the fungi being analyzed.

Molecular phylogenetic analyses

Partial sequences of rRNA (SSU and LSU) and β-tubulin genes were analyzed independently to reconstruct the phylogeny of the *Gigasporales*. Only β-tubulin exon regions were analyzed, with introns excluded.

Sequences (all obtained from the National Center for Biotechnology Information–NCBI) were aligned with ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999) to obtain a final alignment.

Prior to phylogenetic analysis, the nucleotide substitution model was estimated using Topali 2.5 (Milne et al. 2004). Bayesian (two runs over 1×10^6 generations with a burnin value of 2500) and maximum likelihood (1000 bootstrap) analyses were executed, respectively, in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), launched from Topali 2.5. Neighbor-joining (established with the same model used to construct the Bayesian tree) and maximum parsimony analysis were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications. *Pacispora scintillans* sequences were used as outgroup for the SSU and LSU rRNA analyses, and *Acaulospora mellea* was outgroup for β-tubulin analysis.

Results

Molecular phylogenetic analysis

All LSU, SSU (rDNA), and β-tubulin gene trees support the families described in the *Gigasporales* (Figs 1–3). The LSU rDNA sequence-based phylogenies support all ten genera (Fig. 1), and the β-tubulin tree supports all genera with species included in the analysis (Fig. 2). Only the SSU rDNA phylogenetic tree did not fully support a few taxa in the *Dentiscutataceae* and *Racocetraceae* (Fig. 3) with no separation shown between *Dentiscutata* and *Fuscutata* or *Racocetra* and *Cetraspora.*

Morphological phylogenetic analyses

The morphology-based phylogeny supported *Gigasporaceae* and *Dentiscutataceae* (Fig. 4). However, *Scutellosporaceae* and *Racocetraceae* grouped together in a monophyletic clade. Our morphological analyses, which included only the same data and taxa used by Morton & Msiska (2010), did excluded taxa described since 2010: *Orbispora*, *Intraornatospora, Paradentiscutata*, and *Intraornatosporaceae*. Of all genera considered, only *Cetraspora* was not supported. *Fuscutata*, *Gigaspora*, *Racocetra*, and *Scutellospora* were monophyletic, and *Quatunica* formed a monophyletic group with *Dentiscutata* (Fig. 4).

Discussion

All LSU, SSU (rDNA) and β-tubulin phylotrees supported the existence of the gigasporalean families with strong support for all genera shown by the LSU rDNA and β-tubulin analyses. As the LSU region is believed to give the best resolution for *Gigasporales* (Souza et al. 2005, Oehl et al. 2008, 2011b),

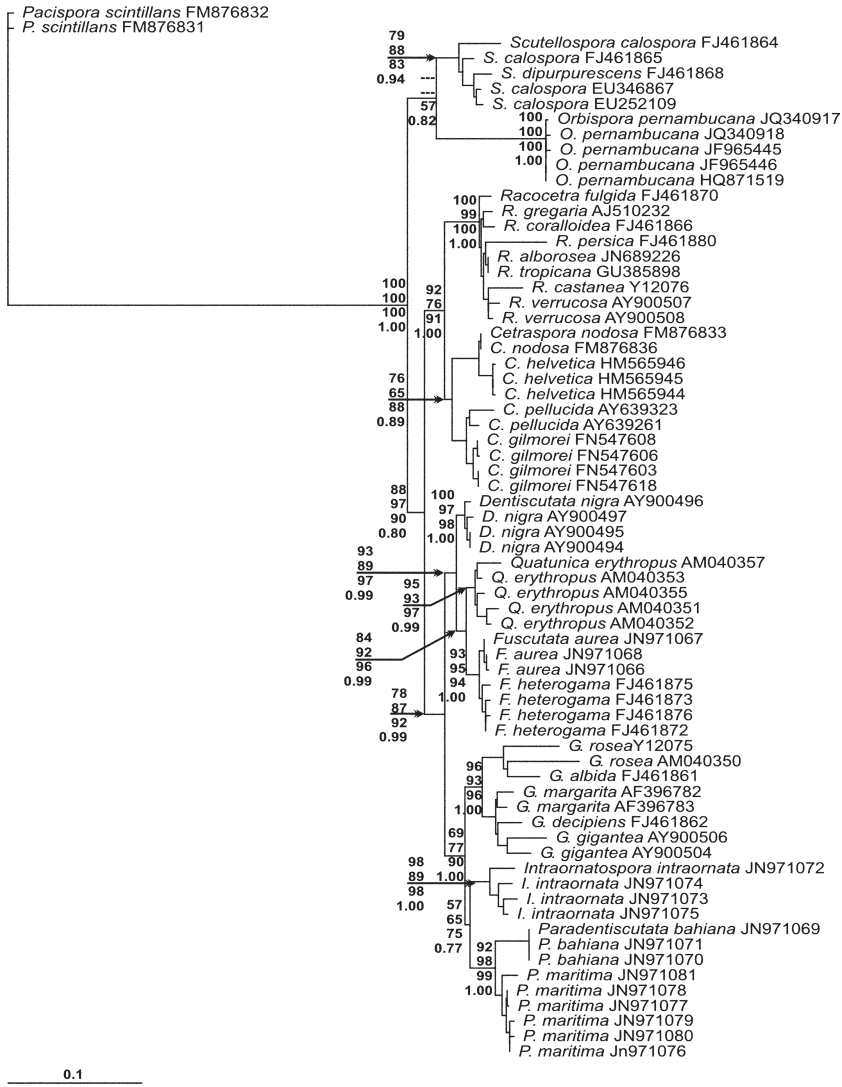

Fig. 1. Phylogenetic reconstruction of the *Gigasporales* obtained from partial LSU rDNA sequences. The NJ, ML, and Bayesian analyses were performed with GTR+G nucleotide substitution model. Sequences are labeled with database accession numbers. Support values are (from up to down) from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. Only topologies with ≥50% bootstrap values are shown. (Consistency Index = 0.55; Retention Index = 0.84).

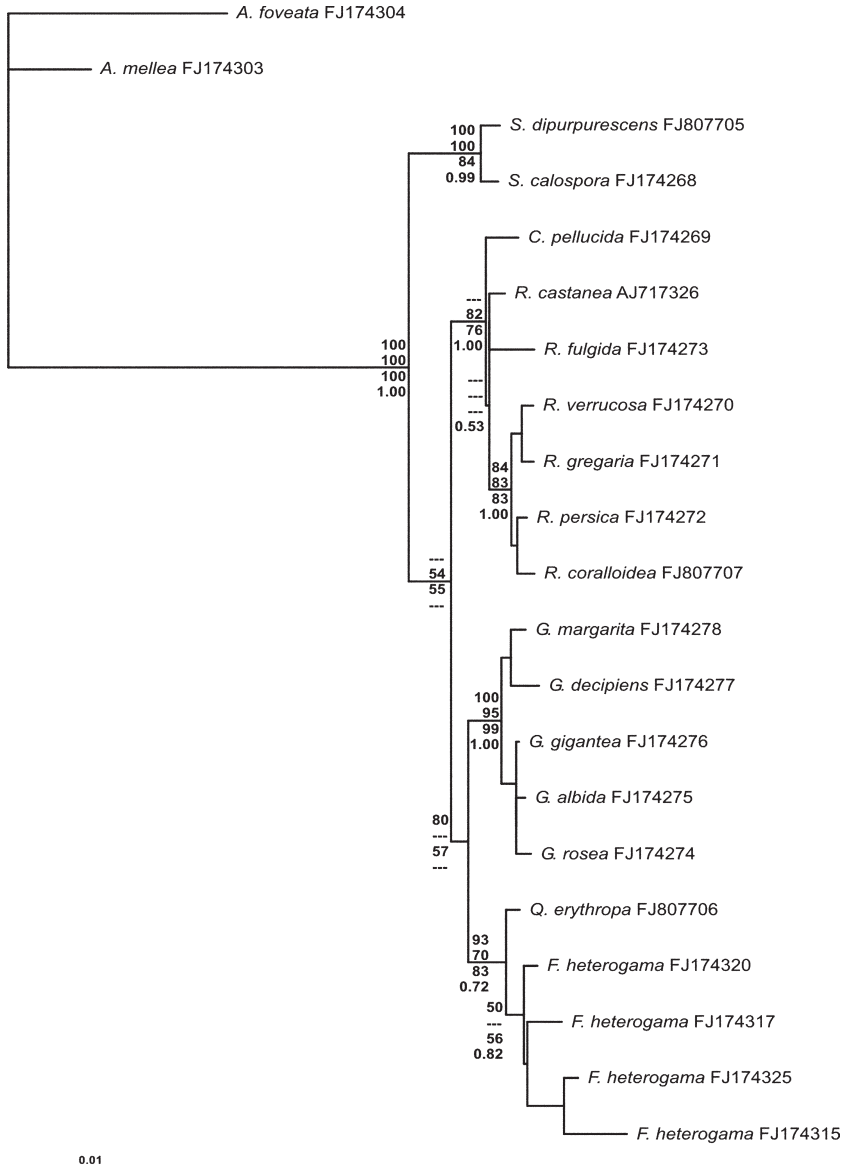

Fig. 2. Phylogenetic reconstruction of the *Gigasporales* obtained from partial β-tubulin sequences. The NJ, ML, and Bayesian analyses were performed with GTR+G+I nucleotide substitution model. Sequences are labeled with database accession numbers. Support values are (from up to down) from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. Only topologies with ≥50% bootstrap values are shown. (Consistency Index = 0.63; Retention Index = 0.68).

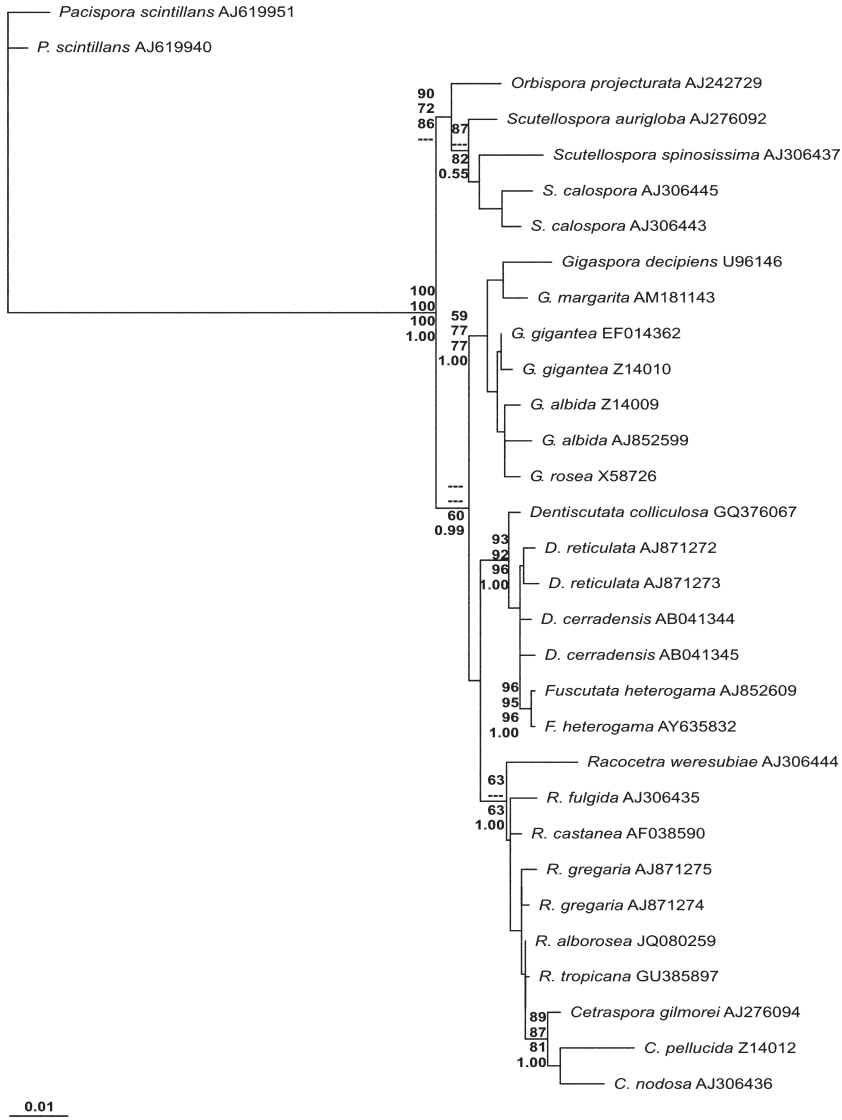

Fig. 3. Phylogenetic reconstruction of the *Gigasporales* obtained from partial SSU rDNA sequences. The NJ, ML, and Bayesian analyses were performed with GTR+G+I nucleotide substitution model. Sequences are labeled with database accession numbers. Support values are (from up to down) from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. Only topologies with ≥50% bootstrap values are shown. (Consistency Index = 0.68; Retention Index = 0.74).

we conclude that the current classification by Oehl et al. (2011c) and Goto et al. (2012) has a strong morphological and molecular congruency and support. The SSU rDNA phylogenetic analysis did not completely support only a few taxa — *Dentiscutata* and *Fuscutata* (*Dentiscutataceae*) and *Racocetra* and *Cetraspora* (*Racocetraceae*), which confirms the analyses of Oehl et al. (2008) using a similar database. In the morphology-based tree, only *Cetraspora* was not supported while *Quatunica* formed a monophyletic group with its sister genus *Dentiscutata*. We argue that this might correspond to the fact that *Cetraspora* lacks any unique morphological character and shares (for example) the number of spore walls with several other genera as well as the germination shield color and structure with related genera. Thus far, *Quatunica* has only one important character (spore wall numbers) that separates it morphologically from *Dentiscutata* species. The fact that *Quatunica* is monospecific gives it a low analytical weight.

Of the 23 morphological characters analyzed by Morton & Msiska (2010), we regard eight (1, 2, 3, 4, 6, 8, 11, 12) as synapomorphies that are identical for all gigasporalean species, thereby leaving just 15 characters to divide clades in the order. Among these, Morton & Msiska (2010) report variation in character 21 (germination shield color) between isolates from the same species, which could prevent using this character in phylogenetic analyses, due to its instability. However, we argue that these authors might have included immature spores with germination shields that were not completely differentiated or very old spores in their observations. They might also have considered similar spores from more than one species that represent different phylogenetic clades (e.g. *Cetraspora pellucida*, *Fuscutata savannicola,* and *Dentiscutata scutata*). We also believe that although species show a typical germination shield pattern, natural exceptions may occur in some individuals, due to several reasons such as a malformation.

Morton & Msiska's (2010) characters 9 (spore size), 10 (spore color), and 13 (outer layer surface of the gigasporoid spore wall) do not pass a homology test, because there is no congruence with other characters among the taxa (de Pinna 1991). Moreover, characters 9 and 10 are quantitative, and the most obvious character filter for cladistic analysis rejects continuous or quantitative attributes. Bentivenga et al. (1997) reported significant variation in the average of spore size and color in a single *Glomus clarum* isolate after generations of selection pressure for some phenotypic characters. Bever & Morton (1999) also observed average variation in *Cetraspora pellucida* spore size and shape in five single-spore cultures from a single isolate population. None of these characters contributed significantly to synapomorphies within the *Gigasporales*, being homoplastic with a consistency index (CI) below 0.4. Character 14 was synapomorphic for the genus *Dentiscutata*, although the CI was also below 0.4.

Fig. 4. Phylogenetic reconstruction of the *Gigasporales* based on 23 morphological characters. (Tree length = 63 steps; Consistency Index = 0.49; Retention Index = 0.77).

Some characters (7 and 19) that serve to separate *Gigaspora* from other gigasporalean taxa do not help solve the polyphyletic former *Scutellospora* groups. Thus, of 23 characters used by Morton & Msiska (2010), just ten are phylogenetically helpful in separating monophyletic groups. Most of these characters are related to germination shield, hyphal color, and germinal wall characteristics.

Despite the Morton & Msiska (2010) criticism of the LSU–SSU interdependence, they used two interdependent morphological characters

(16—number of germinal walls and 20—germination shield position) to group *Racocetra* taxa. In addition, this genus is grouped by the more ancestral character state (as coded by the authors) that clearly represents a symplesiomorphy, which does not provide evidence of common ancestry (Wiley et al. 1991). We conclude that Morton & Msiska (2010) erred when coding these character states.

Evolutionary line in the *Gigasporales*

Ontogenetic data (Franke & Morton 1994; Morton 1995; Bentivenga & Morton 1995, 1996) led readers to believe that *Gigaspora* might be basal to the former *Scutellospora*. However, several molecular studies (Oehl et al. 2008, 2010, 2011b, Goto et al. 2010; 2012) demonstrated that the former *Scutellospora* evolved first in the *Gigasporales*. The report of 400 million year-old fossil specimens with germination shields by Dotzler et al. (2006) suggested that the presence of germination shield is ancestral in *Gigasporales*. The low number of gigasporoid species related to scutellosporoid (sensu lato) species also suggests that *Gigaspora* diverged after other members. Finally, we understand the capacity of repeated germination of *Gigaspora* species (Maia & Kimbrough 1993) from ≤1000 germ warts randomly distributed on the spore wall inner surface as a evolutionary progression compared with single germination events from 1–24 germ tube initiations found on the germination shield periphery of scutellosporoid (sensu lato) species.

The molecular phylogenetic trees suggest that spore wall loss has occurred three times during the evolutionary history of *Gigasporales* [*Scutellospora*, *Cetraspora* (3) → *Racocetra*, *Intraornatospora* (2) → *Gigaspora* (1)], while at least once an additional wall was acquired [*Dentiscutata* (3) → *Quatunica* (4)]. However, even the germinal wall was lost in *Gigaspora* leaving only the innermost warty germination layer, and germination in this genus is related to this specific layer. *Intraornatospora* with one germinal wall with a rudimentary appearing germination shield (Goto et al. 2012) is close phylogenetically to *Gigasporaceae*. This genus forms a particular ornamentation on the inner surface of the outer spore wall (Goto et al. 2009) in the form of tubercular projections resembling the germ warts in *Gigaspora*. Since *Intraornatospora* germination has not yet been observed, it is not possible to infer whether germination occurs from the germination shield or tuberculate projections.

The increasing complexity of the germination shield structure can be observed in the gigasporalean evolutionary line. Clearly, the simple shield states (germ orbs = mono-lobed, germ violins = bi-lobed) are ancestral. The presence of color in the germination shield is a derivative state (shared by *Dentiscutataceae* species and the related *Paradentiscutata*), whereas this structure is clearly hyaline in the basal branch (*Orbispora*, *Scutellospora*).

Final considerations

The classification proposed by Morton & Msiska (2010) was based solely on morphological data that were not correctly interpreted. The authors did not consider their own molecular phylogenetic data, which supported almost all families and genera proposed by Oehl et al. (2008). Moreover, the *Gigasporales* classification suggested by Morton & Msiska (2010) does not reflect natural groups and leaves *Scutellospora* polyphyletic. Thus, we do not accept the analysis and assumptions by Morton & Msiska (2010), who misinterpreted some morphological characters, leading to misanalysis in the morphological phylogeny of the *Gigasporales.*

The low number of informative morphological characters in *Gigasporales* (until now) have not permitted a reliable morphology-based phylogeny. While we work to find more informative phylogenetic characters and reconstruct a reliable evolutionary history for *Gigasporales,* we should investigate the gigasporalean phylogeny using molecular tools.

Our current phylogenetic analyses demonstrate that all families and almost all genera proposed by Oehl et al. (2008) are monophyletic. Molecularly, generic relationships in the *Dentiscutataceae* are not yet completely understood but will be clarified once sequences of more species from this family are available. We believe that the classification by Oehl et al. (2008; 2011b) with implementation of Goto et al. (2012) is reliable and indicate the need for clarification of generic relationships in the *Dentiscutataceae*.

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