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Phylogenies from genetic and morphological characters do not support a revision of Gigasporaceae (Glomeromycota) into four families and five genera

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Abstract The family Gigasporaceae consisted of the two genera *Gigaspora* and *Scutellospora* when first erected. In a recent revision of this classification, *Scutellospora* was divided into three families and four genera based on two main lines of evidence: (1) phylogenetic patterns of coevolving small and large rRNA genes and (2) morphology of spore germination shields. The rRNA trees were assumed to accurately reflect species evolution, and shield characters were selected because they correlated with gene trees. These characters then were used selectively to support gene trees and validate the classification. To test this new classification, a phylogenetic tree was reconstructed from concatenated 25S rRNA and β-tubulin gene sequences using 35% of known species in Gigasporaceae. A tree also was reconstructed from 23 morphological characters represented in 71% of known species. Results from both datasets showed that the revised classification was untenable. The classification also failed to accurately represent sister group relationships amongst higher taxa. Only two clades were fully resolved and congruent among datasets: *Gigaspora* and *Racocetra* (a clade consisting of species with spores having one inner germinal wall). Other clades were unresolved, which was attributed in part to undersampling of species. Topology of the morphology-based phylogeny was incongruent with gene evolution. Five shield characters were reduced to three, of which two were phylogenetically uninformative because they were homoplastic. Therefore, most taxa erected in the new classification are rejected. The classification is revised to restore the family Gigasporaceae, within which are the three genera *Gigaspora*, *Racocetra*, and *Scutellospora*. This classification does not reflect strict topology of either gene or morphological evolution. Further revisions must await sampling of additional characters and taxa to better ascertain congruence between datasets and infer a more accurate phylogeny of this important group of fungi.

Keywords Classification · Nomeclature · Phylogeny · 25S rRNA gene · β-tubulin gene · Arbuscular mycorrhizal fungi

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Introduction

Plant communities are supported in almost every habitat by symbiotic fungal associations in roots that collectively are termed mycorrhizae. Depending on the group of fungi involved in the mycorrhizal association, uptake of nutrients in the matrix around roots is facilitated and plant as well as community sustainability is accrued via other benefits. The most widespread of these groups are arbuscular mycorrhizal (AM) fungi in the phylum Glomeromycota (Schüβler et al. 2001), not only as a result of their panglobal distribution (Morton et al. 1995) but also in the breadth of associations encompassing a majority of terrestrial plant species (Öpik et al. 2006). Understanding the biology and ecology of these fungi requires a robust classification that reflects their evolutionary history. Only then can hypotheses be devised, tested, and explained which truly represent the different and unique genetic backgrounds and histories of the fungi being investigated. Each new classification of any taxonomic group should be an improvement on the existing one by incorporating new characters or reinterpreting old ones, improving delimitation of monophyletic groups that strengthen phylogenetic underpinnings or resolving paraphyletic and/or polyphyletic groups.

Classification of Glomeromycota still is in a formative stage, but it already has passed through several significant iterations. Initially, these fungi were grouped in the Zygomycota and affiliated with fungi in the Endogonales (Gerdemann and Trappe 1974). A cladistic analysis of the group using morphological characters provided the next pivotal revision by establishing the unique evolutionary origin of this group, which was recognized formally by elevating the group to the order Glomales (Morton 1990; Morton and Benny 1990). Membership in Zygomycota remained unchanged because comparative morphology could not determine a priori homology of characters between zygomycotan clades or assign character polarity. An analysis of a near full-length 18S rRNA (SSU) gene led to another momentous revision, which established that AM fungi comprised a more distant monophyletic group that warranted separation from Zygomycota and a promotion in rank the phylum, Glomeromycota (Schüβler et al 2001). This analysis revealed the true power of a molecular character by providing sequence information comparable across phylogenetically distant lineages.

Alternative classifications arise when there are disagreements as to how to interpret the data on which a classification is based. While all available data supports the monophyletic origin of Glomeromycota (Redecker and Raab 2006), the phylogeny of clades within the phylum has been controversial because morphological evolution conflicts with the SSU gene phylogeny at the deepest nodes (Morton 2009). Much of the tension between datasets resides in the absence of any concerted effort at integration. The morphological tree of Morton (1990) did not include any molecular characters, and the SSU tree of Schüβler et al. (2001) did not consider morphological data.

Neither dataset can stand alone because all conserved traits, regardless of the scale at which they are measured, provide phylogenetic information and must be given due consideration in elucidating the one true evolutionary tree (Hillis 1987; Patterson et al. 1993). A recent reclassification of Gigasporaceae into four families and seven genera (Oehl et al. 2008) failed to meet that standard. Characters were not well defined within a phylogenetic context, and systematic principles and methodology were disregarded. The fundamental assumption of these workers was that SSU and 25S rRNA (LSU) gene phylogenies, reconstructed from only 30% of known taxa, were isomorphic with speciation events and thus accurately reflected species evolution in Glomeromycota. Whereupon they examined morphological characters in most of the 39 species currently described in this family and concluded that properties of the germination shield represented "....common morphological features of spores for the species that were congruent with the rRNA-based molecular phylogenetic reconstruction" (Oehl et al. 2008, p. 312). Tests were not performed to determine if shield characters reflected homologous variation, nor was a phylogeny reconstructed from the totality of known morphological characters to validate homology by congruence in the most parsimonious tree. Moreover, information content of the large and small rRNA genes was inflated by erroneously treating each gene as evolving independent of any other (Hillis and Dixon 1991).

Revisions by Oehl et al. (2008) also produced a proliferation of ranks that, in the absence of a sound foundation, potentially can create instability in a classification (Hibbett and Donoghue 1998). A taxonomic hierarchy should reflect as much as possible a hierarchy of monophyletic clades so that they, in turn, portray the phylogeny of a group of organisms. Each rank in this hierarchy should have meaning. In other words, the various characters defining a clade should offer a hypothesis of history that may be tested using other putative conserved traits, whether they be molecular, biochemical, ecological, physiological, or structural. When ranks are created in error or so many ranks are erected that inter-rank distinctions become blurred or misleading, then the classification loses all meaning and becomes merely an indented list of names.

In the phylum Glomeromycota, a number of recent revisions have led to inflation in the number of taxonomic groups, not because of any new discoveries but rather by mixing methodologies and concepts, often without considering underlying processes at the level of the genome (e.g., lineage sorting, gene duplication) or at the level of the organism (e.g., ontogeny) (Morton 2009). Some revisions in either classification or nomenclature have been based on a combination of morphological and molecular data (Morton and Redecker 2001; Walker et al. 2007), some from morphology (Oehl and Sieverding 2005; Spain et al. 2006) and others from rRNA sequences (Walker and Schüβler 2004). Despite unresolved issues with some of these studies, only the reclassification of Gigasporaceae by Oehl et al. (2008) is addressed in this paper.

In this study, partial sequences of the LSU from the D1–D2 domains (van Tuinen et al. 1998) and an approximate 600 bp region of the β-tubulin gene (TUB2) after excluding three introns (Msiska and Morton 2009a) were concatenated and used to reconstruct a gene phylogeny. While the LSU gene is organized as multiple copies in tandem repeats (Hillis and Dixon 1991), the TUB2 gene exists as only one copy in Gigasporaceae (Msiska and Morton 2009b). Both datasets reflect independent tests of phylogeny. A phylogenetic tree also was reconstructed from 23 discrete morphological characters and compared with the hypothesis of gene evolution. Lastly, the problem of proliferation of higher taxa in Glomeromycota and its impact on systematic analyses is discussed.

Evolution of morphological and molecular characters is discussed separately because each has unique ontological and epistemological considerations. Since the final conclusion of this paper is that this revision is fatally flawed and must be rejected, nomenclature will reflect the prior classification of two genera, *Gigaspora* and *Scutellospora*, in the family Gigasporaceae (Schüβler et al. 2001; Redecker and Raab 2006).

Materials and methods

For molecular analysis, a majority of sequences were obtained from fungal germplasm in the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, West Virginia University, USA). They included 27 LSU sequences from 15 species and 26 TUB2 sequences from 15 species. Another 14 LSU sequences of seven species were obtained from GenBank. Methods for DNA extraction, nested-PCR, cloning, and sequencing of the TUB2 gene is reported in Msiska and Morton (2009a). For the LSU gene, the first round of PCR amplification employed the primer pair 1TS1 and NDL22, followed by a second round using primers LR1 and NDL22 (Van Tuinen et al. 1998). Each PCR consisted of 20 μL of 1× PCR buffer (Promega), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 5 pmol of each primer, and 0.1 μL of Taq polymerase (Promega). Amplification sets consisted of an initial denaturation at 94°C for 3 min., 35 cycles at 94°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplicons were sequenced by Davis Sequencing (Davis, CA, USA) using the T7 primer. Nucleotide and amino acid sequences were aligned using ClustalX (Thompson et al 1997) followed by manual adjustments in MacClade 4.0 (Maddison and Maddison 2005). PAUP 4.0* (Swofford 2002) was used to perform neighbor-joining and maximum parsimony analyses of sequences; an online version of the RAxML (Stamatakis et al. 2008) via CIPRES (http://www.phylo.org) was used to generate a maximum likelihood tree. *Acaulospora mellea* was chosen as outgroup to permit direct comparisons with phylogenetic trees reported by Oehl et al. (2008). Gene trees were reconstructed by maximum parsimony analysis for direct comparison with a morphological hypothesis. Topology of neighbor-joining, maximum likelihood, and bayesian trees did not differ significantly in clades with high statistical support (not shown).

For morphological analysis, selection and definition of characters is the most crucial task. In this study, all possible characters that appeared to discriminate groups more inclusive than species were identified from prior ontogenetic and comparative studies. Methods of culturing, morphological analysis, and resolution of discrete ontogenetic stages comparable between taxa in Gigasporaceae are described by Franke and Morton (1994), Bentivenga and Morton (1995) and Morton (1995). Briefly, ontogeny of spores could be divided into three stages that differentiated three distinct components: (1) the spore wall, (2) germinal walls if present, and (3) germination shield if present. Since these stages were discrete from each other and always appeared in a linear sequence (from 1 to 3), they were directly comparable between species (Fig. 1).

Criteria for selection of characters were: (1) stability (inferring high heritability and conservativeness through geologic time), (2) a priori tests of homology, (3) directionality in ontogeny, and (4) position in gene trees when ontogeny was ambiguous. In addition to superficial similarity (correspondence in appearance without other considerations), tests of homology were conducted that measured similarity in: (1) origin relative to adjacent structures, (2) temporal and spatial position in an ontogenetic sequence in relation to adjacent characters, and (3) transformational states of the character phenotype (Doyle 1992; Morton et al. 1995; Patterson 1982). Ontogeny is a most effective tool when characters arise by terminal addition in a linear series (de Queiroz 1985; Meier 1997). The only exception to this pattern was spore characters in *Gigaspora*. Ontogeny inferred that these characters were ancestral to those in *Scutellospora*, but gene trees (Schüβler et al. 2001; Msiska and Morton 2009a) suggested they evolved more recently. Thus, these characters were coded as derived (Table 1).

Of 23 defined characters (Table 1), seven pertained to features and behavior of external and internal hyphae and structures produced from them (arbuscules, vesicles, spores). These vegetative structures are polymorphic because they form repeatedly with no discrete termination. Since each of these characters did not have a single discrete and stable phenotype, they were coded by proportional variation. In other words, the dominant or "majority" phenotype was interpreted as the phylogenetically significant heritable trait (Wiens 1995). In addition to observations of fungi in INVAM, data also were obtained from a detailed analysis by Dickson (2004). The significance of these characters was validated by complete congruence, as well as a consistency index of 1.0 (100% homology) following phylogenetic analysis. Spore size and color clearly were homplastic characters, but they were included to provide apomorphies that reduced polytomies among terminal species in the tree-building steps. The remaining 14 characters were associated with spores. Gigasporoid and acaulosporoid (outgroup) spore characters were coded separately because each group has a different ontogenetic (and, hence, phylogenetic) history and, therefore, were not homologous. Fourteen characters were unordered; seven were interpreted as irreversible (no reversion to primitive

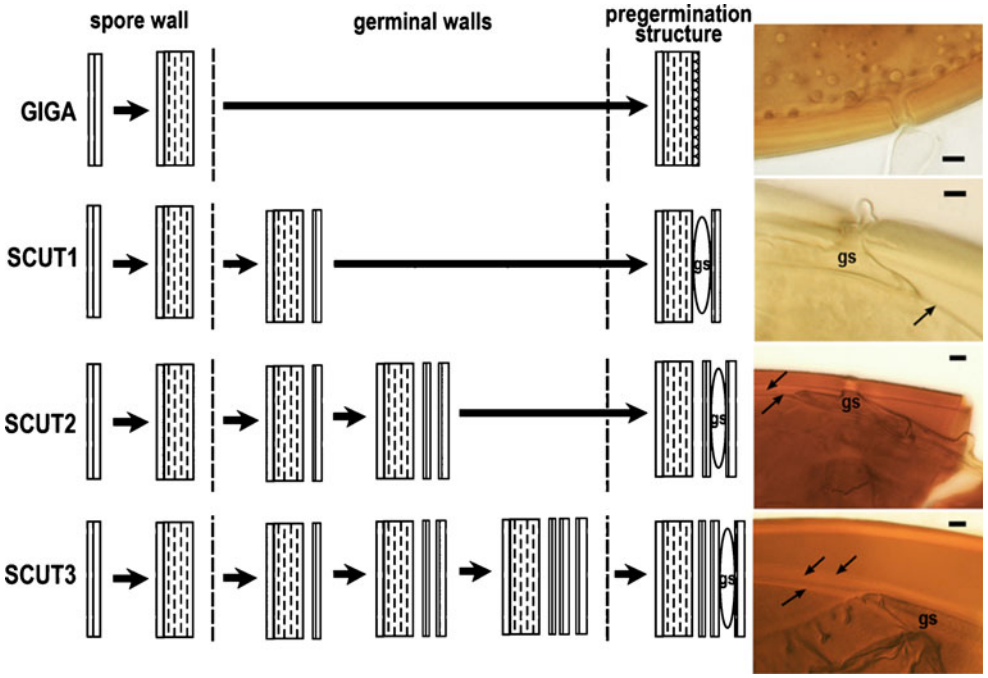

Fig. 1 Illustration of the ontogenetic sequences in the synthesis and maturation of subcellular elements in spores in *Gigaspora* (*GIGA*) and three groups of species in *Scutellospora* distinguished by hyaline bilayered flexible inner (germinal) walls numbered from 1 to 3 (*SCUT1*, *SCUT2*, *SCUT3*, respectively). The spore wall, germinal walls, and pre-germination structure are discrete and independent of each other because variation in any one element does not influence variation in the others. Each column represents a discrete homologous stage that can be compared directly amongst all taxa. Photographs exemplify germinal walls and germination structure in a member fungus of each group. Fill patterns in murographs denote conserved property of a particular layer: *no fill* unspecified properties because they vary among species in each group, *vertical dashes* "laminae" or sublayers, *scallops* warty; *gs* germination shield. *Arrows in photos* point to discrete flexible germinal walls. *Scale bar*=10 μm

state); and two were organized in a step matrix to define an ordered transformation series and also to preserve homology. Three germination shield characters also were coded as polymorphic, as discussed further below.

Germination shield characters require further discussion, because those described by Oehl et al. (2008, Table 2, p. 316) were not well defined. The characters they delimited were: (1) color, (2) shape, lobe curvature, and dentation of margins ("complex structured"), (3) compartment or lobe number, (4) number of folds from margin to interior of shield, and (5) number of germ tube initials (gtis). These definitions pose several problems. First, different putative characters were lumped together as one character even though linkages were not defined. For example, a change in one of the three descriptors for character 2 above did not result in the same change for either of the other two, as can be seen in different spores of *Scutellospora erythropa* and *Scutellospora pellucida* (Fig. 2a–d). Moreover, shield color, shape, or dentation pattern varied greatly between spores and isolates of these two species. Second, terms were used interchangeably so it was unclear whether they were meant to be distinct or equivalent, such as "compartments or lobes." Third, variation within each defined character overlapped, so that discontinuities could not be identified consistently. For example, *S. erythropa* presumably has its own unique set of characters, but these characters can be applied to other groups as well. This species is characterized as having many (8–20) compartments, several to many folds, and several to many gtis (8–20) (Oehl et al. 2008, Table 2, p. 316; Fig. 2a). However, these same character states apply to other species with different definitions (8–30 compartments and gtis), such as *Scutellospora scutata* (Fig. 2e) and *Scutellospora biornata* (Fig. 2f, g). Fourth, some characters either were not defined correctly or applied correctly but resolvable only at the species level. For example, the number of folds in shields of *S. erythropa* rarely exceed 10–12 (defined by Oehl et al. 2008 as several to many) and number of gtis rarely exceed four (instead of 8–20). In another example, the same character states were applied to *Scutellospora coralloidea*, *Scutellospora persica*, and *Scutellospora fulgida*; yet, the shield of *S. fulgida* has a different phenotype (Fig. 2h–j). Fifth, all character states can exist in the same species. For example, character 1 consists of only two discrete states (colorless to pale yellow; yellow-brown to brown), but both are present in different isolates of a well-supported *S. pellucida* subclade (Figs. 2b, c and 3). Variation was extensive even within spores derived from a single spore inoculation (Fig. 2d,

Table 1 Morphological characters and character states used to reconstruct a phylogeny of glomeromycotan species in the family Gigasporaceae

No.	Character: states	CI
1	Arbuscule topology: majority with narrow trunk, branches narrow incrementally (0), majority with wide trunk, branches narrow abruptly (1)	1.0
2	Intraradical hyphae, shape: majority cylindrical and <8 μm wide, (0), majority with inflated regions, >8 μm wide (1)	1.0
3	Intraradical hyphae, growth behavior: majority straight with angular branching (0), majority irregular with extensive coiling	1.0
4[a]	Intraradical hyphae, vesicle formation: present (0), absent (1)	1.0
5	External hyphae color: hyaline (0), dematacеous (1)	0.25
6[a]	Extraradical hyphae, auxiliary cell formation: absent (0), present (1)	1.0
7[a]	Auxiliary cells, surface ornamentation: knobby (0), spiny (1)	1.0
8	Spores develop from a sporogenous cell: no (0), yes (1)	1.0
9	Spore size: <250 um (0), 251–400 um (1), >400 um (2)	0.23
10	Spore color: colorless to pale yellow, green, or pink (0), yellow to yellow brown (1), orange-brown (2), red-brown (3), red-black to black (4)	0.40
11[a]	Acaulosporoid spore wall, laminate layer with outer sloughing layer that originates from hyphal wall of sporiferous saccule: yes (0), no (1)	1.0
12[a]	Gigasporoid spore wall, two permanent layers: no (0), yes (1)	1.0
13	Gigasporoid spore wall, outer layer surface: smooth (0), ornamented (1)	0.20
14[a]	Gigasporoid spore wall, laminate layer rigidity & reaction to Melzer's reagent: none (0), dextrinoid (1), red-amyloid (2)	0.25
15	Gigasporoid spore, flexible germinal walls: present (0), absent (1)	1.0
16	Gigasporoid spore, number of germinal walls: one (1), two (2), three (3) [step matrix]	–
17[a]	Gigasporoid spore, 2nd germinal wall, reaction of inner most layer to Melzer's reagent: no reaction (0), dextrinoid (1), red-amyloid (2)	0.40
18[a]	Gigasporoid spore, 3rd germinal wall, reaction of inner most layer to Melzer's reagent: dextrinoid (0), red-amyloid (1)	1.0
19[a]	Gigasporoid spore, origin of germ tube: from germination shield (0), from warty layer of spore wall (1)	1.0
20	Gigasporoid spore, germination shield, position: 1st germinal wall (1), 2nd germinal wall (2), 3rd germinal wall (3) [step matrix]	–
21	Gigasporoid spore, germination shield, color: majority subhyaline to light yellow (0), majority yellow-brown to brown (1)	0.33
22	Gigasporoid spore, germination shield, number of folds extending from margin to interior: few <4 (0), moderate 6–15 (1), many, >15 (2)	1.0
23	Gigasporoid spore, germination shield, number of germ tube initials: two (0), variable 3–6 (1), variable >6 (2)	0.33

Characters defined and formatted according to recommendations by Sereno (2007)

CI consistency index where 1.0=100% homology

[a] Irreversible character states

from slides contributed by J. Bever, Indiana University). Sixth, characters must represent synapomorphies (each state representing a unique innovation shared by all members of a clade) to be phylogenetically informative. Many of those defined by Oehl et al (2008) are species rather than clade-specific (e.g., species represented in Fig. 2h–j), so they arose more than once in a clade and thus are homoplastic. Last, and perhaps most important within a phylogenetic context, characters were not tested for homology. Similarity alone can be misleading. An essential test is to assess correspondence of a pertinent character with associated characters that precede it in development. Based on this test, the germination shields in each group of species with a different number of germinal walls have different ontogenetic histories. The germination shield is the terminal event in spore differentiation, regardless of species, and the shield changes position with a linear succession of inner walls evolving in different groups of species (Fig. 1). The shield always will be present on the innermost germinal wall regardless of how many are formed because substrates, enzymes, and cofactors from the cytoplasm are needed for synthesis. Oehl et al. (2008) attempted to integrate germinal wall organization with shield characters, but errors occurred because these workers did not interpret this combination of characters within an ontogenetic or even a phylogenetic context. The approach taken here, then, was to treat germination shield characters equivalently with other morphological characters when reconstructing a phylogenetic tree.

Much of the confusion in character definition appears to derive from a poor understanding of germination shield development and what the different characters represent within the overall structure of the shield. Only one hypothesis has been proposed to date, and it is based on a limited range of observations (Walker and Sanders 1986). Oehl et al. (2008) illustrate characters and relationships between characters in Figs. 1–9, p 319. However, the depicted relationships are oversimplified and do not reflect

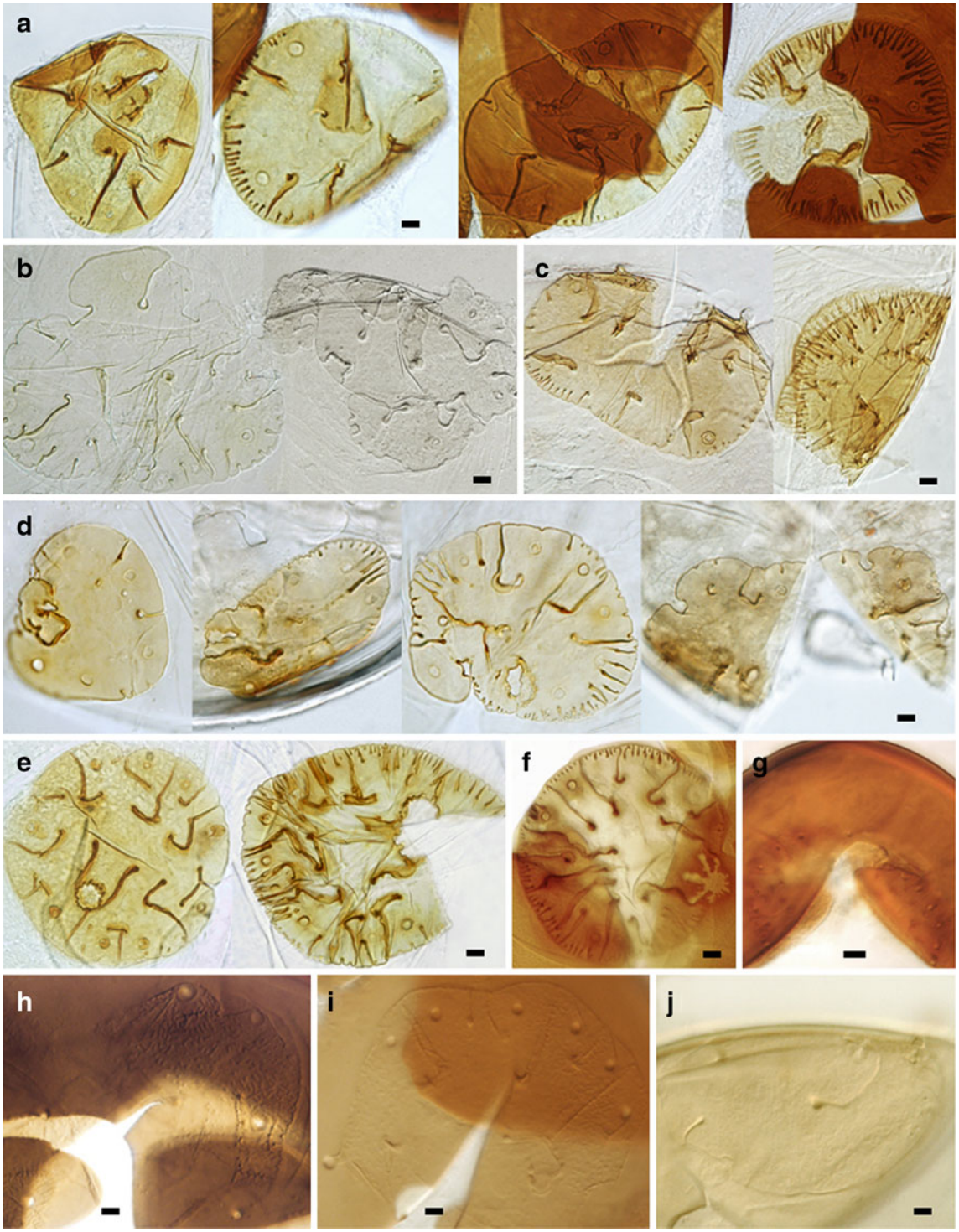

Fig. 2 Variation in germination shield phenotypes of mature spores from selected species in *Scutellospora* permanently mounted in polyvinyl-lactic acid medium on glass slides. *Arrows* point to representative gtis. Shields from four spores of *S. erythropa* MA453A showing a gradation in shape and in complexity of peripheral folds (**a**); unpigmented shields from spores of *S. pellucida* NC155 (**b**); dematoceous shields from spores of *S. pellucida* NC118 (**c**); dematoceous shields from progeny spores of a culture started from one spore of *S. pellucida* NC118 (**d**); shields from two spores of *S. castanea*, showing lack of correlation between complexity of peripheral folds and number of gtis (**e**); shield of *S. biornata* with many gtis and complex peripheral folds (**f**); spore of *S. biornata* showing pattern of holes in the spore wall where multiple germ tubes had emerged (**g**) shield of *S. coralloidea* with rare peripheral folds but multiple gtis (**h**); shield of *S. persica* with phenotype similar to that of *S. coralloidea* (**i**); shield of *S. fulgida*, with smooth surface, few folds, and few gtis (**j**). *Scale bar*=10 μm for all except right photo in **g**, where bar=25 μm

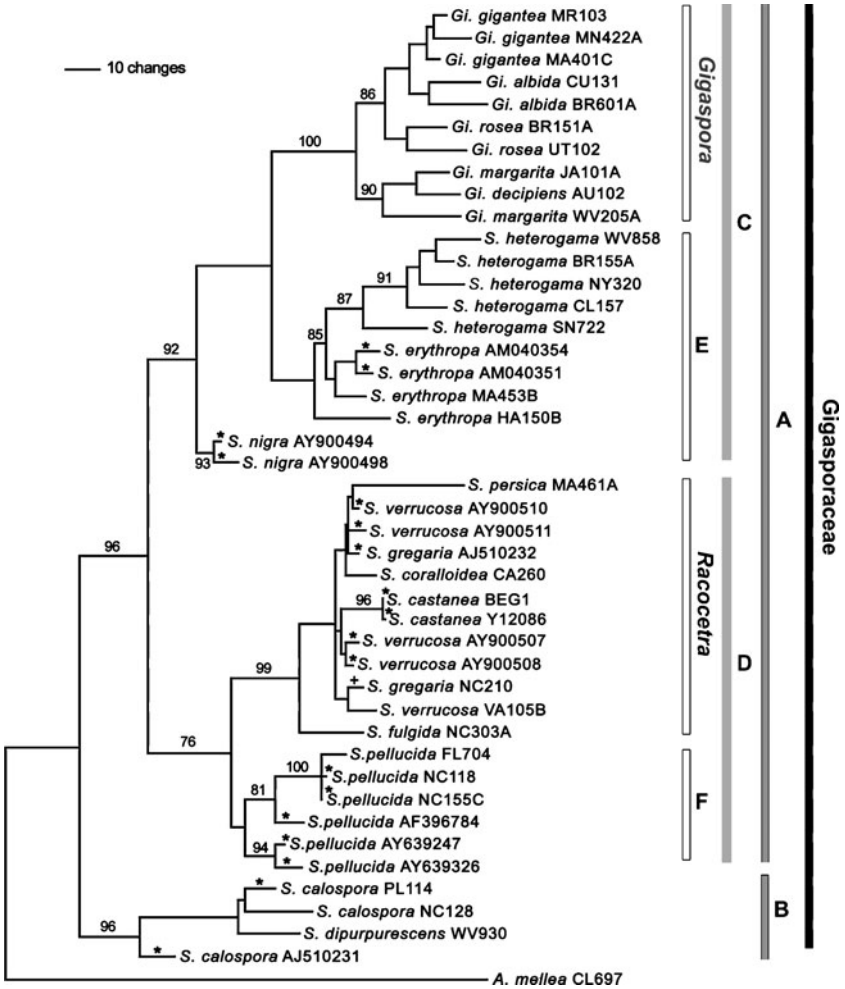

Fig. 3 Phylogenetic tree of concatenated sequences from regions near the 5′ end of the 25S rRNA (LSU) and β-tubulin (TUB2) genes, reconstructed using the parsimony criterion in PAUP*. Total length analyzed was 1,274 bp. *Asterisks* and *cross* indicate taxa in which only LSU and TUB2 sequences, respectively, were used. Only bootstrap values above 70% are reported as indicative of significant support. *Vertical lines* of successively *lighter color* indicate structure of nested clades from most to least inclusive. Genbank accessions for *A. mellea* = FJ461794 (LSU) and FJ174303 (TUB2); Gigasporaceae species = FJ461861–FJ461881 (LSU) and FJ174268–FJ174278, FJ174315–FJ174327, FJ807702–FJ807707 (TUB2)

actual variation within and between species. For example, "compartments" appear to be determined by two criteria: (1) location near folds and (2) an assumption that presence of a gti is indicative of a compartment. In their illustration of shields, gtis are formed uniformly around the shield periphery in close correspondence with folds. Such uniformity is rare except in certain species. Often, gtis are variable in different regions of the same shield (Fig. 2a, c, e) and tend to be more uniform mostly in species which produce many simultaneous germ tubes (Fig. 2f, g).

These and other characters were analyzed for morphological evolution by searching for the most parsimonious trees in PAUP, generating a consensus tree, and then importing a tree with the closest approximation to the consensus tree into MacClade. Character distributions were mapped using the Trace Character option. Tree topology was compared with that of the two gene trees as well as a tree from concatenated sequences. Gene and morphological data were not combined for two reasons. First, each dataset evolves at different scales. Second, each has different strengths and weaknesses in how characters are delimited and how homology is determined (Morton 2009).

Results

Molecular phylogeny

The topology of terminal clades in phylogenetic trees reconstructed separately from LSU and TUB2 gene sequences did not differ appreciably (results not shown). A partition homogeneity test did not reveal any statistically significant incongruence between datasets, so sequences of both genes were concatenated. The aligned sequences consisted of 1,274 characters, of which 251 were parsimony informative. The tree generated from this analysis (Fig. 3) was 939 steps in length, with a consistency index (CI) of 0.60 and a retention index (RI) of 0.75.

The concatenated tree was similar in topology to the LSU tree reported by Oehl et al. (2008; Fig. 10, p. 323) but with some important differences. Their tree consisted of seven clades, all of which showed significant bootstrap support (>70%). The tree in Fig. 3 discriminated five clades, of which only *Gigaspora*, *Racocetra*, and clade B received high bootstrap support (96–100%). Of these, clade B is undersampled, consisting only of isolates of *Scutello-*

spora calospora and *Scutellospora dipurpurescens*. Both of these two species are almost indistinguishable morphologically and thus may constitute a single species. Oehl et al. (2008) classified this clade as Scutellosporaceae, even though its position was distant from that of other clades erected as families (Fig. 4).

Only two of the four clades nested within clade A in Fig. 3 were strongly supported. These clades were populated by species in *Gigaspora* and *Racocetra*. Clade E, which corresponded to three poorly resolved clades named by Oehl et al. (2008) as the genera *Dentiscutata*, *Fuscutata*, and *Quatunica* in the family Dentiscutataceae, lacked statistical support. In their study, each clade translated to a genus consisting of one species. Clade F also was not supported statistically, even though it contained only isolates of *S. pellucida* in both this study (Fig. 3) and the LSU tree from Oehl et al. (2008). Ranking decisions were problematic, and they are addressed later in this paper.

Morphological phylogeny

The most parsimonious tree was reconstructed using 23 characters from 28 species (Fig. 5). The tree consisted of 64 steps, an overall CI of 0.47, and an RI of 0.64. All taxa were united into one clade based on properties of hyphae and associated structures, mode of spore formation, and organization of the spore wall. Characters with a CI of 1.0 signified unambiguous, homologous characters. The two most inclusive subclades consisted of species grouped previously into the genera *Gigaspora* and *Scutellospora*. Characters unambiguously (CI=1.0) discriminating these two clades were (1) auxiliary cell ornamentation, (2) presence or absence of flexible inner (=germinal) walls, and (3) structure from which a germ tube originates. Within the *Scutellospora* clade, two subclades (A and *Racocetra*) were resolved unambiguously by the evolution of one versus two to three germinal walls (character 16) and by position of the germination shield on these walls (character 20). In reality, these two characters are correlated and therefore are not independent tests of clade evolution. Exclusion of character 20 did not alter tree topology (although slightly inflating CI and RI), so it was retained to highlight the linkage between germinal wall and germination shield evolution. Three subclades were discriminated within clade A (clades B–D), but unambiguous characters were too few in number to confidently resolve any of them. Within clade B, a subclade consisting of *S.*

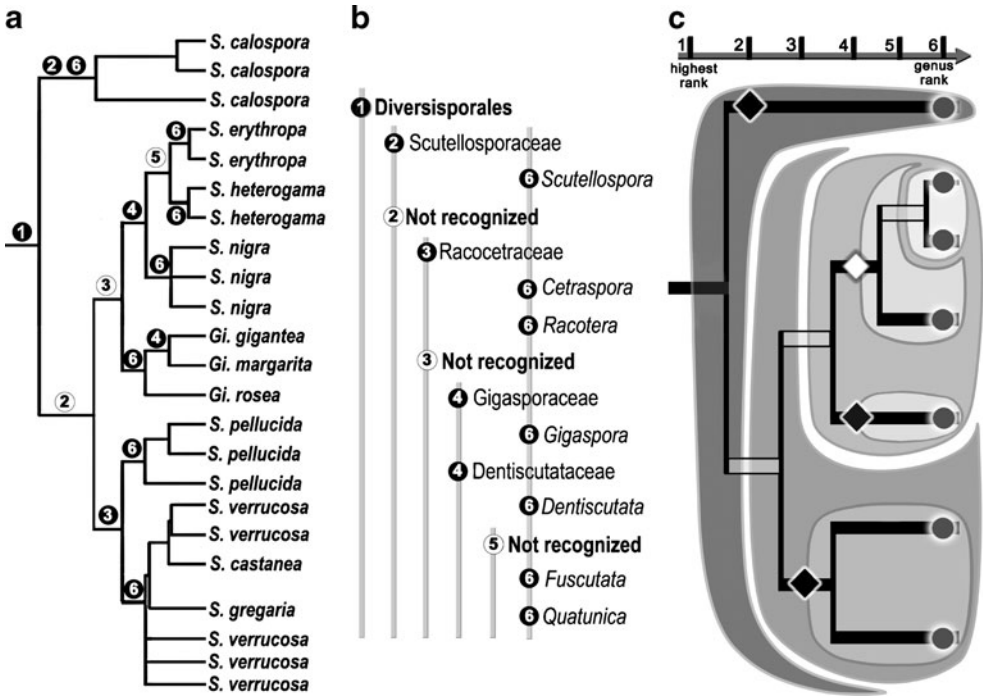

Fig. 4 Relationship between topology of a 25S rRNA gene phylogeny and the classification of fungi in Gigasporaceae erected by Oehl et al. (2008): **a** phylogenetic tree, with *numbers* signifying nodes that discriminate successively nested monophyletic clades. *Filled circles* indicate named taxa and *hollow circles* signify clades not included in the classification; **b** indented classification scheme based on nested clades in the phylogenetic tree; **c** illustration depicting cladistic relationship between groups in the gene tree. *Closed-shaded areas* show properly recognized monophyletic groups. *Open-shaded areas* contain both monophyletic and paraphyletic taxa, where the clade enclosed within shaded area is monophyletic and the clade outside the shaded area shares a common ancestor but is excluded from the classification (paraphyletic). *Filled diamonds* named monophyletic families, *hollow diamonds* named paraphyletic families, *filled circles* named monophyletic genera

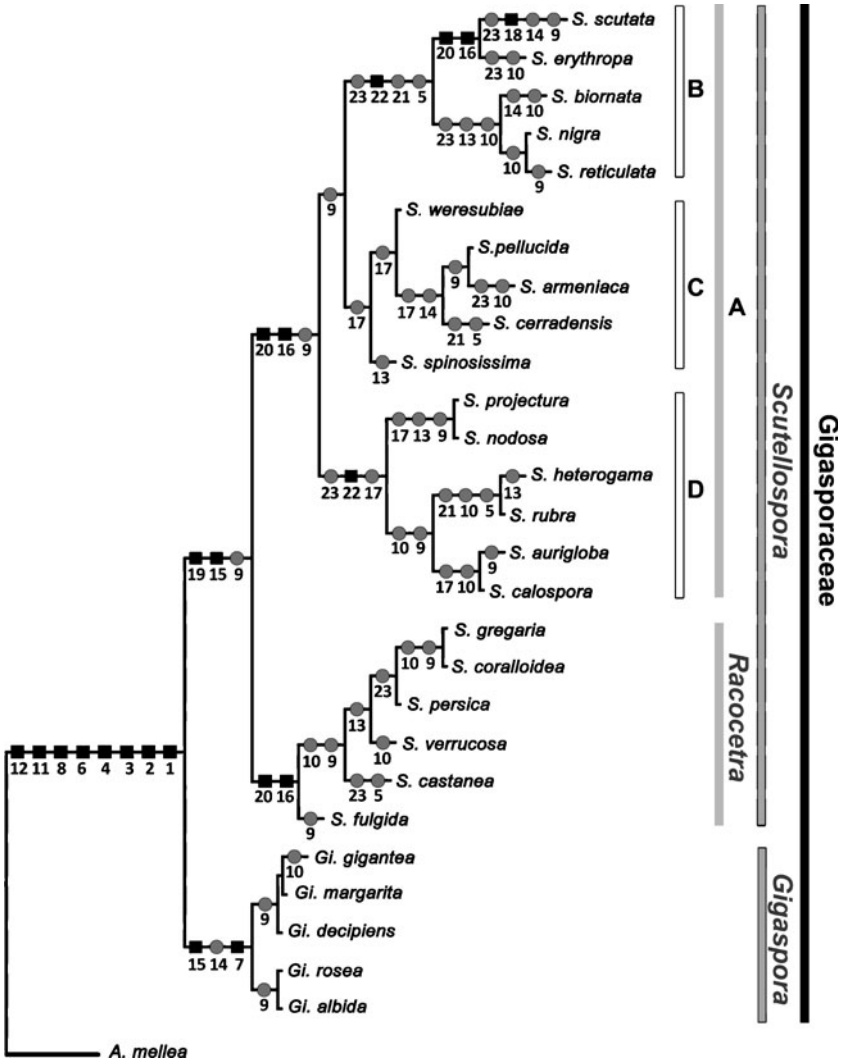

Fig. 5 Phylogenetic tree of 28 species based on evolution of 23 morphological characters defined in Table 1. The tree was reconstructed using the parsimony criterion in PAUP* with manual adjustments in MacClade version 4 after tracing character evolution. Tree length=64 steps, CI=0.47, retention index=0.64. *Dark squares* signify characters where CI=1.0 (Table 1)

erythropa and *Scutellospora castanea* was supported by more derived states of characters 16 and 20 (discussed above).

With the five characters defined by Oehl et al. (2008) recoded as three (characters 21–23), only character 22 was homologous. It resolved clade D, which included species, classified by Oehl et al. (2008) as members of two genera in different families: *Fuscutata* in Dentiscutataceae and *Scutellospora* in Scutellosporaceae. The other two characters evolved more than once in clades B through D. Clearly, the germination shield characters did not resolve the more inclusive groups depicted in the Oehl et al. (2008) classification.

Discussion

Oehl et al. (2008) were the first to formally attempt integration of five putative characters of one structure (germination shield) and sequences from two coevolving rRNA genes to erect a complex classification scheme dividing a morphologically and genetically well-supported group (Gigasporaceae) into four families and five genera. This work is noteworthy in that morphological variation in an underevaluated spore structure, the germination shield, is analyzed exhaustively. However, it also is based on faulty premises, circular reasoning, and imposition of phylogenetic significance to selective characters in the absence of appropriate methodology. First, the rRNA gene trees were presumed to represent the true species phylogeny of this group. Even though morphological characters were not analyzed within a phylogenetic framework, germination shield characters then were implicitly identified as synapomorphies discriminating monophyletic clades (families, genera) because they correlated with the rRNA gene trees. Associated characters, such as germinal walls, were treated secondarily and thus were implicitly homoplastic. Other characters were considered uninformative, apparently because they did not correlate with the rRNA gene trees. Then, the germination shield characters were used to

provide support for gene trees to validate the new classification. If the germination shield is stripped of the bias associated with its presumed importance in defining clades in Gigasporaceae and then analyzed within a developmental and phylogenetic context, two things happen. First, germination shield characters are evaluated as several amongst a pool of 23 equally weighted characters so that bias is eliminated. Second, these characters provide a test of species phylogeny that is independent of gene evolution so that circularity is removed from the analysis. When this is done, only one character of the germination shield (22, Table 1) shows evidence of being a synapomorphy contributing to one clade congruent among data sets (*Racocetra*, erected by Oehl et al. 2008). The other characters are the product of convergent evolution and, therefore, are phylogenetically uninformative at deeper nodes translatable to higher ranks.

An explicit example supporting this conclusion involves *S. erythropa* and *S. scutata*. Oehl et al (2008) populated the genus *Quatunica* (Dentiscutataceae) with the former species only because of (1) a putatively resolved clade in the LSU tree and (2) a perceived complex germination shield associated with three inner germinal walls. These workers placed *S. scutata* in *Dentiscutata*, a different genus of the same family. However, they failed to recognize that *S. scutata* also has three germinal walls (see photos at http://invam.caf.wvu.edu). When the two species are grouped together based on number of germinal walls, germination shield characters used to define genera are, in reality, species-specific. Deep folds (signifying compartments) and gtis are more numerous in *S. scutata* than in *S. erythropa* (Fig. 1a, f). A test of this relationship at the level of gene evolution will require sequences from *S. scutata*. Without these data, *S. erythropa* groups with *S. heterogama* in the combined LSU-TUB2 gene tree. Yet *S. heterogama* is a species Oehl et al. (2008) placed in the genus *Fuscutata*.

Congruence between datasets is an essential prerequisite for establishing confidence that the proposed phylogenetic hypothesis infers true species evolution and any resulting classification will be both stable and informative (Patterson et al 1993). Results of the study reported here show that neither rRNA nor TUB2 genes are sufficiently informative with the number of taxa sampled to resolve clades translatable into the many new genera and families proposed by Oehl et al. (2008). Congruence was not well supported even between two coevolving rRNA genes trees reported in that study. One likely reason is sensitivity of tree topology and statistical support to the number of species analyzed (Hillis et al. 1993). In Oehl et al. (2008), only 30% of known species were sampled. In the study reported here, the LSU-TUB2 tree was populated by only a few more species (35% of all species). An analysis of an independent molecular dataset of TUB2 sequences proved to be equally uninformative. The only two monophyletic groups strongly supported by all three genes and morphology were the *Gigaspora* and *Racocetra* clades. In the former, 90–100% of member species are represented, depending on splitting tendencies of different researchers. In the latter, 75% of the species are present. In contrast, the largest group (clade A) is represented by only two of 10 species in any gene tree, and all other clades contain only one species.

Nomenclature and ranking decisions

Any revised classification should attempt to be structured in a way that maximizes phylogenetic information, where only monophyletic clades receive proper names. At present, two nomenclatorial systems exist: the widely used Linnaean system and a Phylocode system (de Queiroz and Gauthier 1992, Hibbett and Donoghue 1998). Which of these systems will provide the most stable and informative classifications is the subject of much debate (e.g., Nixon et al. 2003). Oehl et al. (2008) obviously applied the Linnaean system when erecting their classification. However, ranking decisions did not conform to the pattern of nested clades in the rRNA phylogenetic trees that served as their template, so the result was subjective and arbitrary.

To explore this conclusion, we make the assumption in this section (even though it is erroneous) that the rRNA tree published by Oehl et al. (2008) is a fully resolved hypothesis of species evolution. The classification then should conform at least loosely to the series of nested clades in that tree (Fig. 4). However, only subsets of reconstructed monophyletic groups are recognized. This approach has several negative consequences. First, not all of the named taxa are monophyletic, such as the family Dentiscutataceae. Second, some monophyletic groups are nested within unrecognized clades at three levels that, if named, would be paraphyletic. Third, taxa designated in a given rank are not at equivalent levels in the phylogenetic tree used to erect the classification.

Systematists interested in a phylogeny-based classification generally support the recommendation of Hennig (1966) and Wiley (1981) that only sister groups are assigned the same rank. Less inclusive groups are ranked lower, and more inclusive groups are ranked higher. In that context, the clade consisting of only two almost indistinguishable species, *S. calospora* and *S. dipurpurescens*, is a sister group to all other members of Gigasporaceae. While this clade has strong statistical support in the combined LSU and TUB2 gene tree, its position in the morphological tree is incongruent. These two species have no unique organismal traits that indicate an ancient divergence from all other *Scutellospora* species. In this case, the gene

phylogeny must be questioned, especially since the clade is grossly undersampled. Nested within the sister clade to "Scutellosporaceae" is the monophyletic clade Racocetraceae, which should be ranked lower if the tree is to be strictly interpreted. Here again, its sister clade goes unrecognized. The two families Gigasporaceae and Dentiscutataceae are sister groups in this unnamed clade and warrant equivalent rank. However, because these clades are one level above that of Racocetraceae and two levels above Scutellosporaceae, they are improperly ranked. Dentiscutataceae is paraphyletic because the sister group of *Gigaspora* is not recognized. Such arbitrary ranking decisions result is an unstable classification that distorts putative phylogenetic relationships and violates a basic tenet that taxa of equivalent rank should reflect equivalent relationships.

The exercise above clearly shows that erection of any classification translating strict topology of a phylogenetic tree will result in a plethora of ranks (De Queiroz and Gauthier 1992; Hibbett and Donoghue 1998). Rank proliferation generally is viewed as undesirable because it leads to unwieldy complexity that promotes confusion rather than clarity. Different approaches have been suggested to resolve this problem (see references in de Queiroz and Cantino 2001), each with its own merits and problems. In the reclassification of Gigasporaceae, problems are compounded by a weakly supported gene phylogeny that serves as the template for ranking decisions and by use of germination shield characters outside a phylogenetic context as supporting evidence. Oehl et al. (2008, p 355) go so far as to suggest "the genera presented here may contain additional groups with divergent shield characters and possibly divergent phylogenies that may justify in the future erection of new genera based on the number of lobes and gtis or due to shield composition." In other words, the only morphological characters that provide information on relationships among species in Gigasporaceae will be those expressed in germination shield phenotypes. If new data on rRNA gene evolution reveal further divisions in clades, these authors then consider a search for correlated characters in those phenotypes is justified. Such an exclusionary view is strongly biased and, together with the unjustified proliferation of higher taxa, dooms usefulness of the resultant classification for broader application and interpretation.

In the study reported here, most named genera are at the same level in the gene tree (Fig. 3), with the exception of clade B (*S. calospora*/*S. dipurpurescens*). However, clade B in the gene tree is embedded within clade D of the morphological tree (Fig. 5), and so its true phylogenetic position remains ambiguous. In the morphological tree, all species in the clade labeled *Scutellospora* is the sister clade of *Gigaspora*. Placing these two clades into a higher rank (above the genus level) cannot be supported at this time. Known characters are insufficient to resolve subclades in *Scutellospora* (clades B–D) nor are they sufficiently unique to show that *Gigaspora* evolved from a common ancestor near the same time as all other species grouped in *Scutellospora*.

Conclusions

Charles Darwin states in his book *Origin of the Species* "The value indeed of an aggregate of characters is very evident....a classification founded on any single character, however important that may be, has always failed." In the reclassification of Gigasporaceae by Oehl et al. (2008), only two truly independent characters were employed: the germination shield and co-evolving rRNA genes. The rRNA genes were not phylogenetically informative at nodes signifying clades ranked as higher taxa. Most characters of the germination shield failed rigorous a priori tests of homology as well as an *a posteriori* test of congruence when tested with a suite of other nonweighted morphological characters.

Therefore, all parts of the new classification by Oehl et al. (2008) which do not reflect strongly supported congruent clades based on independent datasets are rejected. Hibbett and Donoghue (1998) have recommended that even when phylogenetic ambiguities exist in some parts of a phylogenetic tree, well-supported clades should be recognized. This approach seems reasonable if any change directs researchers to discover new insights into the evolutionary history of the group. For complex higher organisms, species are more important than higher ranks for hypothesis testing because morphological characters at that level are biologically and/or ecologically relevant. The reverse appears to be true for glomeromycotan fungi and for exactly the same reasons. Biological and ecological phenomena are linked more closely to properties of the fungal thallus, modes of spore formation, and germination which define higher taxa, whereas species-level characters have no proven significance (Morton et al. 1995; Morton 2009; Van der Heijden 2004). Using the LSU-TUB2 phylogeny as a template, *Racocetra* and *Gigaspora* are at equivalent levels, and since both are resolved in the morphological tree as well, important biological differences may exist between the two groups. Therefore, the genus *Racocetra* is retained to promote comparative studies that potentially might reveal novel insights into the evolutionary history of this clade. Morphological characters do not fully resolve all clades in *Scutellospora* even when a majority of species in the genus are used the analysis. Molecular characters are equally ambiguous given the undersampling of taxa. To subdivide this group based on such flimsy evidence is premature at this time and only serves to destabilize the classification.

The present study indicates that a limited molecular dataset should not be treated as the true measure of species phylogeny against which all other datasets are tested to select for correlative characters. Generation of a gene tree alone provides only one test of a hypothesis of species evolution, and data from morphology and other molecular characters provide robust independent tests if analyzed properly. Using this approach, few proposed taxa in the classification by Oehl et al. (2008) can be justified.

A revised classification is recommended below which reduces the number of families to one (Gigasporaceae) and the number of genera to three (*Gigaspora*, *Scutellospora*, *Racocetra*). This classification does not reflect strict topology of either the concatenated gene tree or the morphological tree. Enough ambiguities exist to take a conservative approach and name only those taxa congruent with all datasets. Germination shield characters also are notably absent as indicators of evolution at the genus or family levels because they either are homoplastic or are not congruent with gene evolution. As more species and/or characters are added to the analysis, congruence amongst data may improve sufficiently to justify further revisions to this important group of glomeromycotan fungi.

Revised classification

FAMILY: Gigasporaceae J.B. Morton & Benny

Morton JB, Benny GL (1990) Mycotaxon 37:471

The clade containing all members of this family is fully resolved as a monophyletic group based on 18S and 25S rRNA genes, a single copy β-tubulin gene and some of the following morphological properties resolved as synapomorphies (Table 1, Fig. 5):

Spores are formed singly in soil and within roots for few species, developed from a swollen sporogenous cell growing terminally on a subtending hypha. Spores of all species develop a spore wall consisting of a rigid to semi-rigid laminate layer, a permanent outer layer, and in some species a very thin inner layer. Spores either have only a spore wall or a spore wall with one to three bilayered germinal walls. Germination is through the spore wall from warts that develop around a developing germ tube when no germinal walls are present or from a germination shield that forms on the innermost germinal wall. Germinal walls develop independent of the spore wall and are conserved enough to be shared by two or more species (Morton 1995; Msiska and Morton 2009a, b). Available evidence indicates that spores are the only infective propagules once the host plant has died or gone dormant (Hart and Reader 2002).

Auxiliary cells branch from germ tubes or from extraradical hyphae produced by mycorrhizal colonization, forming loose aggregates. They are globose to irregularly shaped with an almost smooth surface at one extreme to deep convolutions that resemble spines at the other extreme. Auxiliary cells are abundant early in colonization and usually decline as sporulation ensues. Morton et al. (1995) hypothesize that auxiliary cells serve as a source of carbon partitioned to spores as they develop to reduce cost to host plants.

Mycorrhizae consist of intraradical cylindrical hyphae often with inflated regions; staining uniformly dark with acidic stains; often widespread and abundant in pot culture root systems as host plants senesce. Hyphal growth tends to be irregular, often with coils present most frequently at or near infection points and also throughout areas of colonization (e.g., Dickson 2004). Arbuscules often form with swollen trunks, with more *arum* than *paris* types in roots of herbaceous host species in well-aerated soils.

External hyphae hyaline to dark brown, often with irregular to inflated regions; thin and ephemeral hyphae usually formed concurrently in soil and root-organ cultures.

TYPE GENUS: *Gigaspora* (Gerd. & Trappe) C. Walker & F.E. Sanders

Gerdemann JW, Trappe JM (1974) Mycologia Mem 5:25

Walker and Sanders (1986) Mycotaxon 27:179

The subclade containing all species in this genus is fully resolved as a monophyletic group based on 18S and 25S rRNA genes, a single copy β-tubulin gene and some of the following morphological properties:

Spores form only a spore wall organized to consist of two permanent layers: a thin outer layer and a thicker laminate layer, both of which are devoid of any ornamentations and are limited in color from white to light yellow to yellow-green when healthy. Germination is through the spore wall, with one to multiple germ tubes developing from a warty layer on the inner surface of the spore wall.

Auxiliary cells branching from spore germ tube and external hyphae, forming in loose cluster; cell wall surface conspicuously ornamented with deep convolutions, so that each cell appears to have a spiny surface.

GENUS: *Racocetra* Oehl, de Souza, and Sieverd.

Oehl F, de Souza FA, Sieverding E (2008) Mycotaxon 106:311

The subclade of Gigasporaceae containing all species in this genus appears to be fully resolved as a monophyletic group based on 18S and 25S rRNA genes, a single copy β-tubulin gene, and some of the following morphological properties:

Spores consist of a spore wall and one inner bilayered germinal wall. The spore wall has two to three permanent layers, with the outer layer ranging from smooth to various ornamentation patterns. Spore color ranges the full gamut from white to a dark reddish-black. Two or more germ tubes arise from a germination shield that forms on the surface of the germinal wall distal to spore cytoplasm.

Auxiliary cells branching from spore germ tube and external hyphae, forming in loose cluster; cell wall surface ornamented with shallow convolutions, so that each cell appears to have a somewhat smooth to knobby surface.

This genus was erected together with the family Racocetraceae by Oehl et al. (2008), but that family is rejected as a valid monophyletic group and so *Racocetra* is transferred to the family Gigasporaceae. *Racocetra weresubiae* is transferred to the genus *Scutellospora* because spores contain two inner germinal walls.

GENUS: *Scutellospora* C. Walker and F.E. Sanders

Walker C, Sanders FE (1986) Mycotaxon 27:179

The subclade containing all species in this genus is not well resolved as a monophyletic group based on morphological characters or by an undersampling of species when analyzing rRNA or beta-tubulin genes.

Spores consist of a spore wall and two to three inner bilayered germinal walls. The spore wall has two to three permanent layers. The outer layer and/or the laminate layer may be smooth or ornamented. Spore color ranges the full gamut from white to a dark reddish-black. One or more germ tubes arise from a germination shield that forms on the surface of the innermost germinal wall proximal to spore cytoplasm. One or two other flexible walls present between the functional germinal wall and the spore wall do not have the capacity to form a germination shield because they are not in contact with spore cytoplasm. However, they still are termed germinal walls since they appear to be relict structures retained because of their sequential synthesis in a linear ontogenetic trajectory (Morton et al 1995).

Auxiliary cells branching from spore germ tube and external hyphae, forming in loose cluster; cell wall surface almost smooth or ornamented with shallow convolutions, so that cells appear knobby.

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