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Scutellospora alterata, a new gigasporalean species from the semi-arid Caatinga biome in Northeastern Brazil

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Abstract — A new species of *Gigasporales* (*Glomeromycota*) was isolated from soils of the semi-arid Caatinga biome in NE Brazil. It produced spores in the rhizospheres of *Sorghum sudanense*, *Zea mays*, *Panicum maximum*, and *Helianthus annuus* in pot cultures. Spores are triple-walled, roughened, yellow-white to light yellow, and 150–255 µm in diameter. The single germination shield is hyaline, oval to ovoid, and bi-lobed and has the two germ tube initiations (GTIs) typical for *Scutellospora* species. The shields can change shape under light pressure on the cover slide, causing several 'false' lobes to appear that do not bear GTIs but inflate from the shield periphery. Molecular analyses of the partial LSU rDNA gene place the fungus in a clade next to *Scutellospora calospora*, *S. dipurpurescens,* and *S. spinosissima*. Further investigations on germ shield morphology showed that several false lobes also form in other *Scutellospora* species in lactic acid based mountants under pressure on the cover slide. In *Racocetraceae* more than two 'true' lobes form during spore formation, each potentially bearing one GTI.

Key words — *Glomeromycetes*, *Scutellosporaceae*, *Orbispora*, molecular phylogeny, arbuscular mycorrhiza

Introduction

During the last decades a high species richness of arbuscular mycorrhizal fungi (AMF) has been detected in the *Gigasporales* (e.g., Nicolson & Gerdemann 1968, Gerdemann & Trappe 1974, Ferrer & Herrera 1981, Morton

1995, Souza et al. 2005, Silva et al. 2006, Tchabi et al. 2009a). Many species have been described within this fungal group (e.g., Koske & Halvorson 1990, Błaszkowski 1991, Lin & Yen 2011), especially from tropical South America (e.g., Spain et al. 1989, Walker et al. 1998, Cuenca & Herrera-Peraza 2008, Silva et al. 2008, Goto et al. 2012, Mello et al. 2012). The morphological diversity in the *Gigasporales* concerns above all spore sizes and color (Silva et al. 2008, Goto et al. 2011), outer spore wall ornamentation (Goto et al. 2009, 2010, Tchabi et al. 2009b), number of walls (Oehl et al. 2008, Oehl et al. 2011a), germination shield complexity and color, and specific germination characteristics (Oehl et al. 2010, Oehl et al. 2011a,c,d, Goto et al. 2012). Within the *Gigasporales*, major fungal groups have been identified as a result of detailed morphological and molecular phylogenetic analyses. These analyses so far support five families and ten genera (Silva et al. 2013).

A new species of the *Gigasporales* producing spores with an evanescent papillate surface roughening has been isolated from tropical soils of the semi-arid 'Caatinga' biome in NE Brazil. The fungus forms triple-walled spores with hyaline to subhyaline bi-lobed germination shields, clearly suggesting placement in *Scutellospora* (*Scutellosporaceae*) according to Oehl et al. (2011c). However, we observed an atypically highly variable germination shield morphology, especially when we applied pressure on the cover slides in lactic acid based mountants. The aim of this study was to a) describe this unique species, b) study thoroughly its variable shield morphology, and c) analyze its ribosomal gene. Shields in other *Scutellospora* species were reviewed, and their morphological variation is also discussed.

Materials & methods

Study site, soil sampling and chemical soil analyses

The study site was located at 833 m asl at the Research Station of the Instituto de Pesquisas Agronômicas (IPA) in Araripina (7°29′S 40°36′W), Pernambuco State, NE Brazil, in natural vegetation in the semi-arid 'Caatinga' biome. The mean annual rainfall is 742 mm, temperature is 23°C, and humidity is 52%. According to Albuquerque (2008), the native Caatinga vegetation at the site is hyperxerophilic with *Bauhinia cheilantha* (Bong.) Steud., *Croton sonderianus* Müll. Arg., *Spondias tuberosa* Arruda, and *Mimosa malacocentra* (Mart.) Benth. dominating.

Field soil samples (0–20 cm depth) were taken in October 2010 and analyzed for selected chemical soil characteristics as described in Goto et al. (2009). Mean soil pH (H_2O) was 4.7, organic carbon 17.1 g.kg^{-1}, and available P was 63 mg.kg^{-1}.

AMF bait cultures

Soil from Araripina was placed in four 1 L pots under greenhouse conditions at the Department of Mycology, Universidade Federal de Pernambuco (Recife) as described in Mello et al. (2012) with the objective to cultivate and reproduce the native AMF communities. Corn (*Zea mays* L.), *Sorghum sudanense* (Piper) Stapf, *Panicum maximum*

Jacq., and *Helianthus annuus* L. were planted as host plant mixtures in the same pots during three months. For propagation, field samples of the five field soil replicates were combined into one homogenous AMF inoculum. The new fungus sporulated abundantly in two pots co-occurring with AMF species like *Acaulospora sieverdingii*, *A. spinosa*, *Ambispora appendicula*, *Dentiscutata cerradensis*, and *Gigaspora gigantea*. Despite several attempts, single species cultures of the new fungal species could not be established. Voucher specimens were deposited in the herbaria of Eidgenössische Technische Hochschule, Zürich, Switzerland (ZT), and Universidade Federal de Pernambuco, Recife, Brazil (URM).

Morphological analyses

About 200 spores were extracted from the field soils and bait cultures by wet sieving and sucrose centrifugation (Sieverding 1991). The spores were mounted in polyvinyl-alcohol–lactic acid–glycerin (PVLG), in PVLG + Melzer's reagent, and in water, and microscopically examined. The terminology of Oehl et al. (2008, 2011a) for spore morphology and germination of gigasporalean species is followed.

Molecular analyses

PCR reactions were performed on DNA extracted from five surface-sterilized spores (Mosse 1962) isolated from the bait cultures and crushed with a sterile disposable micropestle in an Eppendorf tube with 23 µL of milli-Q water.

A two-step PCR was conducted to amplify the ribosomal fragment consisting of partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA using the primers SSUmAf/LSUmAr and SSUmCf/LSUmBr consecutively according to Krüger et al. (2009). PCR reactions were carried out in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, California) with a pureTaq Ready-To-Go PCR Beads (Amersham Biosciences Europe GmbH, Germany) following manufacturer's instructions with 0.4 µM concentration of each primer. PCR products were analyzed by electrophoresis in 1.2% agarose gels, stained with Gel Red™ (Biotium Inc., Hayward, CA, U.S.A.) and viewed by UV illumination. The expected amplicons were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ), cloned into the pCR2.1 vector (Invitrogen, Carlsbad, California), and transformed into one shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, California). After plasmid isolation from transformed cells, cloned DNA fragments were sequenced with vector primers (White et al. 1990) in both directions by Taq polymerase cycle sequencing on an automated DNA sequencer (Perkin-Elmer ABI Prism 373).

Querying the National Center for Biotechnology Information databases with the BLASTn program, we verified that the sequences obtained from the new fungus were affiliated with the *Gigasporales* (*Glomeromycota*). The AMF sequences (partial LSU rRNA) obtained were aligned with other glomeromycotan sequences from GenBank using the program ClustalX (Larkin et al. 2007) and edited with the BioEdit program (Hall 1999) to obtain a final alignment. The sequences were deposited at GenBank under the accession numbers HF935017, HF935020–HF935022, and HF935024.

Maximum parsimony (MP) and neighbor joining (NJ) analyses with 1000 bootstrap replications were performed using the Phylogenetic Analysis Using Parsimony (PAUP) program version 4 (Swofford 2003). 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. The model of nucleotide substitution (GTR + G) was estimated using Topali 2.5 (Milne et al. 2004). Two sequences from *Pacispora scintillans* (FM876831, FM876832) were used as outgroup.

Results

Taxonomic analyses

Scutellospora alterata Oehl, J.S. Pontes, Palenz., Sánchez-Castro & G.A. Silva, **sp. nov.** FIGS 1–18

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Differs from all other *Scutellospora* spp. by the evanescent papillate surface roughening on the spores.

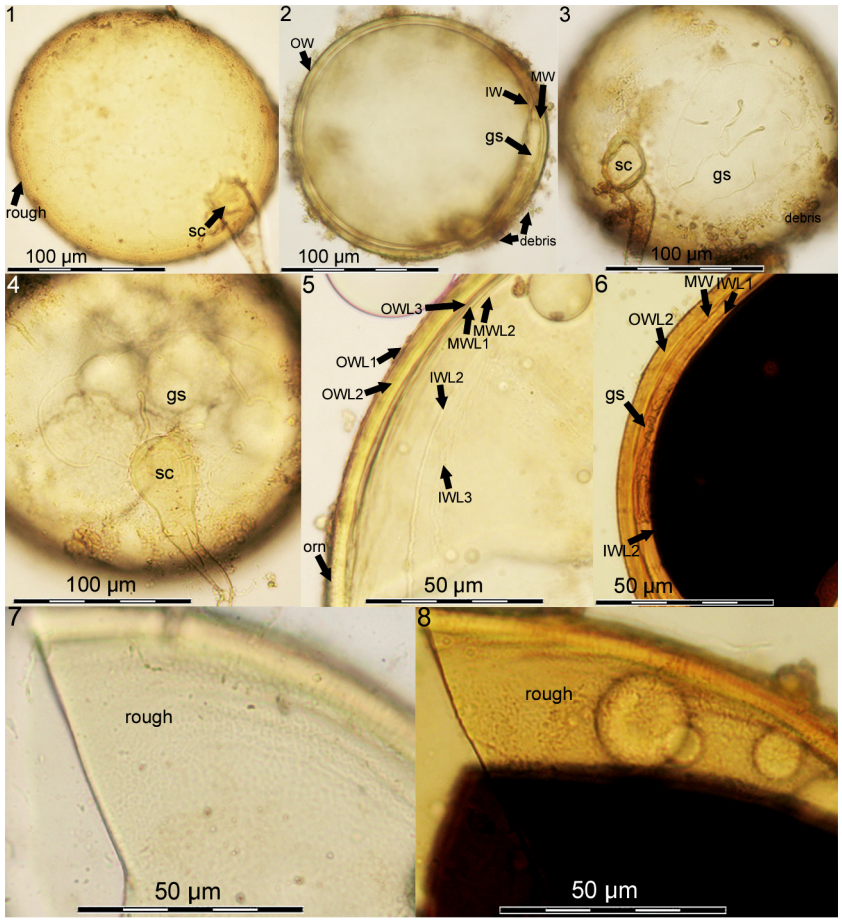
TYPE: Brazil, Pernambuco, Araripina, isolated from pot cultures on plants inoculated with soils from natural Caatinga, 94-9401 (**holotype**, ZT Myc 35647); 94-9402–9410 (**isotypes**, ZT Myc 35648); 94-9411–9413 (**isotypes**, URM 83532–URM 83534).

ETYMOLOGY: *alterata* (altered), referring to the variable germ shield shape, depending on the pressure applied on cover slides.

GLOMEROSPORES are singly formed in soils terminally on single, bulbous suspensor cells (= 'sporogenous' cells). Glomerospores are yellowish white to bright yellow, globose, subglobose to oval or ellipsoid (155–255 × 150–225 µm), and have three walls (an outer, a middle, and an inner wall; OW, MW, and IW) and a bi-lobed, hyaline to subhyaline germination shield.

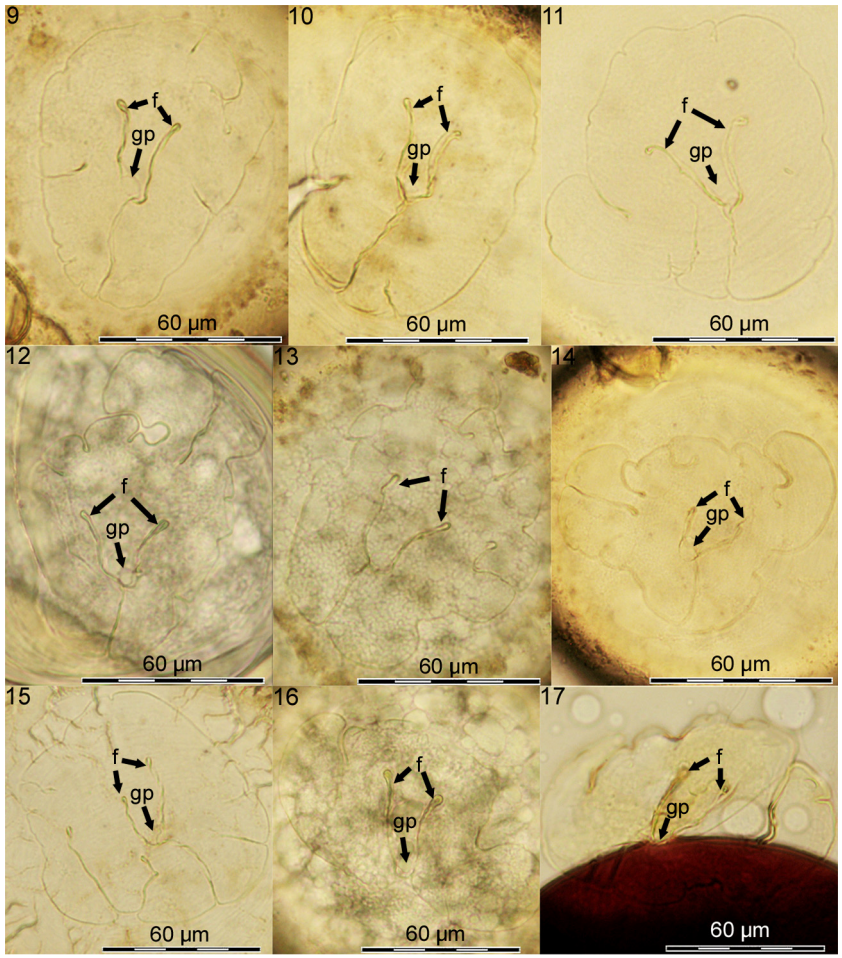
OUTER WALL is 4.0–5.5 µm thick in water, expanding to 7.0–11.0 µm in crushed spores in lactic acid-based mounts when pressure is applied on the cover slide; OW composed of three layers (OWL1–OWL3): the outermost (OWL1) hyaline to subhyaline to light yellow, evanescent to semi-persistent, ~1.0–1.4 µm thick with a papillate surface roughening to which larger debris is often attached, especially while this wall layer is degrading. However, this debris can easily be removed from the spore surface by sonification or slight movements of the spore under the cover slides in liquid or fresh mountants. OWL2 yellowish white to bright yellow, 2.2–3.0 µm thick in H_2O, expanding to 5.2–8.5 µm in lactic acid-based mountants when pressed. OWL3 concolorous with OWL2 or slightly lighter in color, thin, flexible (0.5–1.1 µm thick), and usually difficult to observe as adhering closely to OWL2. OWL2 and OWL3 stain dark yellow in Melzer's reagent. The straight pore channel at the spore base (about 2.8–4.8 µm broad) might be closed by a plug, but often appears to be open.

MIDDLE WALL composed of two hyaline flexible to semi-flexible layers (MWL1, MWL2) that are often difficult to separate. In crushed spores they might show several folds in close vicinity to OW. MWL1 and MWL2 are each about 0.6–1.2 µm thick and may slightly expand in PVLG based mountants.



Figs 1–8. *Scutellospora alterata*, spore morphology: **1–4**. Whitish yellow to light yellow spores formed on sporogenous cells (sc), with three walls (OW, MW, IW) and a single germination shield (gs) on the IW surface. Spore surfaces with papillate roughened (rough) surface often capturing large amounts of debris that can easily be removed through sonification or moving the spores slightly under the cover slides in the first hours after mounting in PVLG. The gs can usually be positioned in planar view through such movements, but often deforms when the oval to ovoid shield shapes (Fig. 3) become irregular and variable (Fig. 4) through pressure. **5–6**. OW triple-layered (OWL1-3), MW bi-layered (MWL1-2), and IW triple-layered (IWL1-3). OW staining dark yellow, and IWL2 purple to deep purple in Melzer's reagent. **7–8**. Papillate roughened spore surface in PVLG and in Melzer's.

Inner wall triple-layered (iwl1- iwl3), bearing a germination shield on the outer surface. Iwl1 hyaline, flexible, 0.4–1.0 µm thin, sometimes difficult to observe; Iwl2 united to finely laminate, 1.4–2.0(–2.7) µm thick; Innermost



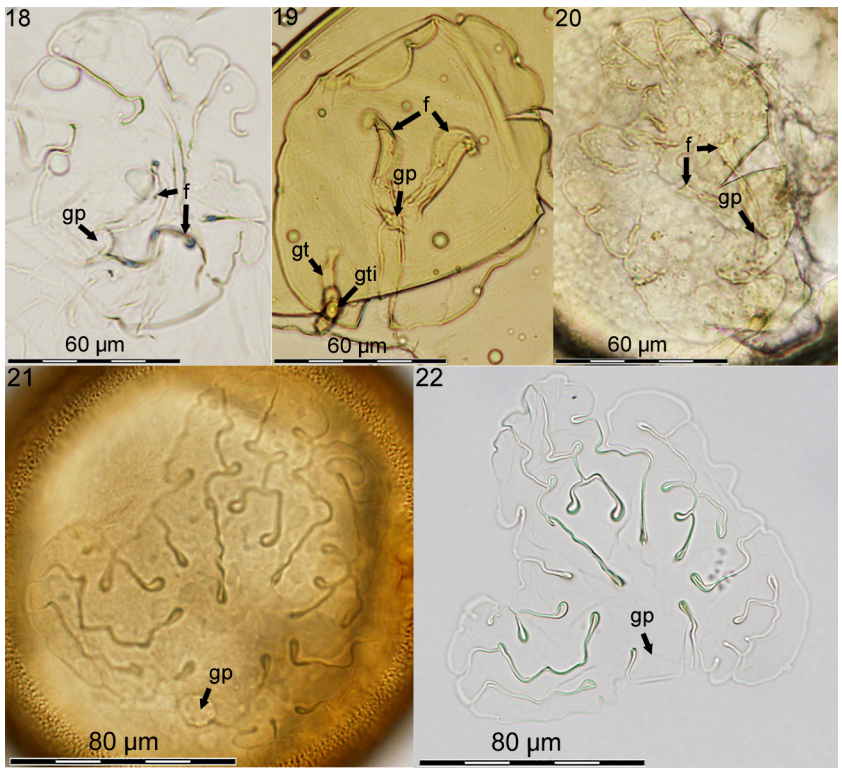
Figs 9–17. *Scutellospora alterata*, variable germination shield morphology. Germ shield is bi-lobed, often oval to ovoid to violin-shaped, bearing a central 'germ pore' (gp) and a 'fork' of two parallel folds (f) separating the lobes. A slight pressure on the spore may cause a few to several 'false' lobes anywhere on the shield periphery. Apparently lobe terminals sometimes do not stop growing close to the central fork and germ pore, but continue recurving laterally (Figs 16–17). However, these processes may also be caused by shield deformation after pressure applied on the spores.

iwl3 flexible, thin (0.4–0.8 µm thick), and often separable from iwl2 when pressure applied on the cover slide and then forming several minute folds. Only iwl2 stains purple to dark purple in Melzer's reagent.

Sporogenous cell globose to elongate, concolorous with or slightly lighter than the spore, 32–50 × 25–41 µm, with 2 wall layers generally visible

(continuous with spore OWL1 and OWL2); OWL1 0.6–1.3 µm thick, evanescent to semi-persistent, roughened as the upper surface of the spore outer wall; OWL2 1.2–2.5 µm thick. Sporogenous hypha bi-layered, 10–26 µm diam tapering to 5–10 µm within 30–120 µm from the sporogenous cell; hyphal wall concolorous with or darker yellow than spore OW, 1.2–2.2 µm tapering to 0.5–1.5 µm. A few (2–5) septa originating from the inner layer might be visible in the hypha.

GERMINATION SHIELD hyaline to subhyaline to (rarely) light yellow, bi-lobed, oval to ovoid (80–100 × 62–80 µm) in uncrushed spores, under pressure becoming irregular (80–130 × 65–125 µm) in crushed or uncrushed spores. Shield has one initial germ pore (GP) at the shield center (in planar view), with two lobes surrounding the two folds of the GP center (= 'fork'). One germ tube initiation (GTIS, 2.2–4.0 µm diam, often difficult to show in photographs) generally positioned towards the end of each lobe. Shield wall thin (0.8–1.6



FIGS 18–22. Deformed bi-lobed germination shields, either on intact or slightly crushed spores or shields completely separated from the spores. **18.** ***Scutellospora alterata***. **19–20**. ***S. calospora*** germination with germ tube (gt) arising from germ tube initiation (gti). **21–22**. ***S. spinosissima*** showing many folds on the shield but no clearly visible gti.

μm), highly flexible, with the shield periphery regularly deforming under light pressure on the cover slide, sometimes even in uncrushed spores, when at any position additional 'false' lobes may arise that are regular to highly irregular in shape but always lacking GTIS.

DISTRIBUTION: Known only from the semi-arid Caatinga biome, in the Municipality of Araripina, in Pernambuco State (NE Brazil).

Spore development

The major stages of spore development could be deduced from clearly identified spores of *S. alterata* found in the bait cultures. First the outer spore wall differentiates one evanescent to semi-persistent outer layer (OWL1), a laminate layer (OWL2), and the adherent thin inner layer (OWL3). The bi-layered middle wall (MW), and later the three-layered inner wall (IW), develop de novo without visible connection with the outer wall. Finally, the germination shield develops on the outer surface of the inner wall as in other *Scutellospora* species (e.g. FIGS 19–22).

Molecular analyses

Phylogenetic analyses of the partial LSU rDNA gene place the fungus within *Scutellospora* in a clade next to *S. calospora*, *S. dipurpurescens*, and *S. spinosissima* (FIG. 23). For the ITS region, the species closest to *S. alterata* were *S. calospora* (92% identity, BLASTn analysis) and *S. spinosissima* (90%). The LSU rDNA sequences were closest to *S. calospora* and *S. dipurpurescens* (both 96% identity). Environmental ITS sequences with closest match to *S. alterata* were found in roots of *Lobelia dortmanna* L. (JN581980, 94% identity; Kohut et al. 2012). The *S. alterata* LSU region was most similar to environmental sequences amplified in roots of *Phellodendron amurense* Rupr. (Heilongjiang, China—EU252109, 96%) and (*Miscanthus sinensis* Andersson (Hokkaido, Japan—AB561124, 96%) and to some sequences obtained from a grassland ecosystem soil (DQ400293, 99%; DQ400277, 97%; Hempel et al. 2007).

Discussion

Scutellospora alterata has two surprising diagnostic features: a) the presence of an evanescent, papillate surface roughening on the spores instead of a smooth surface or persistent surface ornamentation and b) a simple germination shield that may erroneously be interpreted as complex due to the emergence of several 'false' lobes, especially after pressure on the spore. The new fungus can easily be distinguished from all known *Scutellospora* species, since *S. arenicola* (Koske & Halvorson 1990), *S. aurigloba* (Hall 1977, as *Gigaspora aurigloba*), *S. calospora* (Nicolson & Gerdemann 1968, as *Endogone calospora*), *S. dipurpurescens* (Morton & Koske 1988), and *S. tricalypta* (Ferrer & Herrera 1981, as *Gigaspora*

Fig. 23. *Gigasporales*. LSU rDNA-based phylogenetic tree rooted by *Pacispora scintillans*. Sequences are labeled with database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. *Scutellospora alterata* sequences are in bold. Only bootstrap values ≥50% are shown. (Consistency Index = 0.53; Retention Index = 0.82).

tricalypta) all have smooth spore surfaces, and *S. crenulata* (Herrera-Peraza et al. 2001), *S. dipapillosa* (Koske & Walker 1985, as *Gigaspora dipapillosa*), and *S. spinosissima* (Walker et al. 1998, Silva et al. 2013) have either double ornamentations or permanent spines on the spore surfaces (Oehl et al. 2008, 2011e).

Roughened spore surfaces occur quite commonly in glomoid species (e.g., *Viscospora viscosa*; Oehl et al. 2011b) but have rarely been found in other glomeromycotan groups, e.g., in *Ambispora granatensis* (Palenzuela et al. 2011). In the *Gigasporales*, *Dentiscutata cerradensis* is known to have papillae on the spore surface (Spain & Miranda 1996) that become invisible in lactic acid-based mountants, whereas the *S. alterata* papillae generally remain visible on spores immersed in lactic acid-based mountants.

Scutellospora species are characterized by hyaline shields that possess only two 'true' lobes, each generally bearing one GTI when mature. The observation that additional, so-called 'false' lobes may arise on germination shield peripheries through pressure on the spore surface is interesting, because the phenomenon has not been reported previously. Certainly, such a feature has never been so obvious and the extent and variability of the shield alterations might be unique for *S. alterata*. Nonetheless, this distortion can also be recognized to a lesser extent in type specimens of *S. dipurpurescens* (OSC), and we also found such characteristics in a *S. calospora* isolate from Germany (FIGS 19–20) and in *S. spinosissima* (FIGS 21–22), which Walker et al. (1998) and Oehl et al. (2008) at first erroneously interpreted as containing more lobes. Indeed, especially in *S. spinosissima,* multiple folds and 'false' lobes can sometimes be seen beneath cover slides when shields are exposed to pressure or lateral movements in liquid lactic acid mountants. Shield morphology has not yet been analyzed sufficiently in the *Scutellosporaceae* (including *Orbispora*), and we feel that such investigations should be carried out soon.

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