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Orbispora gen. nov., ancestral in the *Scutellosporaceae* (*Glomeromycetes*)

Fritz Oehl[1*], Danielle Karla Alves da Silva[2], Leonor Costa Maia[2], Natália Mirelly Ferreira de Sousa[2], Helder Elísio Evangelista Vieira[2] & Gladstone Alves da Silva[2]

[1]*Federal Research Institute Agroscope Reckenholz-Tänikon ART, Ecological Farming Systems, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland*

[2]*Departamento de Micologia, CCB, Universidade Federal de Pernambuco, Av. Prof. Nelson Chaves s/n, Cidade Universitária, 50670-420, Recife, PE, Brazil*

* Correspondence to: *fritz.oehl@art.admin.ch*

Abstract — *Scutellospora pernambucana* and *S. projecturata* are transferred into *Orbispora,* our proposed new genus in the arbuscular mycorrhiza-forming *Scutellosporaceae* (*Glomeromycota*). *Orbispora* is recognized by spores formed terminally on sporogenous cells and three spore walls with single mono-lobed hyaline to subhyaline germination orbs on the inner 'germinal' wall that appear identical to the orbs in *Kuklospora colombiana*, *K. kentinensis,* and a few *Acaulospora* species. DNA sequence analyses show the two *Orbispora* species as ancestral within the sporogenous cell-forming *Glomeromycetes.* An updated key summarizes the morphological differences among species in the *Scutellosporaceae*.

Key words — evolution, *Gigasporineae*, *Gigasporaceae*, molecular phylogeny, rDNA

Introduction

Recently, the genus *Scutellospora* C. Walker & F.E. Sanders 1986 was revised, and *Gigasporaceae* sensu lato was divided into four families based on phylogenetic data congruent with spore wall morphology (especially that of germination shields) and spore germination: *Gigasporaceae*, *Scutellosporaceae*, *Racocetraceae* and *Dentiscutataceae* (Oehl et al. 2008, Goto et al. 2010a). *Scutellosporaceae* comprised a single genus, *Scutellospora.* Two species of the revised genus were found to have three spore walls and mono-lobed, orbital, hyaline to subhyaline germination orbs on the inner, 'germinal' wall, with a single germ tube initiation from where one to (rarely) two germ tubes emerge during germination to penetrate the outer walls (Silva et al. 2008; Oehl et al. 2008). The fact that such germ orbs and germination features were known previously only

for a few ancestral species in the *Acaulosporaceae* — e.g., *Kuklospora colombiana* (Spain & N.C. Schenck) Oehl & Sieverd. 2006, *K. kentinensis* (C.G. Wu & Y.S. Liu) Oehl & Sieverd. 2006, *Acaulospora mellea* Spain & N.C. Schenck 1984 (Spain 1992, Schenck et al. 1984, Sieverding & Oehl 2006, Silva et al. 2008) — suggesting a close relationship between *Acaulosporaceae* and *Scutellosporaceae* sensu Oehl et al. 2008 (e.g. Silva et al. 2008).

During the current study we investigated whether these two species, *Scutellospora pernambucana* and *S. projecturata*, might be ancestral within *Scutellosporaceae*. To test this hypothesis, partial LSU rDNA sequences were generated for *S. pernambucana*, and the phylogenetic position of *S. projecturata* was analyzed from almost complete 18S rDNA sequences obtained from public databases. Because our hypothesis was confirmed and morphological and phylogenetic analyses are congruent, we segregate the two species from the other *Scutellospora* species that also have hyaline to subhyaline, but bi-lobed and generally violin-shaped, germination shields, and we transfer both species into a new genus within the *Scutellosporaceae*.

Material & methods

Morphological analyses

Oehl et al. (2008) and Silva et al. (2008) published the morphological analyses relevant for this study. *Scutellospora pernambucana* (type species for the new genus) was found in several sites of the Caatinga and Mata Atlantica biomes (e.g. Goto et al. 2010b) in Igarassu, Araripina, São Bento do Una, and Abreu e Lima (all Pernambuco State), São Luis (Maranhão State), and Mataraca (Paraíba State) in NE Brazil. Spores were extracted from several recent collections between 2008 and 2010 at the type locality in the 'Zambana' tropical forest fragment (Usina São José, Igarassu, Pernambuco State, Silva et al. 2008) as described in Sieverding (1991). Although single species cultures were established as described in Tchabi et al. (2010), Palenzuela et al. (2010), and Goto et al. (2011), all trials failed so far. Thus, spores isolated directly from type collection field samples were used for molecular analyses. Neither living spores nor type material of *S. projecturata* were accessible to us.

Molecular analyses

DNA extraction: DNA was extracted from spores of *S. pernambucana* collected from the type location as described in Goto et al. (2011). Spores were rinsed in distilled water, sonicated 2–3 times for 1 min, crushed in 50 µl 10× Taq™ polymerase chain reaction (PCR) buffer (750 mM Tris–HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20; Fermentas), centrifuged at 5,000 rpm for 5 min, and the supernatant incubated at 95°C for 10 min. After extraction, the DNA was stored at –20°C.

Amplification and sequencing: DNA extract was used as template for a semi-nested PCR using primers ITS3 (White et al. 1990) - 28G2 (Silva et al. 2006) and LR1 (van Tuinen et al. 1998) - 28G2 consecutively. PCR reactions were carried out in 50 µl batches of 75 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 200 µM

each dNTPs, 1μM of each primer, and 2 units of Taq™ DNA polymerase (Fermentas); cycling parameters were 5 min at 95°C (1 cycle), 45s at 94°C, 1 min at 55°C, 1 min at 72°C (40 cycles), ending with a final 7-min elongation at 72°C. The amplified products were purified with a PureLink PCR Purification Kit (Invitrogen), sequenced directly or cloned with a CloneJET™ PCR Cloning Kit (Fermentas) following the manufacturer's instruction, and sequenced. Sequencing was provided by the Human Genome Research Center (São Paulo, Brazil).

Sequence alignments: A BLASTn search of the National Center for Biotechnology Information databases verified that the sequence obtained from *S. pernambucana* was affiliated to the *Scutellosporaceae* (*Glomeromycota*). AM fungal sequences (partial LSU rRNA) obtained in our laboratory were aligned with other glomeromycotean sequences from GenBank using ClustalX (Larkin et al. 2007) and in BioEdit (Hall 1999) to obtain a final alignment. The sequences were deposited at GenBank under the accession numbers HQ871519, JF965445, and JF965446. Partial SSU rRNA sequences (obtained from National Center for Biotechnology Information-NCBI) were also aligned as described above.

Phylogenetic analyses: The phylogeny was reconstructed by independent LSU and SSU rRNA analyses. The nucleotide substitution model was estimated using Topali 2.5 (Milne et al. 2004). Maximum likelihood (ML) analysis was performed in PhyML (Guindon & Gascuel 2003), launched from Topali 2.5, using the GTR+G and GTR+G+I models for LSU and SSU rRNA, respectively. Neighbor-joining analysis (established with the same model used to construct the ML tree) was performed using PAUP*4b10 (Swofford 2003). *Pacispora scintillans* (S.L. Rose & Trappe) Sieverd. & Oehl ex C. Walker et al. 2007 was used as outgroup.

Results

Molecular analyses

The LSU and SSU rRNA phylogenetic analyses (Figs. 1–2) support two clear clades in *Scutellosporaceae*. The molecular trees show the clade containing *S. pernambucana* and *S. projecturata* basal to the *Scutellospora* clade, supporting the hypothesis that species with orb-like, coiled germination shields represent an ancestral genus within the sporogenous cell-forming *Glomeromycota*.

New genus in *Scutellosporaceae*

Orbispora Oehl, G.A. Silva & D.K. Silva, **gen. nov.**

MycoBank MB 519533

Sporae terminaliter efformatae anguste adiacetae ad cellulas sporogeneas; cum tunicis tribus; scutellum germinale coniunctum ad tunicam interiorem, orbiforme, hyalinum ad alboflavum, mono-lobatum cum una depressione germationis; formans structuras mycorrhizarum arbuscularum.

Type species: *Orbispora pernambucana* (Oehl et al.) Oehl et al.

Etymology: *orbis* (Latin: circle, orb), and *spora* (Latin: spore) referring to the mono-lobed, coiled, orb-like germination shield of the spores.

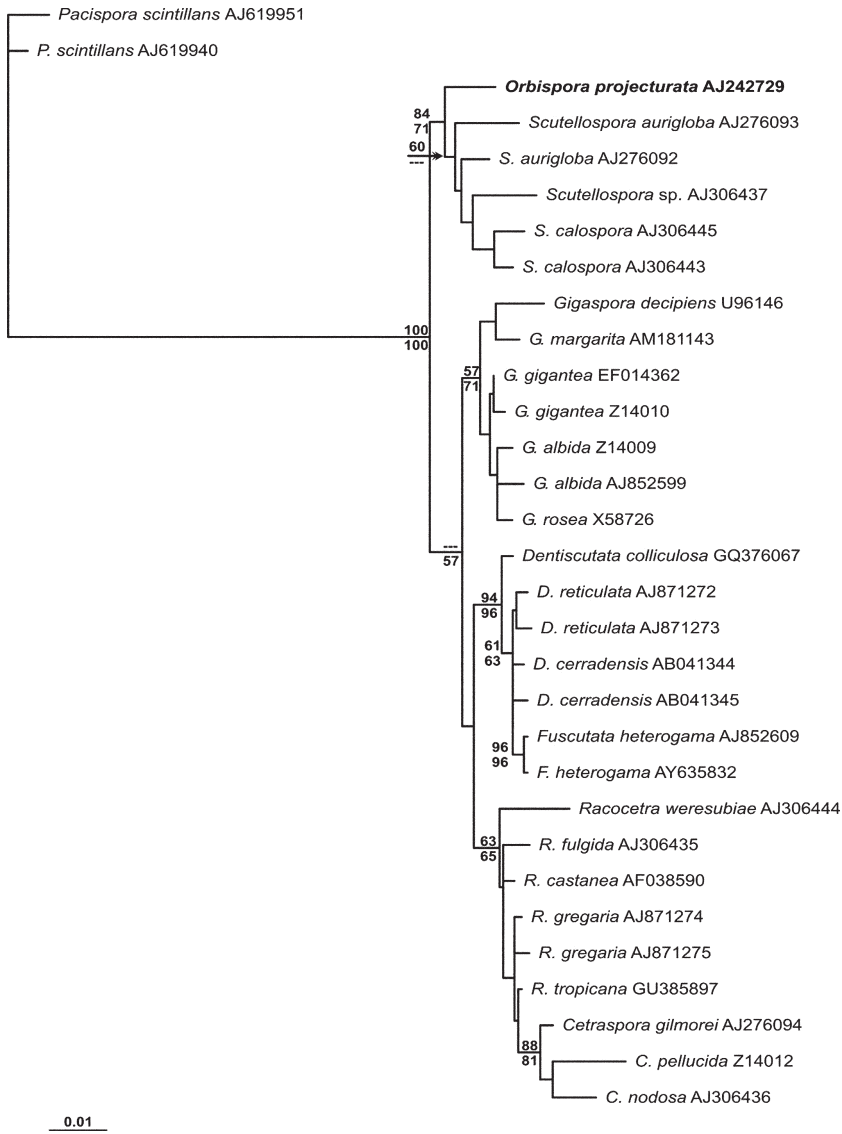

Fig. 1. Phylogenetic reconstruction of the *Gigasporales* obtained from partial SSU rDNA sequences (~1800 bp). Bootstrap values (in %) are from neighbor-joining (NJ) and maximum likelihood (ML) analyses (1000 bootstraps). Only topologies with bootstrap values of at least 50% are shown.

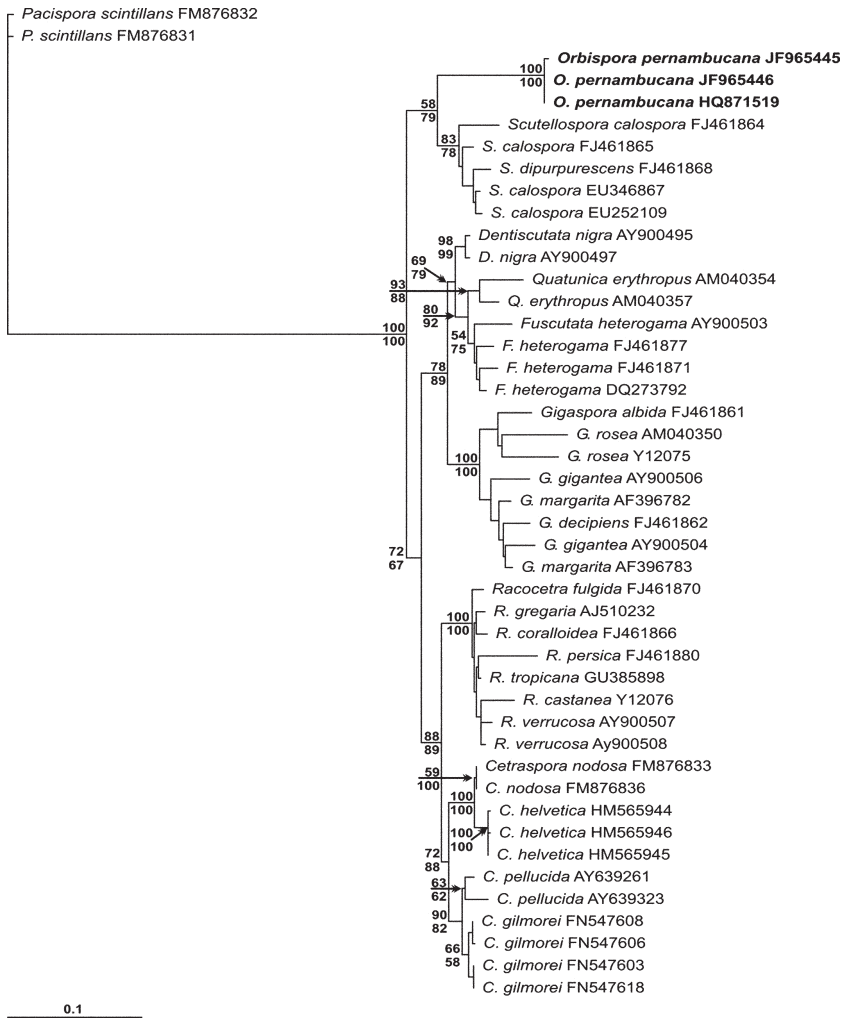

Fig. 2. Phylogenetic reconstruction of the *Gigasporales* obtained from partial LSU rDNA sequences (~700 bp). Bootstrap values (in %) are from neighbor-joining (NJ) and maximum likelihood (ML) analyses (1000 bootstraps). Sequences are labeled with their database accession numbers. Only topologies with bootstrap values of at least 50% are shown.

Key characters: Sporocarps unknown. Spores formed on sporogenous cells that form terminally on hyphae arising from mycelia in soil. Outer spore wall (ow) generally (2–)3-layered and continuous with the wall of the sporogenous cell. Two hyaline walls (middle wall 'mw' and inner wall 'iw') form de novo during spore formation and have 1–2 and 2–3 layers, respectively. A germination orb is formed on the outer iw surface or between the outer and the subsequent layer of iw. Germination orb is transparent, or hyaline to subhyaline, seldom light yellow, mono-lobed; coiled and then, either circular or apparently broad ellipsoid to rarely irregular; with one rounded germ tube initiation in the outer periphery of the lobe. One (rarely two) germ tube arises from this gti to penetrate the outer spore wall layers. Forming typical arbuscular mycorrhizae.

Orbispora pernambucana (Oehl, D.K. Silva, N. Freitas & L.C. Maia) Oehl, G.A. Silva & D.K. Silva, **comb. nov.**

MycoBank MB 519535

≡ *Scutellospora pernambucana* Oehl, D.K. Silva, N. Freitas & L.C. Maia, Mycotaxon 106: 363. 2009 ['2008'].

Orbispora projecturata (Kramad. & C. Walker) Oehl, G.A. Silva & D.K. Silva, **comb. nov.**

MycoBank MB 519550

≡ *Scutellospora projecturata* Kramad. & C. Walker, Annals Bot. 86: 22. 2000.

Dichotomous key to species in *Scutellosporaceae*

1. Spores with mono-lobed, coiled germination orbs 2
1. Spores with non-coiled germination shields 3
2. Spore wall non-ornamented, outer wall strongly expanding in lactic acid based mountants; spores dark yellow to brown yellow (to yellow brown), 105–150 µm in diam ..... *Orbispora pernambucana*
2. Outer spore wall with 2.0–4.0 µm long protuberances formed by the structural, laminated layer, spores golden yellow to ochraeous to sienna, 100–180 µm in diam ..... *O. projecturata*
3. Spores with bi-lobed, violin-shaped to oval germination shields 4
3. Germination shield 1–(2)-lobed, oval to ellipsoid to irregular, with infolds of the same lobe; spores with warted projections having central secondary projections in the tip; spores cream to yellow, 100–170 µm in diam ..... *S. crenulata*
4. Spore walls not ornamented 5
4. Spore walls with two types of ornamentation: small conical warts and blunt, bacilliform larger projections; spores pale orange brown to dark orange brown, 130–180 µm diam ..... *Scutellospora dipapillosa*
5. Spores light colored: subhyaline, pink, creamy, straw to greenish or brownish yellow 6
5. Spores yellow brown to black 7

6. Spores subhyaline to creamy to pale straw to greenish yellow 8
6. Spores dark yellow to golden yellow to brown yellow; spores golden yellow to dull yellow, (130–)200–420(–520) µm diam . . . . . *S. aurigloba*
7. Spores yellow brown to orange-brown, 160–360 µm diam, outer and innermost walls staining strongly in Melzer's reagent . . . . . .*S. arenicola*
7. Spores dark brown to black, 300–460 µm diam . . . . . *S. tricalypta*
8. Spores subhyaline to pale straw to pale greenish-yellow, (100–)150–300(–500) µm diam, generally ellipsoid to oblong, with 2-layered middle wall . . . . . .*S. calospora*
8. Spores yellow to greenish-yellow; 140–240 µm diam, generally globose to subglobose, with one layered middle wall. . . . . .*S. dipurpurescens*

Discussion

Orbispora species can easily be separated from other species in the *Scutellosporaceae* by the germination shield that is coiled, orb-like, and mono-lobed with one gti, in contrast to *Scutellospora* germination shields, which are regularly bi-lobed, and generally violin-shaped to (rarely) oval. Morphological analyses (Kramadibrata et al. 2000, Silva et al. 2008, Oehl et al. 2008) are congruent with our phylogenetic analyses, supporting our hypothesis that *Orbispora* is ancestral within *Scutellosporaceae*, and thus also ancestral within all families of the sporogenous cell-forming *Glomeromycetes,* which have been transferred in this volume to the new order *Gigasporales* (Oehl et al. 2011).

This study renders the family *Scutellosporaceae* (sensu Oehl et al. 2008) bi-generic based on molecular and spore morphological observations. The phylogenetic position of some *Scutellospora* species, however, remains obscure due to lack of molecular data, the rudimentary descriptions of the germ shields, and the unavailability of good type material (e.g., for *S. tricalypta* (R.A. Herrera & Ferrer) C. Walker & F.E. Sanders 1986, *S. arenicola* Koske & Halvorson 1990, and — above all — *S. crenulata* R.A. Herrera et al. 2001). We retain these species in *Scutellospora*, as they all appear to have hyaline and bi-lobed germ shields. However, this might be a mistake, at least for *S. crenulata* whose germination shield, although still insufficiently described, appears to be mono- rather than bi-lobed, but not orb-like or coiled (Oehl et al. 2008).

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