


Sacculospora felinovii, a novel arbuscular mycorrhizal fungal species (Glomeromycota) from dunes on the west coast of India

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Abstract During an arbuscular mycorrhiza fungal spore survey on a primary coastal sand-dune system in Goa on the west coast of India, entrophosporoid spores tightly covered with a dense hyphal mantle were recovered. When intact, the spores, at first sight, seemed to be identical in morphology to those of *Sacculospora baltica* (originally described as *Entrophospora baltica*) extracted from Polish maritime sand dunes and, to date, the sole member of the recently described genus *Sacculospora* in the new family Sacculosporaceae, phylum Glomeromycota. Later detailed morphological studies indicated that both fungi produce two-walled spores but the structure and phenotypic features of components of the outer spore wall in the novel fungus differ considerably from those of *S. baltica*. Differences between the fungi were subsequently confirmed in the phylogenetic analysis of SSU–ITS–LSU nrDNA sequences. Consequently, we describe the novel species as *Sacculospora felinovii* **sp. nov.**

Keywords Entrophosporoid spore · Molecular phylogeny · Morphology · Tropical coastal dunes

Introduction

Of the ca. 280 described arbuscular mycorrhizal fungal (AMF) species of the phylum Glomeromycota C. Walker & A. Schüßler, only five form spores inside the neck of a sporiferous saccule (Błazkowski 2012). From one side, the neck is continuous with a mycorrhizal extraradical hypha and from the other side, the neck passes into a globose to ellipsoidal saccule. Contents from both structures are used in spore genesis. In fully developed specimens, the saccule is usually empty and frequently detached from spores and the neck wall is continuous with the outermost spore wall layer, forming the spore surface (Błazkowski 2012). This mode of spore synthesis was first described by Ames and Schneider (1979) in a species originally named *Glomus infrequens* I.R. Hall (Hall 1977). Ames and Schneider (1979) subsequently transferred *G. infrequens* to a newly erected genus *Entrophospora* R.N. Ames & R.W. Schneid., with *E. infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid. as type species. The other four species accommodated in *Entrophospora* were *E. baltica* Błazk., Madej & Tadych, *E. colombiana* Spain & N.C. Schenck, *E. kentinensis* C.G. Wu & Y.S. Liu and *E. schenckii* Sieverd. & S. Toro (Schenck et al. 1984; Sieverding and Toro 1987; Wu et al. 1995; Błazkowski et al. 1998). Such a mode of spore formation and the spores themselves have been named entrophosporoid (Goto and Maia 2006).

From observed differences in the subcellular structure of spores and the phenotypic and histochemical characters of components of spore walls of *Entrophospora* species, Sieverding and Oehl (2006) transferred three of the species to two newly erected genera, *Intraspora* Oehl & Sieverd., with

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I. schenckii (Sieverd. & S. Toro) Oehl & Sieverd. (formerly *E. schenckii*) in the family Archaeosporaceae J.B. Morton & D. Redecker, and *Kuklospora* Oehl & Sieverd., with *K. colombiana* (Spain & N.C. Schenck) Oehl & Sieverd. (formerly *E. colombiana*) and *K. kentinensis* (C.G. Wu & Y.S. Liu) Oehl & Sieverd. (formerly *E. kentinensis*) in the family Acaulosporaceae J.B. Morton & Benny. Kaonongbua et al. (2010) and Schüßler and Walker (2010) rejected *Kuklospora* and *Intraspora*, respectively, later substantiated by the phylogenetic analyses of Krüger et al. (2012). Oehl et al. (2011) concluded from phylogenetic analyses of nrDNA sequences that *E. infrequens* and *E. baltica* differ at the family level and, therefore, erected *Sacculosporaceae* Oehl et al. fam. nov., with *Sacculospora* Oehl et al. gen. nov. and *S. baltica* (Błaszcz., Madej & Tadych) Oehl et al. comb. nov.

To date, the genus *Sacculospora* has been represented by *S. baltica* alone. Morphologically, *S. baltica* differs clearly from all other known species forming entrophosporoid spores. The most conspicuous structure is the hyphal mantle tightly covering spores. The spore subcellular structure, which is complex and difficult to define, further distinguishes the species. According to Błaszczowski et al. (1998), *S. baltica* spores contain two spore walls: an outer wall with four layers and an inner three-layered wall. Oehl et al. (2011) stated that layer 1 of the inner wall sensu Błaszczowski et al. (1998) clearly separates from the other layers of the wall and, therefore, redefined the subcellular structure of *S. baltica* spores as three-walled. However, neither Błaszczowski et al. (1998) nor Oehl et al. (2011) performed ontogenetic studies of the species, characterising the subcellular structure of *S. baltica* spores based only on the spatial grouping of its components in crushed specimens. Importantly, the spatial arrangement of spore subcellular components in AMF may be influenced by the vigour of spore crushing (Błaszczowski, pers. observ.). Study of the ontogeny of *S. baltica* spores is severely hindered for two reasons. First, the fungus is difficult to grow in culture. In the literature, there is no report that the species has been grown in single-species cultures and numerous efforts to obtain such cultures by J. Błaszczowski failed (Błaszczowski et al. 1998; Błaszczowski 2012). The ontogeny of any AMF may be known with certainty only by the examination of specimens originating from single-species cultures. Second, the disclosure of sequences of differentiation and organisation of the spore subcellular components may be difficult, or impossible, because of the obscuring influence of the dense and coloured hyphal mantle.

Whilst investigating AMF spore density and diversity on an interrupted belt transect across a primary dune system on the coast of Goa, India (73°40'33" E, 14°53'54" N), AMF spore specimens were found that seemed identical to those of *S. baltica*. Detailed morphological studies of the spore subcellular structure, however, and the phenotypic and histochemical characters of components of spore walls of the

specimens, indicated that traits in the outer spore wall differed. Unsure if the differences represented intraspecific variability of one organism, a comparison was made using molecular methods. The molecular analyses confirmed the previous conclusions resulting from morphological observations that the novel fungal species differs considerably from *S. baltica*. Consequently, the fungus is described as *Sacculospora felinovii* sp. nov.

Materials and methods

Extraction of spores, establishment and growth of trap and single-species cultures

Spores were extracted by the wet-sieving and decanting method (Gerdemann and Nicolson 1963) from field-collected samples of rhizosphere soil of the perennial C₄ *Zoysia matrella* (L.) Merr. (Poaceae) and incorporated into pot trap cultures, 12 cm diam., 9 cm high (1018 cm³). The pots were maintained outdoors at temperatures of 18–30 °C for 10 months at Goa University. Host plants were *Z. matrella*, rhizomes transferred from the dunes, along with stem cuttings of *Solenostemon scutellarioides* (L.) R.Br. (Lamiaceae), cultured in unsterilised *Z. matrella* rhizosphere soil. Plants were watered 2–3 times a week. No nutrients were applied during the growing period. Subsequently, spores (ca. 200) extracted from the trap culture were placed in pots at the base of holes into which stem cuttings of *S. scutellarioides* were inserted. The medium was sterilised (180 °C for 12 h × 3) dune sand. The plants were raised in a 'closed' greenhouse (i.e. no ventilation, reducing risk of outside contamination) at Goa University. Hoaglands solution minus P was applied fortnightly. After 8 months, single-species spore cultures were achieved and harvested. This two-stage method follows that of Dr. B. F. Rodrigues, Goa University (unpublished), demonstrating successful single-species culture where, all too often, novel species culture fails. Nevertheless, only one of two initial cultures sporulated and only one of two subsequent cultures has sporulated but in less abundance. The morphological and histochemical features of mycorrhizal structures revealed following staining in Trypan blue (Phillips and Hayman 1970) testified that they represent one species of AMF. In addition, in the cultures, no other spore morphotypes were found among the characteristic spores of the new species.

Microscopy and nomenclature

The morphological features of spores and the phenotypic and histochemical characters of spore wall layers were determined after examination of at least 100 spores mounted in water, lactic acid, polyvinyl alcohol/lactic acid/glycerol (PVLG; Omar et al. 1979) and a mixture of PVLG and Melzer's

reagent (1:1, v/v). The preparation of spores and mycorrhizal structures for study and photography is described by Błaszowski (2012) and Błaszowski et al. (2012). Descriptions of spore wall layers follow definitions from Błaszowski (2012), Stürmer and Morton (1997) and Walker (1983). Colour names are from Kornerup and Wanscher (1983). Nomenclature of fungi and the authors of fungal names are from the Index Fungorum website <http://www.indexfungorum.org/AuthorsOfFungalNames.htm>. Voucher specimens were mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides and deposited at ETH Zurich, Switzerland (Z + ZT; holotype), the Botany Department, Goa University, the Department of Ecology, Protection and Shaping of Environment (DEPSE), West Pomeranian University of Technology, Szczecin, and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA (isotypes).

DNA extraction, polymerase chain reaction, cloning and DNA sequencing

Crude DNA was extracted from eight single spores. Procedures prior to polymerase chain reactions (PCRs), the conditions and primers used in the PCRs to obtain SSU–ITS–LSU nrDNA sequences, and cloning and sequencing were as those described in Błaszowski et al. (2013). The sequences were deposited in GenBank (KX345938–KX345943).

Sequence alignment and phylogenetic analyses

Comparisons made between sequences of the novel fungus and those listed after BLAST enquiries indicated that the species belongs in the Glomeromycota. The closest relatives are firstly *S. baltica*, and then a number of *Acaulospora* spp. and *Pacispora scintillans* (S.L. Rose & Trappe) Sieverd. & Oehl. A set was, therefore, established comprising six sequences each of the new species and *S. baltica* and three to four sequences each of seven *Acaulospora* spp., including two forming entrophosporoid spores, i.e. *A. colombiana* and *A. kentinensis*. *Pacispora franciscana* Sieverd. & Oehl and *P. scintillans* served as the outgroup taxa. The sequences covered the SSU–ITS–LSU nrDNA segment in all species except for two LSU sequences of *A. colombiana*, two SSU sequences of *P. franciscana* and one SSU sequence of *P. scintillans*. The sequences were aligned and analysed phylogenetically by the method described and justified by Błaszowski et al. (2015a, b). In Bayesian (BI) phylogenetic analysis, GTR + G and two-parameter Markov (Mk2 Lewis) models were applied for the established nucleotide partitions and indel matrices, respectively. GTR + G was estimated as the best-fit model by Modeltest 3.7 (Posada and Buckley 2004). Maximum likelihood (ML) analysis was carried out with the raxmlGUI

(Silvestro and Michalak 2012) implementation of RAxML (Stamatakis 2006), with GTRGAMMA for DNA and the default set for binary (indel) characters. The generated phylogenetic trees were visualised and edited in MEGA6 (Tamura et al. 2013).

Results

General data and phylogeny

The established set of SSU–ITS–LSU nrDNA sequences, including those of the putative new species, had a length of 1929 characters, 100 % of which were informative. The six PCR-generated new-species sequences showed only 1 % variability. Bayesian and ML phylogenetic analyses of the sequence set fully supported the morphological indication that the fungus is a glomeromycotean species not previously described, and the closest relative is *S. baltica* (Fig. 1). The clade with the new species described below as *S. felinovii* and the clade with *S. baltica*, as well as the node linking them, received full (1.0 and 100 %) supports. The node linking the Sacculosporaceae and the Acaulosporaceae clades was also fully supported in both analyses. The trees generated following the BI and ML analyses had identical topologies.

Taxonomy

Sacculospora felinovii Willis, Błasz., T. Prabhu, Chwat, Góralaska, Sashidhar, Harris, J. D'Souza, Vaingankar & Adholeya, **sp. nov.** Figs. 2 and 3

Mycobank No. MB 817258

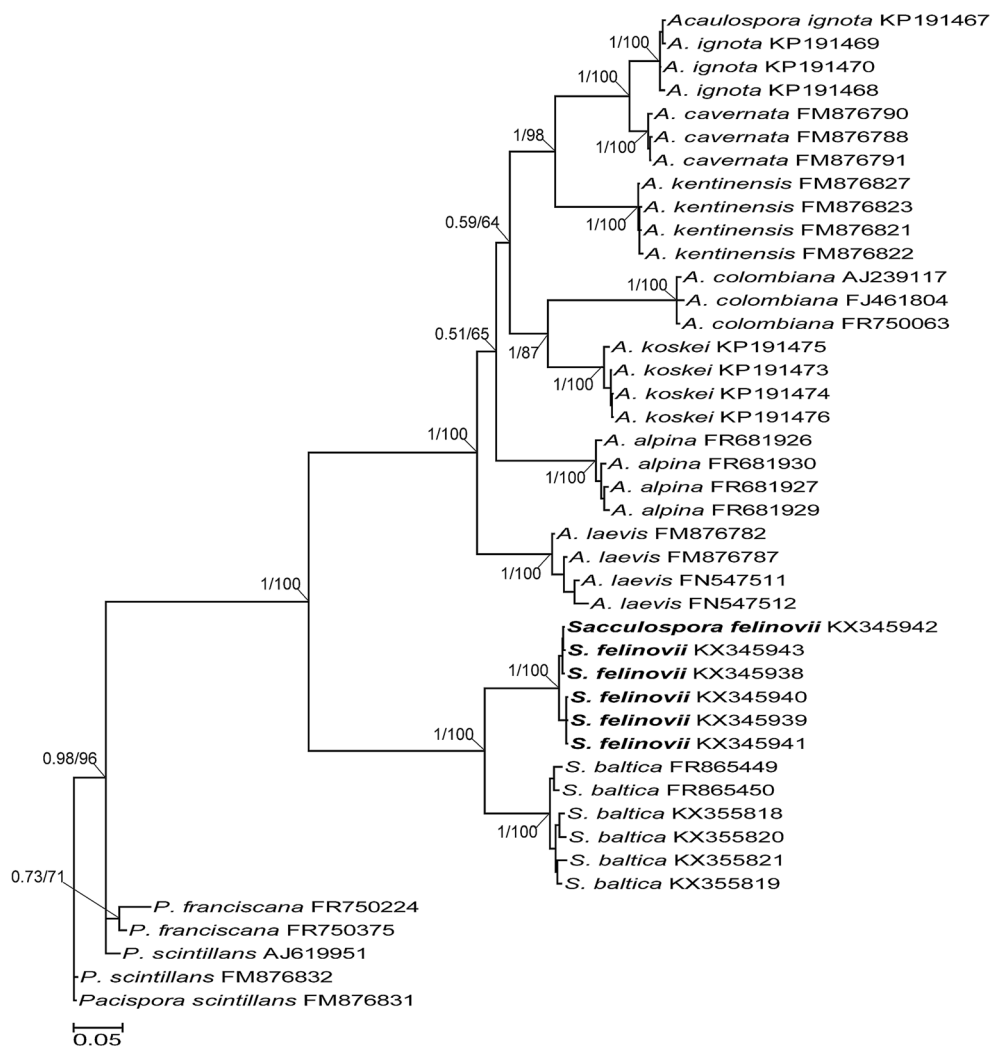
Holotype: ZT Myc 55704 (Z + ZT), isotypes: 3517–3530 (DEPSE), and OSC 154006, OSC 154007 (OSC).

The holotype and isotype spore specimens come from single-species culture with *S. scutellarioides* as host plant grown at Goa University, India. The spores were collected on 14/04/2015 by Ms. Prabhu.

Etymology The specific epithet is respectfully given in honour of Prof. Bernard Felinov Rodrigues, Botany Department, Goa University, India, who has dedicated more than 25 years to the study of AMF.

Sporocarps unknown. *Spores* are entrophosporoid arising inside the neck of a sporiferous saccule, occurring singly in soil, covered with (less often the saccules) a hyphal mantle (Fig. 2a); pale yellow (4A3) to greyish orange (5B5); globose to subglobose (100–)171(–230) µm diam; rarely ovoid (140–170 × 190–210 µm). *Mantle* hyaline to brownish yellow (5C8), 15–50 µm thick when seen in cross view, composed of hyaline to pale yellow (4A3) tightly interwoven hyphae; hyphae straight or twisted with numerous branches inclined at different angles, 4.0–8.0 µm wide, walls 1.3–2.3 µm thick

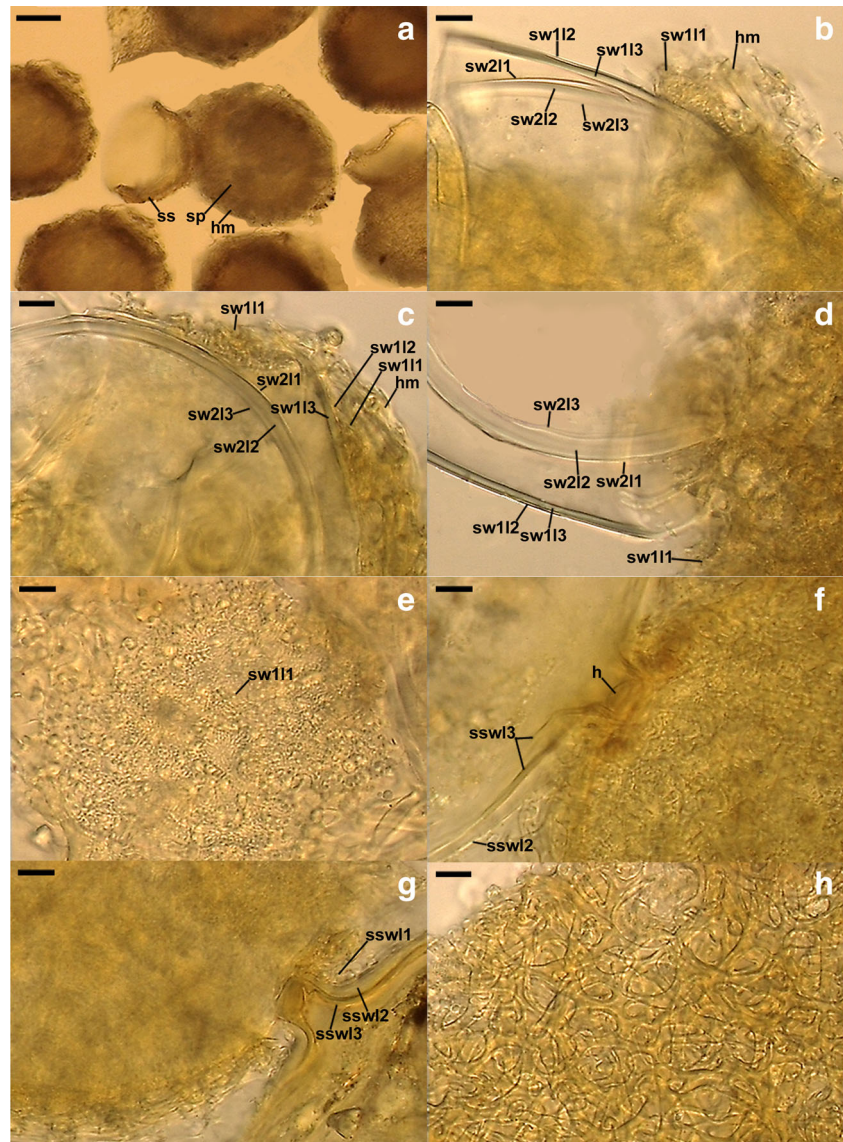
Fig. 1 50 % majority rule consensus phylogram inferred from a Bayesian analysis of SSU–ITS–LSU rDNA sequences of *Sacculospora felinovii* among seven known *Acaulospora* spp. and *S. baltica*. *Pacispora* spp. served as the outgroup. Sequences of *S. felinovii* are in **bold** and are followed by their GenBank accession numbers. The Bayesian posterior probabilities ≥ 0.50 and ML bootstrap values ≥ 50 %, respectively, are shown near the branches. The bar indicates 0.05 expected change per site per branch



(Fig. 2a–c, h). *Spore wall structure* composed of two walls, spore walls 1 and 2 (Figs. 2 and 3). *Spore wall 1* (Fig. 2b–d) consists of three layers (spore wall 1 layers 1–3). Layer 1 permanent, flexible to semi-flexible, pale yellow (4A3) to pastel yellow (4A4), (1.3–)1.5(–1.8) μm thick, the outer surface ornamented with evenly distributed warts, 1.0–4.0 μm wide at the base, 1.8–3.3 μm high; layer 1 loosely associated with layer 2, frequently easily separating from it in crushed spores (Fig. 2b–e). Layer 2 uniform (not divided into visible sublayers), semi-flexible, smooth, (0.8–)1.1(–1.5) μm thick, rarely separating from layer 3 in even vigorously crushed spores (Fig. 2b–d). Layer 3 permanent, uniform, rigid, sometimes cracking in vigorously crushed spores, (1.5–)2.6(–4.0) μm thick, edges almost black in polarised light (Fig. 2b–d). *Spore wall 2* (Fig. 2b–d) composed of three uniform, smooth, hyaline layers (spore wall 2 layers 1–3). Layer 1 flexible to semi-flexible, (1.0–)1.3(–1.5) μm thick, loosely associated with layer 2 in intact spores, always separating from it in crushed spores (Fig. 2b–d). Layer 2 laminate, coriaceous, (1.0–)3.5(–5.8) μm thick (Fig. 2). Layer 3 flexible,

(1.0–)1.1(–1.3) μm thick, always tightly adherent to the lower surface of layer 2 in intact spores, frequently separating from it in crushed spores (Fig. 2b–d). *Sporiferous saccule* hyaline to light yellow (4A4); egg-shaped; 104–116 \times 123–148 μm ; connecting with a spore by a short stalk (Fig. 2a, f, g). *Sporiferous saccule wall* consists of three layers (sporiferous saccule wall layers 1–3; Fig. 2f, g), of which layer 1 is continuous with the coloured and ornamented spore wall 1 layer 1 and is present only in the first, short, up to 12.5 μm long, part of the sporiferous saccule wall (Fig. 2g), and layers 2 and 3 are probably continuous with spore wall 1 layers 2 and 3; the connection is invisible as it is tightly covered by the hyphal mantle. Layer 2 is hyaline, 1.8–3.5 μm thick at the spore, gradually reducing to no more than ca. 1.0 μm thick (Fig. 2f, g). Layer 3 is pale yellow (3A3) to brownish yellow (5C7), 2.8–4.0 μm thick at the spore, gradually becoming colourless and reducing to ca. 1.0 μm thick distally (Fig. 2f, g). *Stalk* cylindrical to funnel-shaped, 10.5–25.0 μm wide, surrounding a circular hole, 10.0–22.5 diam. when seen in cross view (Fig. 2f, g); hole closed by a hyphal plug, 4.3–

Fig. 2 *Sacculospora felinovii* spores. **a** Intact spores (*sp*) with sporiferous saccule (*ss*) and hyphal mantle (*hm*). **b–d** Spore wall 1 layers (*sw1l*) 1–3, spore wall 2 layers (*sw2l*) 1–3 and hyphal mantle (*hm*). **e** Warts ornamenting the upper surface of spore wall 1 layer 1 (*sw1l1*) seen in plan view. **f** Sporiferous saccule wall layers (*sswl*) 2 and 3 and hole (*h*) connecting the interior of the spore with the interior of the sporiferous saccule. **g** Sporiferous saccule wall layers (*sswl*) 1–3. **h** Hyphae of the hyphal mantle seen in plan view. **A** Spores in lactic acid. **B, C, E–H** Spores in PVLG. **D** Spores in PVLG + Melzer's reagent. **A–H** Differential interference microscopy. Bars: **A** = 50 μm , **B–H** = 10 μm



7.8 μm thick (Fig. 2g). It is likely the plug is formed in the final stage of spore development. Connection to mycelium was not visible. *Germination shield* not observed to date. Spores and hyphal mantles do not react in Melzer's reagent (Fig. 2d).

Mycorrhizal associations In single-species cultures with *S. scutellarioides* as host plant, *S. felinovii* formed mycorrhiza with arbuscules and intra- and extraradical hyphae (Fig. 3a, b). No vesicles were found. All the structures stained very faintly in 0.1 % Trypan blue and, hence, were difficult to visualise but there probably was a patchy distribution along the examined root fragments.

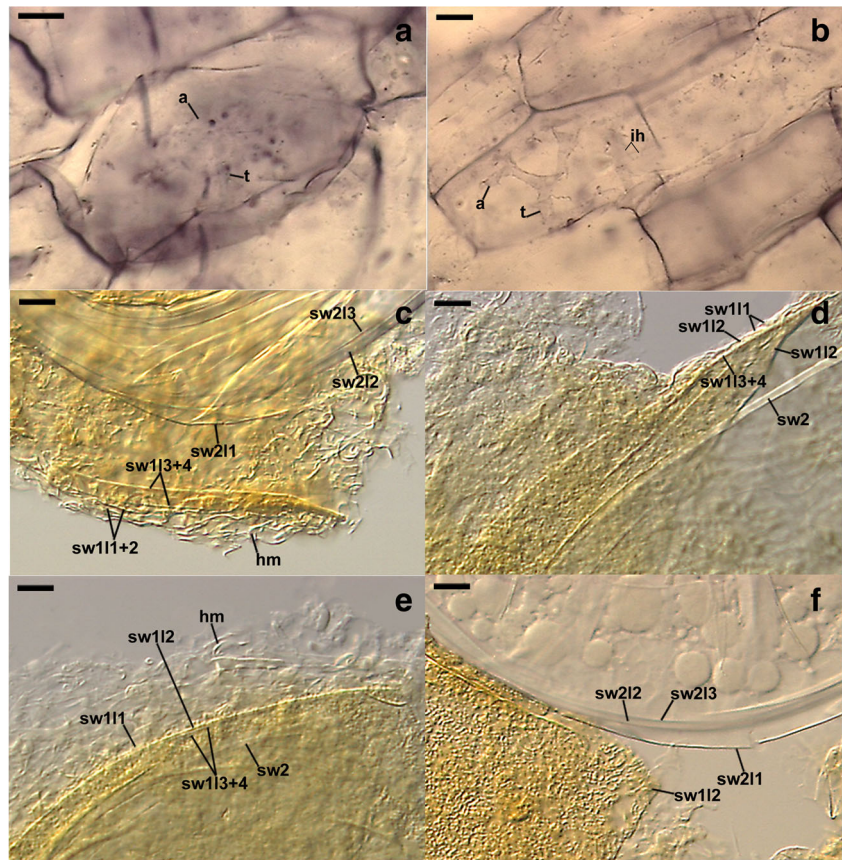
Distribution and habitat In the field, *S. felinovii* was likely associated with roots of *Z. matrella*. No molecular analysis on DNA extracted from roots was

performed to confirm this supposition however. Spores were most abundant (ca. 35 ± 7 100 g^{-1} soil) in the regions of the transect where *Z. matrella* was the dominant species, 101–138 m from the strand. BLAST searches revealed no sequence with similarity ≥ 97 % to SSU–ITS–LSU sequences of *S. felinovii*.

Discussion

Sacculospora baltica has, so far, been the only member of the recently newly erected monospecific genus *Sacculospora* and the family Sacculosporaceae (Oehl et al. 2011). Intact *S. felinovii* entrophosporoid spores, always tightly surrounded by a dense hyphal mantle (Fig. 2a–c, h), are indistinguishable from those of *S. baltica* (Błaszowski et al. 1998; Błaszowski 2012). However, the unique

Fig. 3 **a, b** Mycorrhizal structures of *Sacculospora felinovii* in roots of *Solenostemon scutellarioides* stained in 0.1 % Trypan blue: arbuscule (*a*) with trunk (*t*) and intraradical hyphae (*ih*). *Sacculospora baltica* spores: **c** Spore wall 1 layers (*sw1l*) 1–4, spore wall 2 layers (*sw2l*) 1–3 and hyphal mantle (*hm*). **d, e** Spore wall 1 layers (*sw1l*) 1–4, spore wall 2 (*sw2*) and hyphal mantle (*hm*). **f** Spore wall 1 layer 2 (*sw1l2*) and spore wall 2 layers (*sw2l*) 1–3. *A–F* in PVLG, differential interference microscopy, bars = 10 μ m



feature of *S. felinovii* is its spore wall 1 layer 3 that has birefringent properties in polarised light, where the edges of the wall, despite being colourless, turn almost black (Fig. 2b–d). Further, it is comparatively thick, fragile and is covered with a thin layer (spore wall 1 layer 2). This layer rarely separates from the upper surface of spore wall 1 layer 3 in even vigorously crushed spores (Fig. 2b–d).

Morphological differences clearly separating *S. felinovii* and *S. baltica* reside in the structure and phenotypic features of spore wall 1. First, spore wall 1 of *S. felinovii* consists of three layers (Fig. 2b–d; four layers in *S. baltica*, Fig. 3c–e), lacking the evanescent hyaline spore wall 1 layer 1 of *S. baltica* (Błaszowski et al. 1998; Błaszowski 2012). Secondly, spore wall 1 layer 1 of *S. felinovii* (Fig. 2b, c, e), where in *S. baltica* it is spore wall 1 layer 2 (Fig. 3c–f), is permanent and similarly coloured, and on the upper surfaces ornamented with warts. The warts in *S. felinovii* are 3–4-fold higher and, hence, more visible. Thirdly, in *S. felinovii*, the main structural layer of spore wall 1 is the thickest innermost layer 3 that is covered with a thin layer 2 (Fig. 2b–d). In *S. baltica*, layer 3 is also the thickest but, in comparison, it is 1.2–1.5-fold thinner than spore wall 1 layer 3 of *S. felinovii* and covers a thin innermost layer 4 (Fig. 3c–e). Finally, in polarised light, none of the spore wall 1 layers

of *S. baltica* show the birefringent properties of spore wall 1 layer 3 of *S. felinovii*.

Further differing structures are spore wall 2 layer 2 and hyphae of the hyphal mantle. Spore wall 2 layer 2 of *S. felinovii* is 1.3–1.8-fold thicker than that of *S. baltica*. Hyphae of the hyphal mantle in both species are similar in size and colour, but those of *S. felinovii* are usually straight and have numerous branches of different inclinations relative to the parent hypha (Fig. 2h), whereas those of *S. baltica* are generally sinuous (Błaszowski et al. 1998; Błaszowski 2012).

The birefringent property of spore wall 1 layer 3 in polarised light is the most important differential feature. Of members of the Glomeromycota, this property has, so far, been found only in *Ambispora appendicula* (Spain, Sieverd. & N.C. Schenck) C. Walker, *Am. fennica* C. Walker, Vestberg & A. Schüßler, *Am. gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) C. Walker, Vestberg & A. Schüßler and *Am. granatensis* Palenz., N. Ferrol & Oehl of the order Archaeosporales C. Walker & A. Schüßler (Spain et al. 2006; Walker et al. 2007; Palenzuela et al. 2011; Błaszowski 2012). However, *Ambispora* spp. produce acaulosporoid spores, i.e. laterally from the sporiferous sacculule neck, whereas *S. felinovii* produces entrophosporoid spores (Fig. 2a, f, g). Furthermore, *Ambispora* spp. are

genetically very distant from the Diversisporales C. Walker & A. Schüßler, to which *S. felinonii* belongs.

Finally, the uniqueness of *S. felinonii* is shown in the phylogenetic analyses of SSU–ITS–LSU nrDNA sequences (Fig. 1). The SSU–ITS–LSU nrDNA sequences of *S. felinonii* differed by an average of 10.1 % from those of *S. baltica*.

The mycorrhizal structures of *S. felinonii* grown in single-species culture stained exceptionally faintly (Fig. 3a, b) and many may not have stained at all in 0.1 % Trypan blue. Unfortunately, comparison cannot be made with *S. baltica* where, as mentioned above, pure culture has not been attained. Therefore, currently, it is possible to only conjecture that the faint or no staining is a family-level synapomorphy, as is likely in members of Ambisporaceae, Archaeosporaceae and Paraglomeraceae (Morton and Redecker 2001; Spain et al. 2006; <http://invam.wvu.edu/>).

The literature and our observations, and the lack in public databases of uncultured AMF sequences with a similarity of ≤ 97 % to those of *S. baltica* and *S. felinonii*, suggest that these species occur rarely in the world. However, it is likely they are widely distributed on the Earth and are adapted to different soil and climatic conditions, despite *S. felinonii* being, so far, identified only in one dune site of the west coast of India. In Poland, *S. baltica* was found in dunes of the Baltic Sea but also in inland dunes of the Błędowska Desert and in the Tatra Mountains, ca. 600 and 700 km away from the sea, respectively (Tadych and Błaszczkowski 2000; Błaszczkowski et al. 2002; Zubek et al. 2008; Błaszczkowski 2012). In addition, *S. baltica* was recorded at several Alpine elevations in Switzerland and in forest ecosystems near Valdina in southern Chile (Sieverding and Oehl 2006; Oehl et al. 2011). The rare records of both fungi may also result from there being few mycologists dealing with the morphology of AMF and weaknesses of molecular methods applied in recognition of intraradical AMF (Oehl et al. 2010; Wetzel et al. 2014).

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