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ORIGINAL ARTICLE

Serendipita restingae sp. nov. (Sebacinales): an orchid mycorrhizal agaricomycete with wide host range

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Abstract

The Serendipitaceae family was erected in 2016 to accommodate the Sebacinales 'group B' clade, which contains peculiar species of cultivable root–associated fungi involved in symbiotic associations with a wide range of plant species. Here we report the isolation of a new *Serendipita* species which was obtained from protocorms of the terrestrial orchid *Epidendrum fulgens* cultivated in a greenhouse. This species is described based on phylogenetic analysis and on its microscopic and ultrastructural features in pure culture and in association with the host's protocorms. Its genome size was estimated using flow cytometry, and its capacity to promote the germination of *E. fulgens* seeds and to associate with roots of *Arabidopsis thaliana* was also investigated. *Serendipita restingae* sp. nov. is closely related to *Serendipita* sp. MAFF305841, isolated from *Microtis rara* (Orchidaceae), from which it differs by 14.2% in the ITS region and by 6.5% in the LSU region. It produces microsclerotia formed of non-monilioid hyphae, a feature that was not reported for the Sebacinales hitherto. *Serendipita restingae* promoted the germination of *E. fulgens* seeds, forming typical mycorrhizal pelotons within protocorm cells. It was also able to colonize the roots of *Arabidopsis thaliana* under in vitro conditions. *Arabidopsis* plants grown in association with *S. restingae* increased their biomass more than fourfold. *Serendipita restingae* is the first Serendipitaceae species described for the Americas.

Keywords Serendipitaceae · Symbiotic · Endophyte · *Epidendrum* · New species · Microsclerotia

Introduction

The Sebacinales are a monophyletic order of Basidiomycetes involved in a broad range of symbiotic associations with the roots of land plants (Weiß et al. 2004, 2016; Oberwinkler et al. 2013). They likely originated 300–400 million years ago (He et al. 2019) and are globally distributed (Garnica et al. 2016), from agricultural to pristine ecosystems (Setaro and Kron 2011; Riess et al. 2014). While most mycorrhizal basidiomycetes form ectomycorrhiza (Garnica et al. 2016), the species within the Sebacinales establish remarkably flexible associations (Selosse et al. 2007). They can also be found as symptomless endophytes (Wilson 1995) within the roots of many plant species or as free-living saprotrophs (Selosse et al. 2009; Garnica et al. 2016; Weiß et al. 2016).

The order is phylogenetically divided into two families: the Sebacinaceae, which often form macroscopically visible basidiomes, and the Serendipitaceae, in which only anamorphic strains were studied in detail hitherto (Weiß et al. 2004, 2016). These two families also have key differences in ecology and biotechnological potential (Weiß et al.

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2004; Oberwinkler et al. 2014). While the first contains species described as root endophytes, ectomycorrhizal or orchid mycorrhizae restricted to fully or partially heterotrophic orchids (Weiß et al. 2016), the second family has a much wider mycorrhizal spectrum, including ericoid (Selosse et al. 2007; Vohník et al. 2016), ectendomycorrhizas from the cavendishioid (Setaro et al. 2006), pyroloid (Setaro et al. 2011), arbutoid (Hashimoto et al. 2012) and jungermannioid (Kottke et al. 2003) types, and orchid mycorrhiza (Warcup 1981, 1988; Suárez et al. 2008; Yagame and Yamato 2008); finally they also occur as symptomless endophytes (Selosse et al. 2009; Riess et al. 2014; Venneman et al. 2017).

Perhaps less anchored in biotrophy than the uncultivable Sebacinaceae, Serendipitaceae maintain genes responsible for saprotrophy in parallel with the evolution of biotrophic ability (Zuccaro et al. 2011). This not only enables their axenic culture but also allows an outstanding capacity to colonize the roots of a wide range of mono- and dicotyledonous plants as endophytes, while promoting many beneficial effects to their hosts (Lahrmann and Zuccaro 2012). This makes them unique models for the study of mutualistic interactions with plants and excellent biotechnological tools for sustainable agriculture (Deshmukh et al. 2006). For example, the colonization of switchgrass (*Panicum virgatum*) roots by *Serendipita vermifera* can considerably increase the host's shoot and root biomass (Ghimire and Craven 2011), an increase that is superior to that obtained by years of switchgrass genetic improvement efforts (Ray et al. 2015).

Although many environmental DNA sequences available in GenBank are assigned to the Serendipitaceae, only four species have been described to date. *Serendipita indica* was isolated from desert soil samples in India under the name *Piriformospora indica* (Verma et al. 1998). *Serendipita vermifera* was first isolated in Germany (Oberwinkler, 1964) and later from the roots of Australian terrestrial orchids (Warcup and Talbot 1967), but it seems that this name has been applied to a wide range of different *Serendipita* spp. in the literature and reflects a species complex (Weiß et al. 2016). *Serendipita williamsii* (Basiewicz et al. 2012), which is closely related to *S. indica*, was also isolated from arbuscular mycorrhiza spores. More recently, *S. herbamans* was isolated from the roots of a Polygonaceae in Europe where it grew as an endophyte (Riess et al. 2014).

Epidendrum fulgens is an abundant and widespread terrestrial neotropical orchid that occurs in shrubby sand dunes and rock outcrops of the Atlantic rainforest in Brazil (Sujii et al. 2019). This orchid occurs in a harsh and stressing environment formed by marine deposits between the sea and the Atlantic rainforest mountain chains in Brazil, where plants are subjected to constant winds, floods, drought, high salinity, and low nutrient levels (Scarano 2002).

This study reports morphological and molecular characteristics of a novel species of *Serendipita* isolated from protocorms of *E. fulgens.* In this species as in other orchids, the small reserveless seeds germinate into an undifferentiated seedling called a protocorm supported by the fungus (Rasmussen 1995; Dearnaley et al. 2017).

Material and methods

Isolation and cultivation of the fungus endophyte

The fungus was isolated from symbiotic protocorms of *Epidendrum fulgens* Brongn growing attached to the roots and over the substrate of potted *E. fulgens* maintained in a greenhouse, at the Federal University of Santa Catarina (Florianópolis, Brazil). These plants were collected without their original substrate from natural populations in Florianópolis, Brazil (27° 37′ 27.9″ S 48° 27′ 25.4″ W), in the restinga vegetation (Araujo 1992), and were maintained in greenhouse conditions for 3 to 8 years. After the observation of naturally occurring symbiotic germination in the greenhouse, a few potted plants were separated and *E. fulgens* seeds were sown at their base. After 4–6 months, young symbiotic protocorms were collected and surface sterilized with 70% ethanol for 1 min and 0.5% sodium hypochlorite for 5 min, followed by three rinses in sterile distilled water. They were longitudinally cut and deposited in Petri dishes containing PDA medium supplemented with 100 mg L^{-1} streptomycin and incubated in the dark at 25 °C. The plates were checked daily under an inverted microscope for the emergence of fungal hyphae. The tips of hyphae were then selected and transferred to new Petri dishes with fresh PDA medium.

For culture before DNA extraction, fungal isolates were transferred to glass flasks containing 100 mL of potato dextrose broth and maintained under constant agitation at 100 RPM for 2 weeks. The mycelia were harvested, filtered through cheesecloth, and rinsed several times with sterile distilled water to remove culture media.

DNA extraction, PCR cloning, sequencing, and phylogenetic analysis

About 50 mg of fresh mycelia from the fungal isolates was ground in a Precellys® homogenizer, and the total DNA was isolated using the CTAB protocol (Doyle and Doyle 1990). The isolated DNA was diluted, and the ITS and D1/D2 regions of the nuclear rDNA were amplified with the primers ITS1F (Gardes and Bruns 1993) and NL4 (O'Donnell 1993). The PCR reaction was carried out in a 20-μL final volume containing 50 ng of template DNA, 0.2 μM of each primer, 0.2 mM dNTPs, 1 PCR buffer, 0.2 mg mL^{-1} bovine serum albumin, and 2.5 mM $MgCl_2$. Thermal cycling parameters were an initial denaturation step at 94 °C for 2 min followed by 35 cycles of denaturing

at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, plus a final extension at 72 °C for 7 min. The resulting PCR products were checked by electrophoresis on a 1.5% agarose gel stained with GelRed® (Biotium™) and visualized through UV light. The PCR products were purified by precipitation with 1 volume of a solution of 20% polyethylene glycol 8000 and 2.5 M NaCl. Purified PCR products were sequenced in both directions with the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990); and NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993), using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Foster City, CA, USA) on an ABI 3500xl genetic analyzer (Applied Biosystems). Sequence chromatograms were assembled and manually edited using CLC Genomics Workbench (Qiagen Bioinformatics; https://www.qiagenbioinformatics.com). The newly acquired DNA sequence was deposited in GenBank under accession number MN595219.

A Blast search against the NCBI database (https://www.ncbi.nlm.nih.gov) was used to check the sequence similarities. A phylogenetic hypothesis based on the ITS plus D1/D2 regions of the nuclear 28S rDNA was made using the newly obtained sequence and 35 GenBank accessions spanning the ITS and D1/D2 regions from taxa representative of Sebacinaceae and Serendipitaceae. The matrix produced by Riess et al. (2014) and sequences from the Sebacinaceae by Oberwinkler et al. (2014) were used as references. Sequences were aligned with MUSCLE (Edgar 2004), and minimal manual adjustments were performed using MEGA7 v7.0.9 software. Four sequences were removed because they lacked partial ITS1 or ITS2 regions, leaving 33 sequences in the final data set. The final alignment length was 1685 bp and was deposited in TreeBase (https://purl.org/phylo/treebase/phylows/study/TB2:S26366).

Phylogenetic analyses were performed using a maximum likelihood (ML) approach in RAxML-HPC2 v8.2.12 software (Stamatakis 2014) with combined rapid bootstrapping and 1000 runs, using the GTRCAT model for DNA substitution. We also performed a Bayesian Markov Chain Monte Carlo (MCMC) analysis in MRBAYES 3.2.7a (Ronquist and Huelsenbeck 2003) using GAMMA distribution within sites, with two replicates and four heated Markov chains. Jmodeltest2 software (Darriba et al. 2012) was used to select the best-fit model of nucleotide substitution. In each run, 200,000 sample generations were used. The first 50,000 were discarded, and one tree every 100 generations was pooled and used to compute the final majority-rule consensus tree to estimate branch support. All phylogenetic analyses were performed on the CIPRES Science Gateway v.3.3 (https://www.phylo.org/sub_sections/portal/). The phylogenetic trees with the best scores were illustrated using FigTree v1.4.4.

Morphological studies

Microscopic observations were made from fresh and fixed culture samples and symbiotic protocorms. To morphologically describe the fungal isolate and its interaction with the host plant, we used light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM and SEM, symbiotic protocorms of *E. fulgens* at the late stage of development, with emerging first leaf, as well as 0.5-cm^2 plugs of 1-month-old colonies growing on PDA, were fixed in 2.5 glutaraldehyde-paraformaldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 48 h (Karnovsky 1965). For SEM, the fixed samples were dehydrated in ethanol series (20%, 40%, 60%, 70%, 80%, 90%, and 100%) for 30 min each, followed by critical point drying in liquid carbon dioxide (EM CPD 030/Leica, Germany). Dried samples were longitudinally sectioned, mounted over aluminum stubs with double-sided sticky carbon tape, coated with gold (EM SCD 500/Leica, Germany), and examined under a JEOL, JSM-6390LV scanning electron microscope (LCME-UFSC). For TEM examinations, we used the methodology described by Suárez et al. (2006), and sections were examined under a JEOL JEM1011 (JEOL, Inc., Peabody, MA) transmission electron microscope of the Central Laboratory of Electron Microscopy at the Federal University of Santa Catarina (LCME/UFSC, Florianópolis, Brazil).

To count the number of nuclei in hyphae, colonies were grown in 300-mL glass flasks with 30 mL of PDA medium and sterile microscopic cover slides were perpendicularly placed on the media surface to allow colonization of the slides. The colonized slides were removed in a flow chamber and fixed and stained for microscopic observations according to the methodology described by Wilson (1992).

Genome size estimation

Flow cytometry was used for nuclear genome size estimation. Initially, we used *Solanum lycopersicum* 'Stupické' (2C = 1.96 pg DNA) as an internal reference standard to estimate the genome size of *Arabidopsis thaliana* 'Col-0'. Seeds of the reference standard were kindly provided by Dr. Jaroslav Doležel of the Institute of Experimental Botany of the Czech Academy of Sciences. Then, we used *A. thaliana* (2C = 0.32 pg) as an internal reference standard for the genome size estimation of the fungal isolate. That was necessary because genome size differences between *S. lycopersicum* and the fungal isolate were too large to allow flow cytometry analysis using internal standardization, i.e., simultaneous isolation, staining, and analysis of the sample and the reference standard. Internal standardization

is recommended to avoid errors due to instrument drift and variation during the sample preparation and staining (Doležel and Bartoš 2005).

Nuclei from the leaves of the reference standard (≈ 25 mg) and the mycelia of the fungal isolate (≈ 5 mg) were simultaneously extracted by chopping with a razor blade (Galbraith et al. 1983) on 2 mL ice-cold Otto I buffer (Otto 1990) containing 0.1 M citric acid (Merck) and 0.5% Tween 20 (Synth). The nuclei suspension was filtered through a 40-µm nylon mesh (BD Falcon) and centrifuged at 150*g* for 5 min. The supernatant was removed with a pipette, and the pellet was resuspended after the addition of 100 µL of fresh ice-cold Otto I buffer. For nucleus staining, the suspension (200 µL) was incubated in the dark for 30 min after the addition of 500 µL of Otto II buffer (Otto 1990) supplemented with 50 µg mL^{-1} of propidium iodide (PI; Sigma-Aldrich) and RNase (Sigma-Aldrich). Fluorescence intensity was measured with a BD FACSCanto™ II flow cytometer, equipped with an argon laser (488 nm) used for PI excitation, at the Multi-users Laboratory of Studies in Biology at the Federal University of Santa Catarina (LAMEB/UFSC). The position of each peak, from fungus and reference standard, was settled by analyzing a first run with each sample separately. The G1 peaks were assigned to a specific channel, and the equipment voltage and gain were kept constant throughout the analyses. Eight independent replicates were performed, and at least 40,000 G1 nuclei from the fungus sample were analyzed for each replicate.

Flowing software 2.5.1 was used to process the data. First, we analyzed dot-plots of fluorescence intensity on a linear scale vs. forward scatter light in a logarithmic scale. A polygonal region including all PI-stained nuclei was created on dot-plots from which gated histograms of fluorescence intensity in linear scale were created. Linear regions were created on histograms to gate and obtain descriptive statistics of only intact nuclei.

The sample genome size was calculated by multiplying the fluorescence intensity ratio between the G1 cell cycle peaks of the fungus and the reference standard by the DNA 2C value of the reference standard (Doležel and Bartoš 2005). To convert DNA content in picograms (pg) to base pairs (bp), we considered that 1 pg = 0.978×10^9 bp (Doležel et al. 2003).

Symbiotic seed germination

In order to test whether the isolated fungus was able to promote seed germination, we performed an in vitro assay with three different treatments. Seeds were harvested from one mature fruit of *E. fulgens*, disinfected with 0.5% NaClO for 10 min, and washed three times in sterile distilled water. Thereafter, they were sown in Petri dishes containing agar/oat medium (4 g L^{-1} oatmeal flour; 7 g L^{-1} agar) either with or without a fungal inoculum. For the inoculated treatment, a 1-cm^2 plug of the fungal isolate was inoculated at the center of the dishes. The fungus had previously been cultivated for 4 weeks on PDA medium, and the inoculum plugs were obtained from the active growing hyphae from colony margins. *Epidendrum fulgens* seeds can be germinated asymbiotically in MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose (Voges et al. 2014). Therefore, seeds were also sown in Petri dishes containing MS medium supplemented with 3% sucrose as a positive control. Dishes were kept at 25 °C, in a 16-h photoperiod.

Each Petri dish was considered as a repetition, with a mean of 217 ± 39 seeds per plate, and three repetitions per treatment were used. Data were collected 12 weeks after sowing by inspecting each plate under a stereomicroscope. All seeds from each plate were accessed, and a germination rate was calculated with the ratio between the number of germinated seeds by the total number of viable seeds. A score was attributed to protocorms according to their developmental stage:

N0 = non germinated seed.
N1 = germinated/embryo swelling;
N2 = protocorm with apical meristem;
N3 = protocorm with first leaf;
N4 = plantlet stage; formation of first root;
N5 = plantlet with more than one leaf and root;

A growth index (GI) was calculated from the developmental scores, as described by Otero et al. (2004). The germination rates and GI between the different treatments were compared with Kruskal-Wallis and *F* test, respectively, ($p = 0.05$). All analyses were performed on the R environment (R core team, 2019).

Interaction with *Arabidopsis thaliana*

To test the interaction capacity and possible biotechnological potential of the fungal isolate, we performed inoculation assays with *Arabidopsis thaliana*. We chose *A. thaliana* because it is a model plant that could help future interaction mechanism studies. The experiments were performed according to method 1 described by Johnson et al. (2011), with modifications. Briefly, *A. thaliana* (Col-0) seeds were surface sterilized in 1% NaClO for 7 min., washed in sterile distilled water, and inoculated in Petri dishes containing 25 mL of half-strength MS medium (Murashige and Skoog 1962), supplemented with 1% sucrose. Petri dishes were kept in the dark at 5 °C for 7 days to ensure homogenous germination and were then transferred to a growth chamber at 23 °C with a 16-h light photoperiod and kept in this condition for 10–12 days. Homogenous seedlings were selected and used for inoculation experiments. Polypropylene dishes of 8-cm width and 4.5-cm height were poured with 25 mL PNM medium (5 mM KNO_3, 2 mM $MgSO_4$, 2 mM

$Ca(NO_3)_2$, 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM Na_2MoO_4, 0.01 mM $CoCl_2$, 0.01 mM $FeSO_4$, 10 mM NaCl, 1% agar, pH 5.8), and a sterile 70-µm nylon mesh was placed on the medium surface. The dishes were inoculated by placing a 0.5-cm^2 PDA medium inoculum plug from a 4-week-old fungal colony on the center of the nylon mesh. For mock treatment, plugs from fresh non-inoculated PDA medium were used. Two seedlings were placed on the nylon mesh in each dish and were kept at 23 °C with a 16-h photoperiod. A total of 26 dishes per treatment were used (inoculated or mock treatment). Two weeks after inoculation, plants were harvested and dried at 50 °C until they reached a stable weight for dry mass measurement. To evaluate root colonization, 1-cm-long root segments were cleared in 10% KOH overnight, rinsed in water, acidified for 3 min in 1% HCl, stained in 0.05% trypan blue in lactophenol solution (lactic acid/phenol/glycerol/distilled water in a 1:1:2:1 mixing ratio), mounted on microscopic slides, and observed with a light microscope.

Results

Phylogenetic position of the isolate

Three isolates, each obtained from a different protocorm, presented homogenous characteristics in culture. The ITS and D1/D2 regions of the 28S rDNA sequences from these three isolates were identical. Therefore, one isolate was chosen to perform the morphological, phylogenetic, and inoculation analyses. We looked for similar sequences using the BLAST search against the NCBI database using sequences from the ITS region, which is the universal barcode locus for fungi (Schoch et al. 2012), and the D1/D2 regions of the 28S rDNA, which is the standard region for Sebacinales phylogenetic studies (Weiß et al. 2004). Sequences retrieved from GenBank shared 88.27–89.25% identity with our sequence, and they corresponded mostly to environmental DNA *Serendipita* spp. sequences from orchid mycorrhizae and from mycothalli of the hepatic *Aneura* (Table S1). This result suggested that our sequence belongs to a species that was neither morphologically nor molecularly described.

The newly generated sequence contains two characteristic introns on the end of the 18S gene: downstream from the end of 18S/beginning of ITS1, a small intron of 35-bp length, from positions 37 to 72; and another one of 228 bp length, from positions 81 to 309. According to the AIC criterion, the alignment best-fit evolutionary model for nucleotide substitution was GTR + I + G. The phylogenetic tree inferred from the concatenated ITS + D1/D2 regions of the 28S rDNA sequences presented clades with high support values. The ML and Bayesian analyses yielded trees with the same topology and similar node support values. The newly isolated fungus clusters together with species within the Serendipitaceae family in the order Sebacinales form a highly supported terminal lineage (Fig. 1). It is most closely related to *Serendipita* sp. MAFF 305841 (KF061292), but it differs from it by 98 bp in the ITS region (14.22%) and by 44 bp in the D1/D2 regions of the 28S rDNA (6.51%). Thus, this isolate represents a new species in the Serendipitaceae that we describe as a new species of the *Serendipita* genus.

Taxonomy

Serendipita restingae Y. Fritsche, Selosse, Guerra, sp. nov.—Figs. 1 and 2 and Fig. S1.

MycoBank no.: MB 835677.

GenBank accession number MN595219.

Diagnosis: The following diagnostic nucleotide characters can be used to distinguish other Serendipitaceae species from *Serendipita restingae*, respectively:

ITS1: upstream from the end of 18S at positions 65 (T:C), 148 (T/C/G:A), and 178 (G/A:T).

ITS2: upstream from the end of 5.8S at positions 16 (A/C/–:G), 48 (G/T/C:A), 49 (T/C:A), 59 (T/C:A), 71 (T:C), 78 (T/C:G), 79 (G:A), 80 (T/C:G), 160 (T/C/G:–), and 161 (T/C/A:–).

28S: upstream from the end of ITS2 at positions 125 (G:T), 137 (C/T:A), 166 (C/T:–), 239 (A/C:G), 250 (G:A), 446 (T/G:C), 452 (T:C), 538 (T/C:–), 539 (G/A:–), 547 (T/C:–), and 548 (A/G:–).

The anamorphic *Serendipita restingae* produces microsclerotia formed from non-monilioid hyphae on water-agar medium (Fig. 2d, e).

Typus: Brazil, Santa Catarina state, Florianópolis (27° 34′ 58.6″ S 48° 30′ 18.7″ W), at sea level, from cultivated *Epidendrum fulgens* protocorms, in September 2018, collected and isolated by Yohan Fritsche (Holotype SR2619, preserved in a metabolically inactive state at the LFDGV Collection from the Federal University of Santa Catarina, Florianópolis SC, Brazil—27° 34′ 56.3″ S 48° 30′ 21.7″ W).

Host: *S. restingae* was isolated from symbiotic protocorms of *Epidendrum fulgens* Brongn. (Orchidaceae). The protocorms were attached to the roots or over the substrate of *E. fulgens* potted plants, which were collected from natural populations and maintained in greenhouse conditions for 3–8 years.

Description

The fungal colony grows 0.19 ± 0.01 mm day^{-1} on PDA medium at 25 °C in the dark. Cultures are white to cream in color and the growth habit is zonate, with submerged edges and an intermediate zone of aerial mycelia, mostly composed of monilioid hyphae (Fig. 2a, b). Mycelia are composed of hyaline, irregularly septate, and thin-walled hyphae, with a

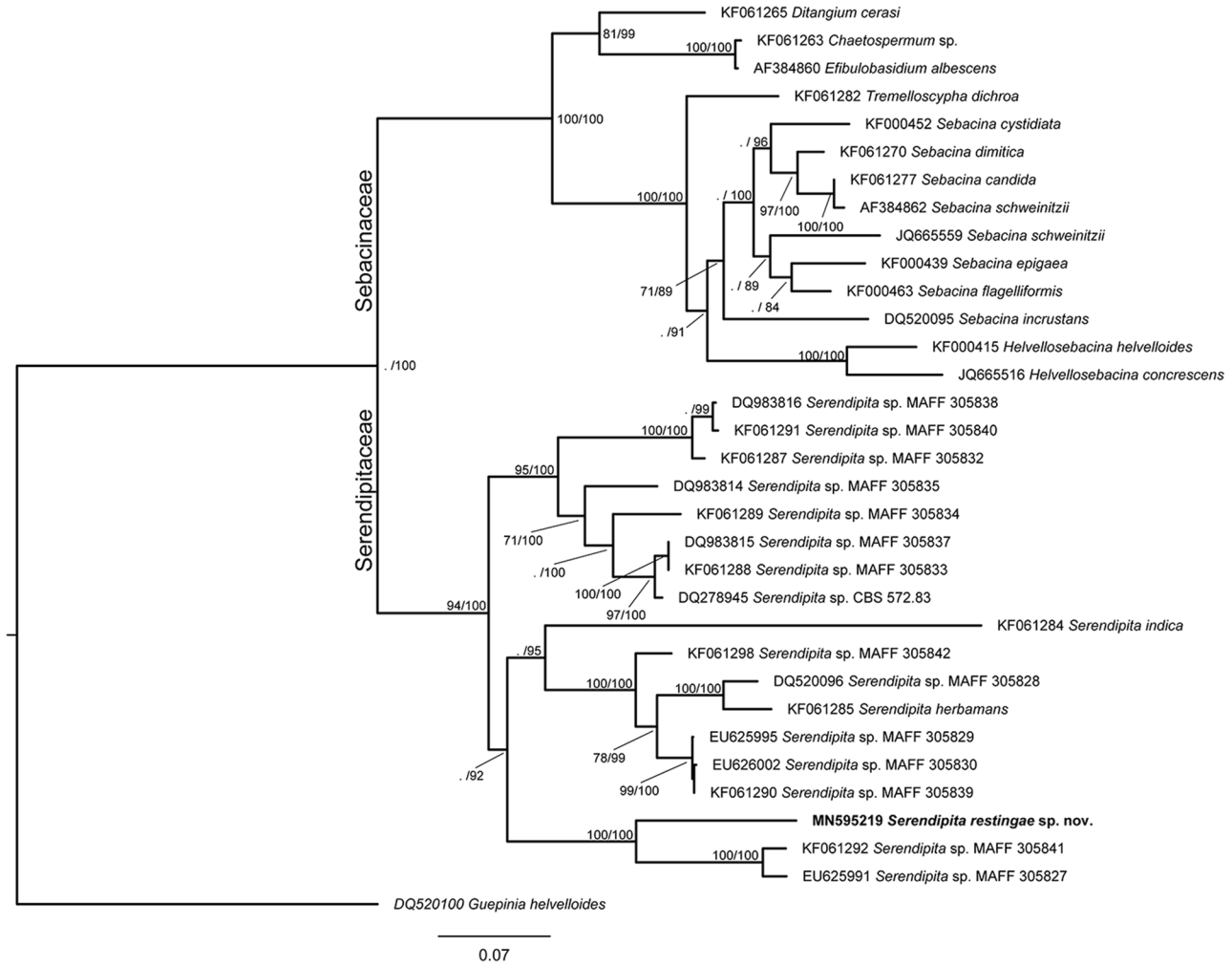

Fig. 1 Midpoint rooted ML tree obtained from ITS + D1/D2 regions of 28S rDNA (1685 bp length alignment) showing the placement of *Serendipita restingae* sp. nov. within the Sebacinales. Numbers on nodes are ML bootstrap support values (≥ 70% are shown) based on 1000 replicates/Bayesian estimates of posterior probabilities in percent (≥ 80% are shown) inferred with MRBAYES. *Guepinia helvelloides* (Auriculares) was used as an outgroup. Note that the name *Serendipita* sp. is given to a wide range of different *Serendipita* spp. in the literature, commonly referred to as *Serendipita vermifera* (Weiß et al. 2016). See Table S2 for a complete description of GenBank accessions used in the analysis

diameter of (0.9)1.4–2(2.4) µm, lacking clamp connections. Hyphal coils were frequently observed (Fig. 2f). Hyphae are multinucleate (Fig. 2g), although, presumably due to irregular septation, the number of nuclei per cell is quite variable. On the surface of the water-agar medium, after 2 months of culture, either in the dark or under indirect light, microsclerotia (72 ± 36 µm) composed of non-monilioid hyphae are abundantly formed (Fig. 2d, e) The fungus produces abundant thin-walled monilioid hyphae, in chains of up to 14 cells, after about 4 weeks of culture in a variety of synthetic culture media (Fig. 2c). Thick-walled chlamydospores (mature monilioid cells) were globose to sub-globose and (5)7–10(11) × (5)7–10(13) µm. Sexual structures were not observed. The ultrastructure analysis of septal pores showed they consisted of dolipores with straight/flat imperforate parenthesomes, composed of two outer electron-dense layers surrounding an inner less electron-dense lumen (Fig. 2h), as typical in Sebacinales. Hyphal cell walls are 47 ± 10 nm thick, while chlamydospore wall thickness is 0.8 ± 0.2 µm.

Geographic distribution

S. restingae is described from a strain isolated from a glasshouse specimen of *Epidendrum fulgens*. Its actual geographic distribution is yet to be defined.

Etymology

Named after the habitat of *Epidendrum fulgens* orchids, the sand dune vegetation within the seashore along the coastal

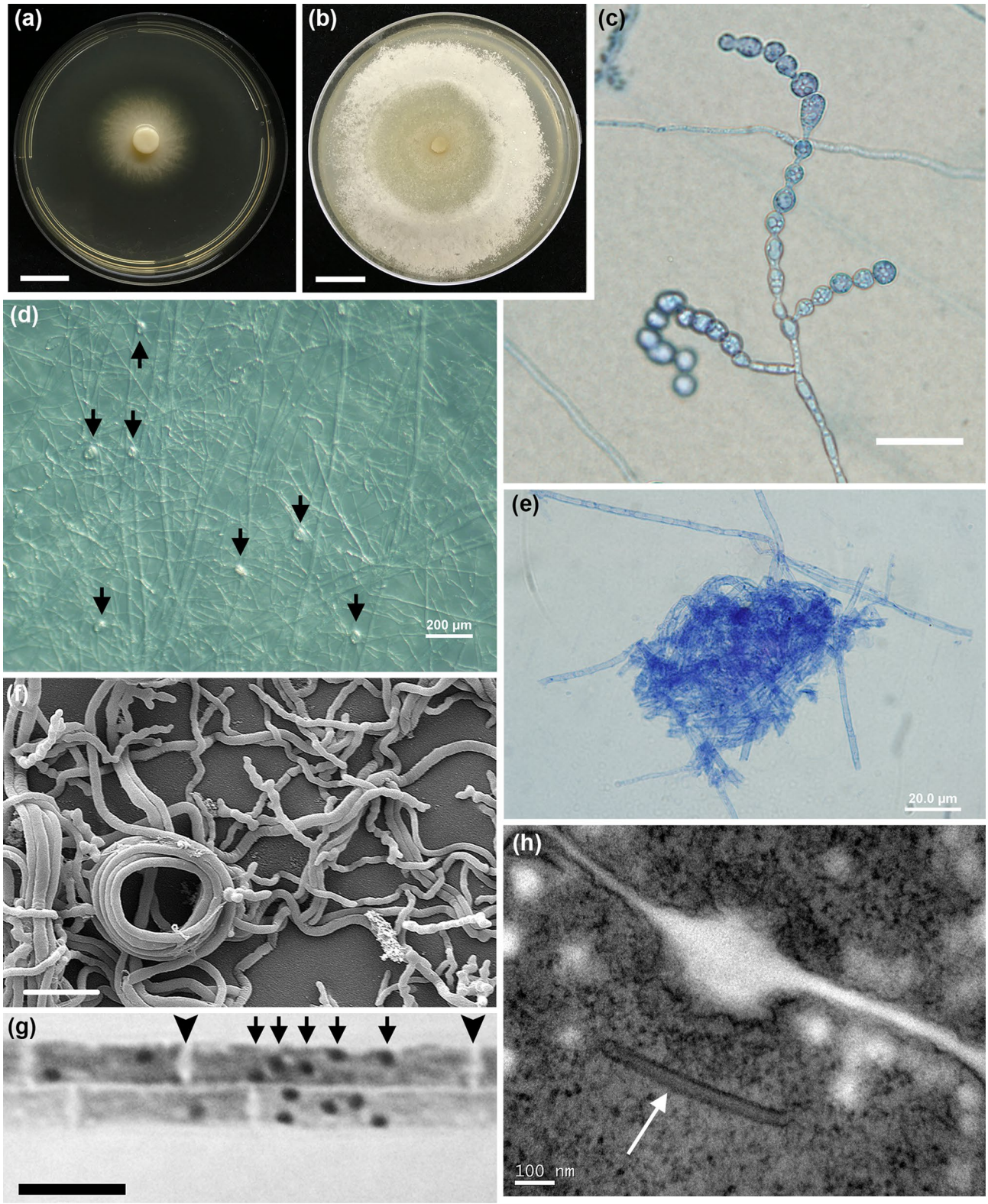

Fig. 2 Morphological features of *Serendipita restingae* sp. nov. **a**, **b** Colonies on PDA after 6 and 40 days, respectively bar = 1.8 cm. **c** Monilioid hyphae stained with 0.05% trypan blue, bar = 25 µm. **d** Microsclerotia (arrows) on the surface of agar-water medium after 2 months of culture. **e** Detail of a microsclerotium stained with 0.05% trypan blue. Note that it is not formed from monilioid hyphae. **f** Scanning electron micrograph of hyphae on the agar surface showing typical hyphal coils, bar = 10 µm; g) Giemsa-stained hyphae showing multinucleate cells. Arrows point to nuclei and arrowheads point to septa, bar = 5 µm; h) Transmission electron micrograph of a side of the dolipore septum with a flat and imperforate parenthesome (arrow)

range of the Brazilian Atlantic Rainforest, known as restinga (Araujo 1992; Scarano 2002).

Genome size

The genome size of *A. thaliana* was estimated using *S. lycopersicum* as an internal reference standard (i.e., the calibration step necessary for further use of *A. thaliana* as an internal reference standard for fungal genome size estimation) (Fig. S1a). The haploid genome size of *A. thaliana* was estimated as 0.16 pg of DNA. With this procedure, we were further able to use it as an internal reference standard for the estimation of *S. restingae* genome size.

The coefficients of variation (CV) from G1 cell cycle nucleus fluorescence peaks of the fungal sample and the internal reference standard were, on average, 9.7% and 4.6%, respectively. The genome size estimation between the eight replicates varied by less than 1.3%. In total, the fluorescence of > 437 thousand nuclei of the G1 peaks of the fungal sample was measured to calculate the genome size, which was estimated as 0.0369 ± 0.0005 pg or 36.10 ± 0.48 Mb (Fig. S1b).

Germination of *E. fulgens* seeds

The germination rate was significantly higher for symbiotic germination in comparison with asymbiotic MS medium (Fig. 3a). While 97.5% (± 0.8%, confidence interval) of seeds germinated on AO medium inoculated with *S. restingae*, only 82.5% (±3.3%) germinated asymbiotically on MS medium. No seed germinated on AO medium in the absence of fungal inoculum. The comparison of the GI from symbiotic and asymbiotic protocorms (Fig. 3b) showed no significant differences.

Symbiotic protocorms developed numerous rhizoids just after emerging from the seed coat (Fig. 4a, b). Such structures were not observed on asymbiotic protocorms (Fig. 4c, d). It was possible to obtain complete plantlets, with more than one root and fully developed leaves after 4 months on AO medium inoculated with the fungus, without any subcultivation (Fig. 4e).

Rhizoids were mainly concentrated at the protocorm base (Fig. 5a), and hyphae were observed inside them (Fig. 5b). Typical pelotons were observed at the basal cells of symbiotic protocorms (Fig. 5c–f). Both active and degenerated pelotons were observed. The hyphae from active pelotons were loosely coiled and cylindrical (Fig. 5d, e), while degenerated pelotons were compact (Fig. 5f).

Ultrastructural analysis of symbiotic protocorms showed a hypha penetrating a rhizoid cell wall (Fig. 6a). The hyphae further invaded the cortical cells and were observed passing from one cortical cell to another, leading to the formation of host cell wall appositions (Fig. 6b). The hyphal diameter

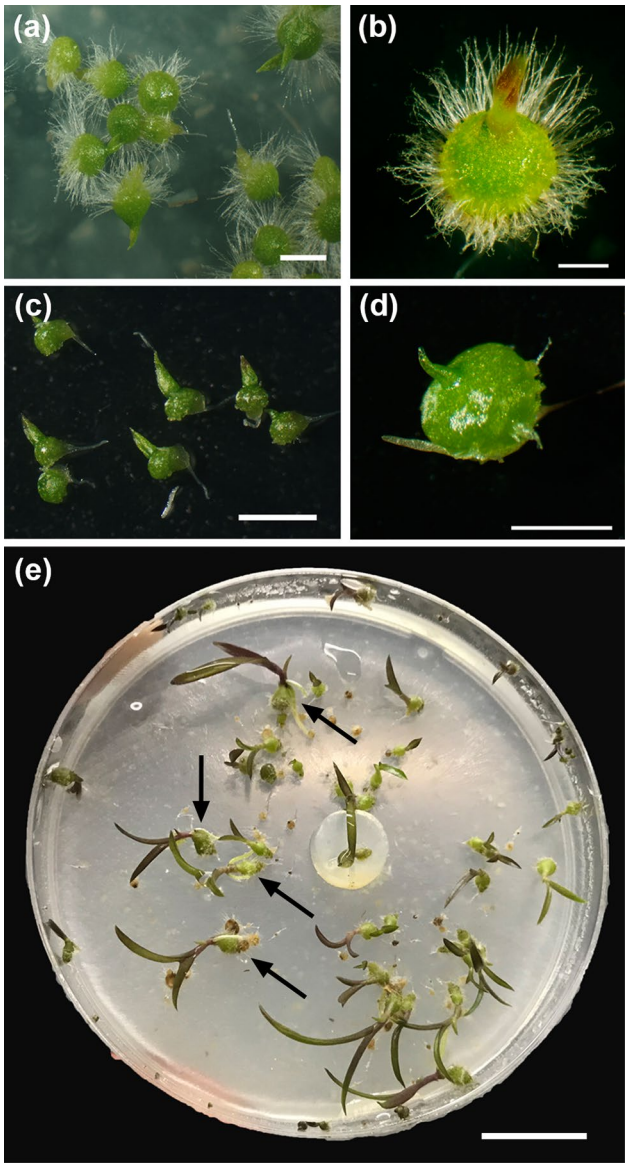

Fig. 3 Symbiotic and asymbiotic seed germination of *E. fulgens* 4 weeks after seed sowing. **a**, **b** Symbiotic protocorms on agar-oat medium inoculated with *S. restingae*. Note the presence of numerous rhizoids in symbiotic protocorms. Bars = 1 mm. **c**, **d** Asymbiotic protocorms on MS medium supplemented with 3% sucrose. Bars = 1 mm. **e** Plantlets (arrows) obtained 12 weeks after seed sowing on agar-oat medium inoculated with *S. restingae*. Bar = 1.8 cm

was narrowed at the penetration sites (Fig. 6a, b). No sign of damage was observed in plant host cells. Hyphae intensively colonized living cortical cells, and typical sebacinoid dolipore septa were observed (Fig. 6c, d).

Inoculation with *Arabidopsis thaliana*

Serendipita restingae was able to colonize *A. thaliana* roots in vitro under experimental conditions. Colonization

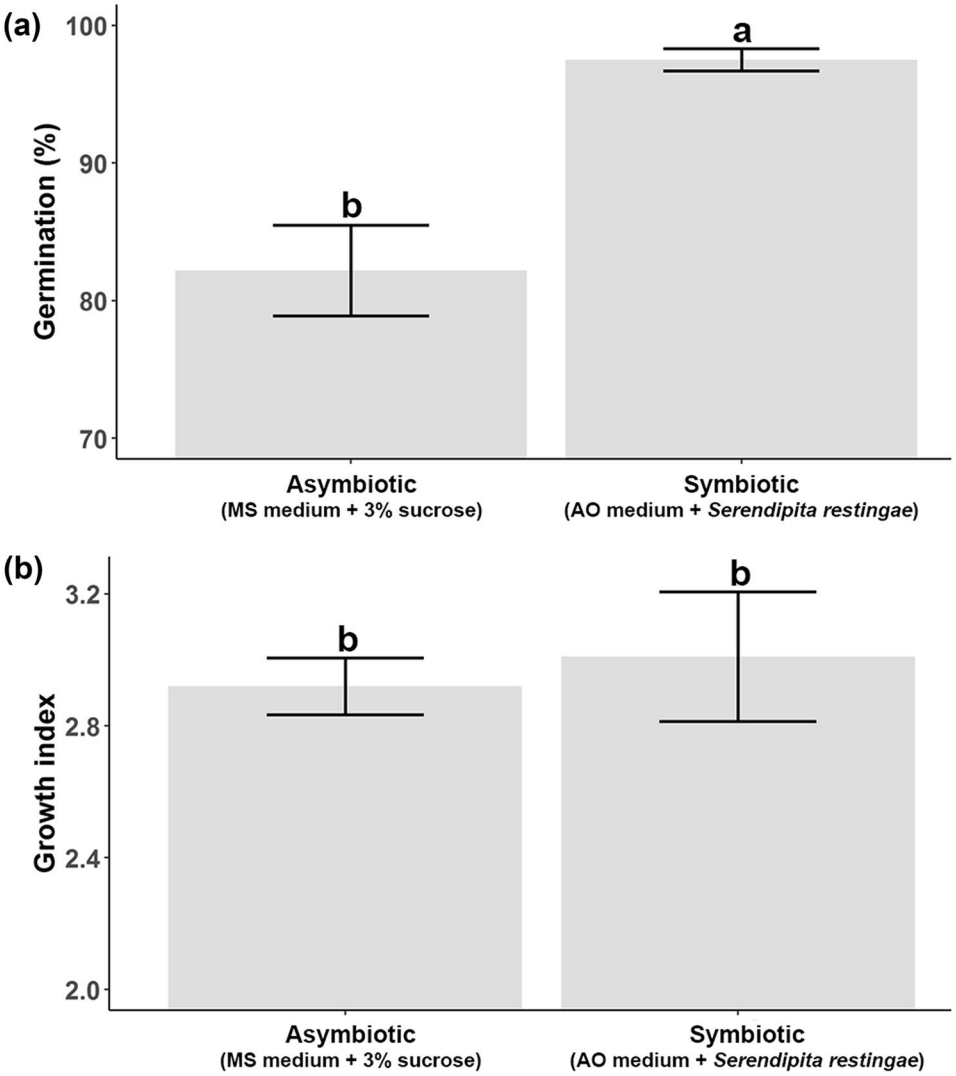

Fig. 4 Comparison between asymbiotic and symbiotic germination of *E. fulgens* seeds, 12 weeks after sowing on respective culture media. **a** Seed germination rates. **b** Growth index. Error bars are confidence intervals ($p = 0.05$). Different letters on the top of bars indicate significant differences ($p = 0.05$) according to Kruskal-Wallis test in (a) and F test in (b)

of host cells was confirmed by examination of cleared and stained roots (Fig. 7). Hyphae were abundant throughout the entire root surface, from distal to proximal regions, where abundant production of monilioid cells occurred (Fig. 7a). Intense colonization of outer cortical cells (Fig. 7b) and root hairs (Fig. 7c) was observed, also with intense monilioid cell production. No coiled hyphae were observed inside the root tissue, although typical hyphal coils, also observed on the agar surface, were frequent on the root surface.

Experimental data suggest strongly beneficial effects of the interaction between *S. restingae* and *A. thaliana*. Both roots and shoots visually developed better in the presence of the fungi (Fig. 8a). Significant differences in dry weight between inoculated and non-inoculated plants were observed. The dry weight of entire plants was more than fourfold higher in seedlings co-cultivated with *S. restingae* (Fig. 8b).

Discussion

A new Serendipitaceae species

After the pioneering work by Warcup (Warcup and Talbot 1967; Warcup 1981, 1988), fungal strains from the Serendipitaceae have been more frequently isolated over the last few years. Vohník et al. (2016) isolated a Serendipitaceae fungus from Ericaceae roots, in Norway, that survived for years in pure culture, although Serendipitaceae associated with such a host are notoriously difficult to isolate (Berch et al. 2002). A strain attributed to the *S. vermifera* species complex was isolated from poplar (Salicaceae) roots in France (Lacercat-Didier et al. 2016). Using sudangrass (*Sorghum sudanense*) roots as traps, Venneman et al. (2017) were able to obtain 51 axenic cultures in the Democratic Republic of Congo, which were closely related to

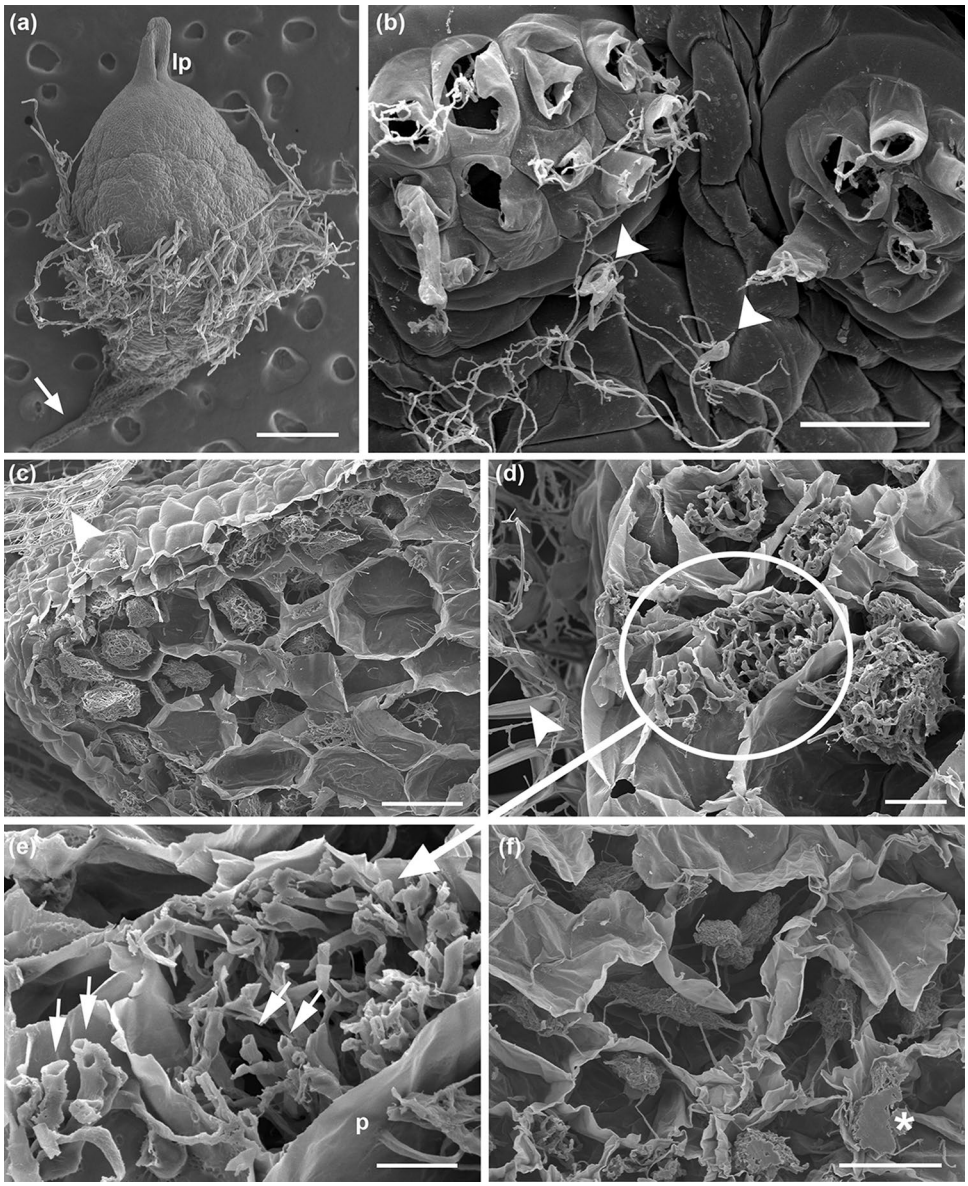

Fig. 5 Scanning electron micrographs illustrating the interaction between *S. restingae* sp. nov. and *E. fulgens*. **a** A 4-week-old symbiotic protocorm with a leaf primordium (lp) at the top and numerous rhizoids on its base. Arrow points to seed coat residue. Bar = 500 µm. **b** Details of collapsed rhizoids showing *S. restingae* internal and external hyphae. Arrow heads point to hyphal coils. Bar = 50 µm. **c** A transverse section of the base of a protocorm showing the formation of pelotons inside colonized cells. The arrowhead points to the seed coat residue. Bar = 100 µm. **d**, **e** Details of pelotons within marginal cells of the basal region of a protocorm. **d** The seed coat residue (arrowhead) in covered with hyphae, and the marginal cells are colonized by the coiled hyphae. Bar = 20 µm. **e** Loose cylindrical hyphae (arrows) and the passage of the hyphae through the cell walls (p) can be seen. Bar = 10 µm. **f** Protocorm cells containing aged collapsed pelotons. An asterisk indicates a sectioned peloton showing the compacted hyphal residue. Bar = 50 µm

S. indica and *S. williamsii*. Novotná and Suárez (2018) isolated *Serendipita* sp. from roots of *Stanhopea connata* (Orchidaceae) in southern Ecuador, which is also closely related to *S. indica*, according to its sequence. Although environmental DNA sequences from Serendipitaceae have been retrieved from American orchids (Suárez et al. 2009; Oliveira et al. 2014) and Ericaceae (Setaro et al. 2006; Selosse et al. 2007), *S. restingae* is the first *Serendipita* species isolated and described for the American continent.

The *Serendipita* genus proposed by Roberts (1993) was lately accepted by the mycological community (Kirk et al. 2013). Although Warcup (1988) suspected that his *S. vermifera* strains could be a complex of species, the first molecular evidence came from 28S rDNA phylogenetic analysis (Weiß et al. 2004). Later, Basiewicz et al. (2012) provided physiological evidence for this species complex and, more recently, Riess et al. (2014) added ITS sequences to resolve its phylogeny, which also rendered a polyphyletic group. Therefore, Warcup's *S. vermifera* strains are currently considered a complex of still undescribed species and should not be designated as *S. vermifera* any more (Weiß et al. 2016). Our description of *S. restingae* makes it formally paraphyletic and calls for more efforts in taxonomic analysis of Serendipitaceae.

Molecular phylogenetic studies showed that morphology is a poor marker to distinguish monophyletic groups in the Sebacinales (Weiß et al. 2016) and that the use of structural characters is practically irrelevant for species-level delimitation (Oberwinkler et al. 2013). For molecular species delimitation within the Sebacinales, a 1% LSU threshold distance was shown to correspond to the widely used 3% ITS dissimilarity (Setaro et al. 2011).

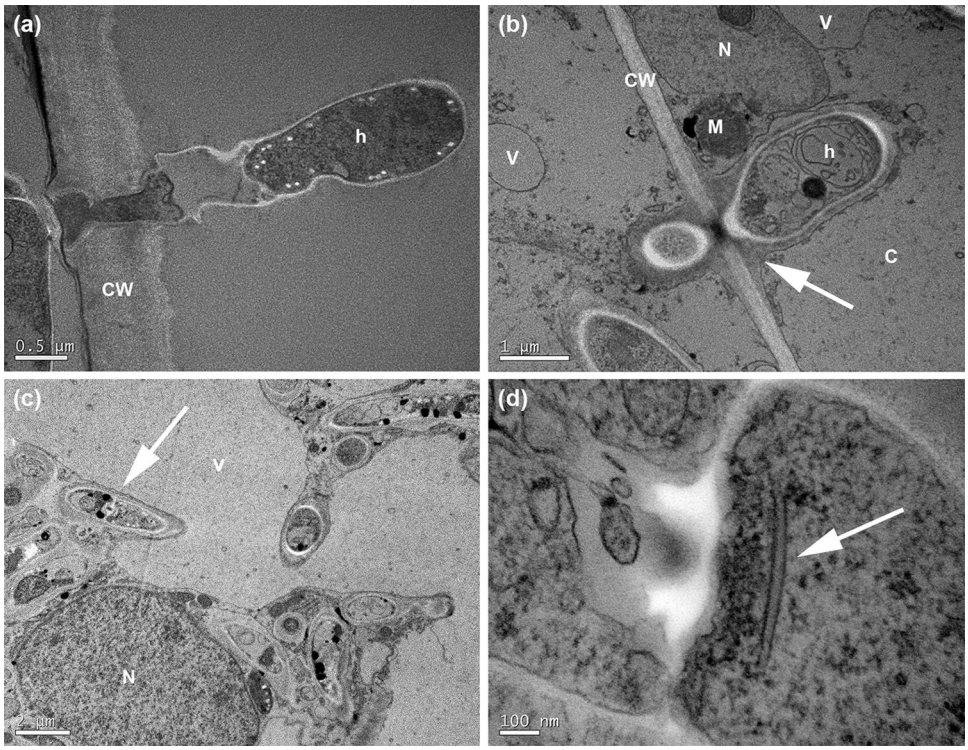

Fig. 6 Transmission electron micrographs showing the ultrastructural features of the mycorrhizal interaction between *S. restingae* sp. nov. and *E. fulgens*. **a** Hyphae penetrating a rhizoid cell. **b** Hyphae passing from one cortical cell to another, and the formation of a host cell wall apposition (arrow). **c** Overview of a colonized cortical cell showing a hypha with dolipore (arrow). **d** Detail of the dolipore with imperforate parenthesome (arrow). Abbreviations: *N*, plant cell nucleus; *CW*, plant cell wall; *V*, plant cell vacuole; *C*, plant cell cytoplasm; *M*, plant cell mitochondria; *h*, hyphae

The dissimilarity index in our study vastly exceeds these threshold values for both markers.

Features of *S. restingae*

Besides molecular differences, *S. restingae* is the first Sebacinales species reported to produce microsclerotia in culture medium. The production of sclerotia by other *Serendipita* spp. should be investigated, as they may serve as a diagnostic feature for the species. This feature can also be favorable for the production of inoculants, since these structures may enable the fungus to survive periods of adverse conditions, which are too severe for the regular mycelium (Townsend and Willetts 1954). They can also be a source for the future study of secondary compounds, which are often produced in sclerotia (Smith et al. 2015). Among orchid mycorrhizal fungi, sclerotia formed by non-monilioid hyphae were previously reported for '*Epulorhiza*' *amonilioides* = *Tulasnella amonilioides* (Almeida et al. 2014), from the Cantharellales order.

Monilioid cells are reported for all described *Serendipita* spp. (Warcup and Talbot 1967; Verma et al. 1998; Basiewicz et al. 2012; Riess et al. 2014). Although differences in the size and number of monilioid cells produced by *S. restingae* could also be highlighted, such phenotypic characteristics are highly variable and are influenced by environmental and cultural factors, so that their use as diagnostic features should be treated with caution (Jeewon and Hyde 2016). The monilioid cells observed in cultures of *S. restingae* are initially thin-walled, and, with maturation, they become thick-walled and could be considered as chlamydospores (Milligan and Williams 1987).

The number of nuclei per cell compartment is also unsuited to distinguishing *Serendipita* species, as, e.g., *S. williamsii* and *S. indica* (Basiewicz et al. 2012), although Verma et al. (1998) also observed many cells with more than one nucleus when they described *S. indica*. According to Milligan and Williams (1988), *S. vermifera* is multinucleate, although there is no information for either of Warcup's Serendipitaceae strains or for the *S. vermifera* sensu Oberwinkler. For *S. herbamans*, the number of nuclei was not provided (Riess et al. 2014).

S. restingae dolipore ultrastructure resembles that of *S. indica* (Verma et al. 1998) and *S. williamsii* (Williams and Thilo 1989), with a flange composed of electron-translucent material. This feature is in contrast to the electron-dense flanges normally observed in other endophytic sebacinoid fungi (Kottke et al. 2003; Setaro et al. 2006; Selosse et al. 2007; Riess et al. 2014).

Scanning electron microscopy clearly shows the presence of pelotons in the protocorm cortical cells (Fig. 4b). Peloton formation is a crucial criterion for the recognition of orchid symbionts (Rasmussen 2002; Rasmussen et al. 2015). These structures are the site of nutrient exchange between orchids and fungi and are recognized as typical of orchid mycorrhiza (Dearnaley et al. 2017). The nutrient exchange between orchid and fungi symbionts occurs in intact pelotons, but a considerable amount of carbon and

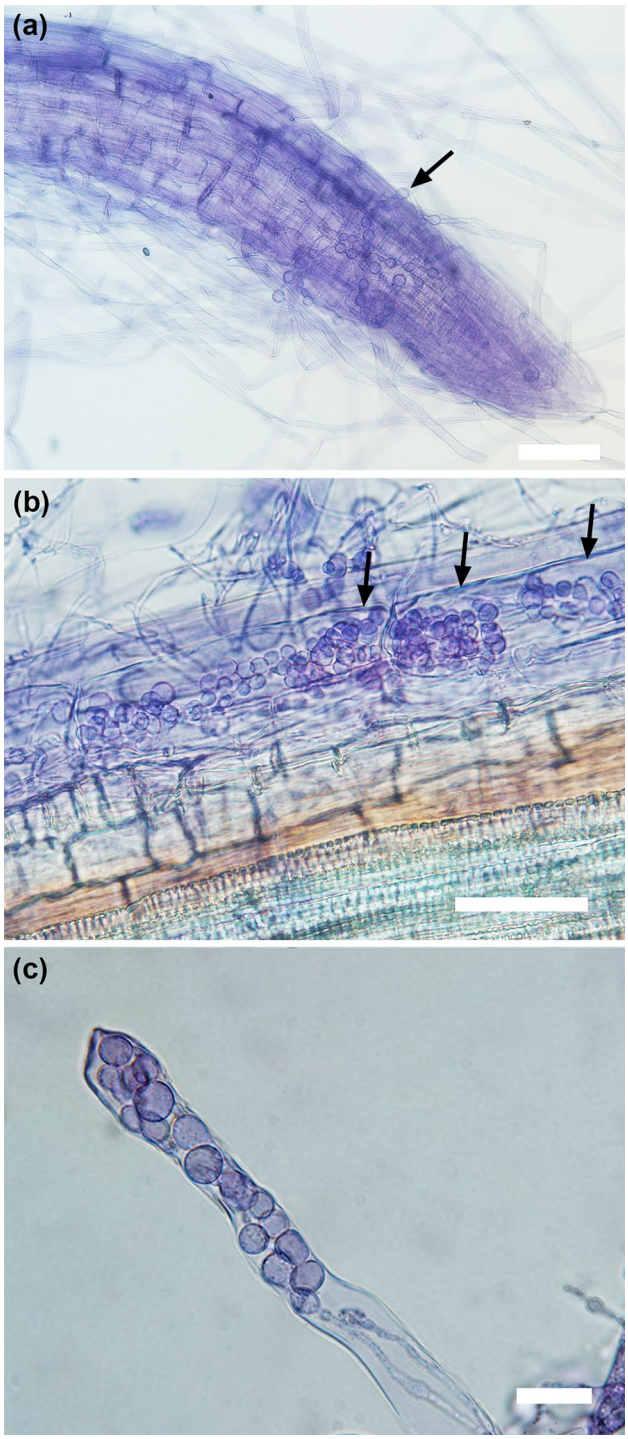

Fig. 7 Methyl blue–stained roots of *A. thaliana* plants inoculated with *S. restingae*. **a** A root tip with external monilioid hyphae. **b** The root cortex cells are densely colonized by monilioid hyphae (arrows). **c** Detail of an *A. thaliana* root hair fully colonized by *S. restingae* sp. nov. monilioid hyphae

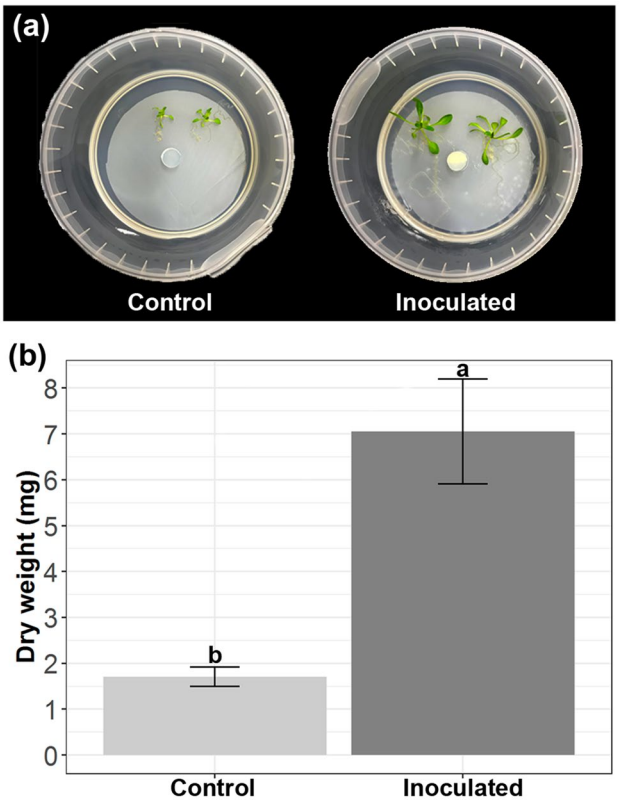

Fig. 8 In vitro inoculation experiment between *Serendipita restingae* sp. nov. and *Arabidopsis thaliana*. **a** The phenotypes of the inoculated and non-inoculated (control) plants. **b** Total dry weight (roots and shoots) of *Arabidopsis* plants 14 days after inoculation in PMN medium at 23 °C with a 16-h photoperiod, either with (inoculated) or without (control) a 0.5-cm^2 *S. restingae* inoculum plug (means of 26 replicates with two plants per dish). Error bars are confidence intervals, and different letters indicate significant differences according to the *F* test ($p = 0.001$)

nitrogen is released to host cells after peloton lysis (Kuga et al. 2014; see Selosse 2014 for a discussion).

Our study provides an accurate estimation of the genome size of *S. restingae*. Our estimation of *A. thaliana* using *S. lycopersicum* as an internal reference standard resulted in a C-value of 0.16 pg DNA, which fits exactly the value of previous measurements (e.g., Bennet et al. 2003; Tavares et al. 2014). Although the genome size data available from other Sebacinales were obtained by different methods, our results are in accordance with these previous estimations. The genome size of *S. indica* was estimated to range from 15.3 to 24.97 Mb (Zuccaro et al. 2009; 2011). Basiewicz et al. (2012) estimated the genome size of *S. williamsii* and *S. vermifera* (stains MAFF 305,828, 305,830, and 305,842) to be, respectively, 22 Mb and 21–26 Mb. More recently, the complete genome sequencing of *S. vermifera* MAFF305830 revealed a 38.1 Mb genome size (Kohler et al. 2015).

Interaction with hosts

One interesting feature of some *Serendipita* spp. is their capacity to colonize living and dead cells of plant hosts. In barley roots, *S. indica* colonizes living cortical cells, establishing a biotrophic interaction, but its proliferation requires host cell death in some cells (Deshmukh et al. 2006). Although this interaction resembles hemibiotrophy, there is no evidence of massive host cell death and, instead of detrimental effects, the plants show growth promotion and increased resistance to biotic and abiotic stresses (Lahrmann and Zuccaro 2012). *Serendipita indica* can thus be seen as biotrophic at tissue level: considering *S. restingae*, it is more difficult to draw a conclusion regarding the colonization pattern from our observations, since we were not able to observe whether colonized cells are alive or not. Future analyses may answer that question, but at least we did not evidence massive cell death in response to colonization.

In the present study, beyond confirming its association with the orchid as a mycorrhizal fungus at the germination stage, we demonstrate that the capacity of *S. restingae* to associate with *Arabidopsis* roots is notable, since both organisms have evolved in completely different habitats. Nevertheless, considering that other *Serendipita* spp. are capable of colonizing *Arabidopsis* roots (Peškan-Berghöfer et al. 2004; Basiewicz et al. 2012; Riess et al. 2014; Ray and Craven 2016), this result was already expected for *S. restingae*. Sebacinales were also shown to associate with *Arabidopsis* under natural conditions (Weiß et al. 2011) as well as with Brassicaceae in general (Selosse et al. 2009). Due to the great availability of mutants, the positive interaction between *S. restingae* and *Arabidopsis* is an excellent model for future studies of plant-fungal interactions.

Serendipita herbamans was detected in the roots of many crop plants collected in crop fields in Germany, including wheat, barley, triticale, sunflower, and pea as well as forage crops as *Lolium perenne*, *Trifolium pratense*, and *Trifolium repens* (Riess et al. 2014). These results show that the interaction between Sebacinales and domesticated plants may not be restricted to in vitro artificial conditions, but are rather common in domesticated landscapes.

We consider that, in the future, more studies should focus on phylogeography and diversity of the Sebacinales in the restinga ecosystem and more generally in South America. Many orchids occur in sympatry in this ecosystem and, whether they share mycorrhizal fungi or have specific associations, identifying their mycorrhizal fungi is an important issue for future study. Indeed, looking at orchid mycorrhizal fungi may help identify many species that also have endophytic abilities (Girlanda et al. 2011; Selosse and Martos 2014). We also consider it important to investigate whether *S. restingae* has dual abilities in nature, i.e., is both orchid mycorrhizal and endophytic with other plant species from the same habitat. Future studies using metabarcoding approaches could also be useful, especially to understand the distribution of *S. restingae* in soil, independently of their symbiont hosts.

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