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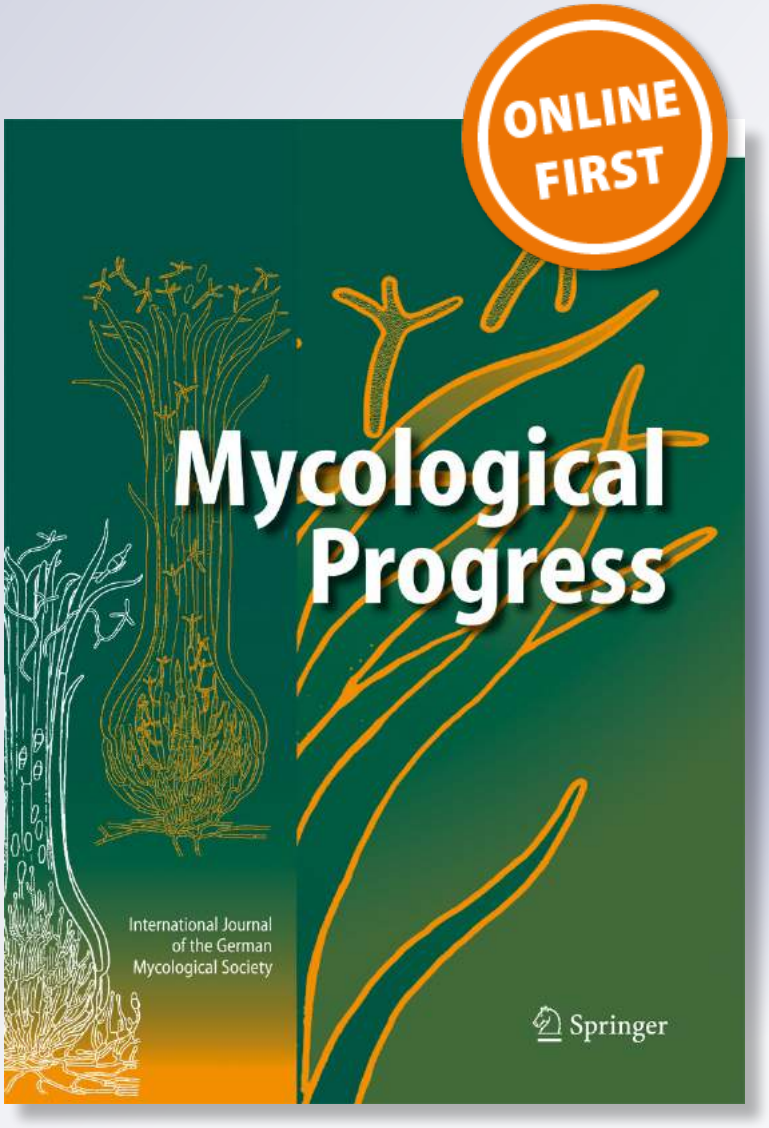
Mycological Progress

ISSN 1617-416X

Mycol Progress
DOI 10.1007/s11557-018-1386-5



Springer

Mycological Progress
https://doi.org/10.1007/s11557-018-1386-5

ORIGINAL ARTICLE

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Four new species of arbuscular mycorrhizal fungi (Glomeromycota) associated with endemic plants from ultramafic soils of New Caledonia

Thomas Crossay[1] · Alexis Cilia[1] · Yvon Cavaloc[1] · Hamid Amir[1] · Dirk Redecker[2]

Received: 13 September 2017 / Revised: 2 February 2018 / Accepted: 8 February 2018

Abstract

Four new species of arbuscular mycorrhizal (AM) fungi (Glomeromycota) were isolated from the rhizosphere of endemic metallophytic plants in ultramafic soils in New Caledonia (South Pacific) and propagated on *Sorghum vulgare*. *Acaulospora saccata* and *A. fragilissima* are placed in the Acaulosporaceae, *Scutellospora ovalis* in the Gigasporaceae, and *Rhizophagus neocaledonicus* in the Glomeraceae. The novelty of these species is supported by morphological characters of spores and phylogenetic analyses of sequences of the rDNA region, comprising partial small subunit rRNA gene, the internal transcribed spacers, 5.8S rRNA gene, and the partial large subunit rRNA gene. New Caledonia is known for its high degree of endemism in plants, which is due to its geographic position and geological history. This is the first taxonomic study exploring local Glomeromycota of this island, which may help to address the question of possible AMF endemism in future studies.

Keywords Arbuscular mycorrhiza · New Caledonia · Ultramafic soils · Glomeromycota · Species description

Introduction

Fungi of the phylum Glomeromycota are ubiquitous microorganisms living in symbiosis with 70–90% of the world's vascular land plants (Smith and Read 2008; Brundrett 2009). Despite their common occurrence and evidence of their association with land plants since their appearance 460 million years ago (Pirozynski and Malloch 1975; Redecker et al. 2000), the number of described arbuscular mycorrhizal fungi (AMF) species is very low compared with other phyla of fungi (Ohsowski et al. 2014). During the recent decades, the knowledge of diversity of AMF has considerably increased due to the use of DNA-based identification tools and continuing progress in morphological identification of AMF taxa (da Silva et al. 2006; Krüger et al. 2012; Błaszkowski et al. 2015). Many AM fungi are known only from environmental DNA sequences and the number of molecular operational taxonomical units or virtual taxa exceeds the number of AMF species described by traditional morphological analyses of soil-borne spores (Öpik et al. 2013, 2014). In 2017, A. Schüßler (http://www.amf-phylogeny.com/) listed 291 species of Glomeromycota. Molecular diversity studies have suggested the existence of 348 to 1600 Glomeromycota species (Ohsowski et al. 2014). Recent studies (Opik et al. 2013; Ohsowski et al. 2014) suggested that targeting previously unstudied or little-studied environments would result in revealing many novel AMF taxa. Furthermore, every year about a dozen of new AMF species have been described through AMF single-species culturing and concomitant morphological and molecular phylogenetic analyses, often, but not only from, thus far "unexplored" habitats (Palenzuela et al. 2013; Symanczik et al. 2014; Błaszkowski et al. 2015).

Section Editor: Marco Thines

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11557-018-1386-5) contains supplementary material, which is available to authorized users.

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Published online: 05 March 2018

New Caledonia, a tropical archipelago in the South Pacific, is known as one of the world's most important biodiversity hot spots, displaying a very high proportion of endemic and endangered plant species (Myers et al. 2000). In New Caledonia, serpentine ecosystems which have developed over ultramafic rocks (peridotites, serpentinites) cover one third of the surface area of the main island called Grande Terre. The originality of these ecosystems, where plant endemism levels reach 82%, is not only due to their geographic isolation, but also to the adaptation of communities to strong edaphic constraints (Jaffré 1993). New Caledonian ultramafic soils have very low levels of major elements (N, P, K, and Ca), with low Ca/Mg ratios and high concentrations of Ni, Co, Cr, and Mn (Brooks 1987; Jaffré and L'Huillier 2010). The stripping of large areas, for the purpose of nickel mining, endangers this unique ecological heritage. Ecological restoration of degraded mine sites is therefore a major concern in New Caledonia. Improvement of currently used techniques for revegetation (Luçon et al. 1997) requires a good understanding of the role of mycorrhizal associations involved in ultramafic soils (Smith and Read 2008; Graham 2009). Previous studies of New Caledonian ultramafic *maquis* (a biome characterized by shrubby and bushy plants with a low growth rate and xeromorphic foliage, equivalent to italian *macchia*) have highlighted that nearly all plant species are arbuscular mycorrhizal (Amir et al. 1997; Perrier et al. 2006), including Ni-hyperaccumulating plants (Amir et al. 2007) and species of Cyperaceae and Proteaceae, two families generally considered as non-mycorrhizal (Amir et al. 1997; Perrier et al. 2006; Amir and Ducousso 2010; Lagrange et al. 2011). AMF isolates from ultramafic soils have been found to be highly tolerant to Ni (Amir et al. 2008), so AM fungi from New Caledonian ultramafic soils are a good target to be used as plant growth promoters. Ultramafic habitats are among those that have received only little attention in AMF diversity, (Fitzsimons and Miller 2010; Schechter and Bruns 2008, 2012; Gensous 2014; Kohout et al. 2015); these studies suggested the presence of several new species of AMF in ultramafic soil. AM fungi from ultramafic soil have never been clearly described and identified.

Our morphological and molecular investigation of fungal communities associated with plants of ultramafic soil of New Caledonia revealed the presence of four undescribed AMF species in pot trap cultures comprising mixtures of rhizosphere soils and roots of plant colonizing the ultramafic soil in New Caledonia. We subsequently grew the fungi in single-species cultures. Two species formed typical acaulosporoid spores, one formed typical gigasporoid spores, and the last species formed glomoid spores. The aim of the present study was to isolate, describe, and identify these new species, based on both morphological and molecular analyses.

Material and methods

Study site

The archipelago of New Caledonia is situated in the South Pacific, approximately 1500 km east of Australia and 1800 km north of New Zealand. The climate is subtropical to tropical, with a rainy season from December to May and a dry season from June to November that can vary in duration and severity. Mean monthly temperature in the capital, Noumea, is highest in February with 26.2 °C and lowest in August with 19.9 °C (Enright et al. 2001). The study site is covered by ultramafic maquis vegetation under annual rainfalls varying from 900 to 4000 mm (Proctor 2003; Read et al. 2006). The study site is situated in the great south ultramafic massif and was in the area at Plum (22° 16′ S, 166° 38′ E). The altitude varies from 34 to 206 m. The topography is rugged with slopes often above 30%. In our study site, the vegetation is a mosaic of maquis and relict forests (paraforest formations). The paraforest formations are restricted to humid or topographically protected sites and form small patches surrounded by maquis. New Caledonian maquis has more than 2150 species with an endemism level of 82% (Jaffré et al. 2010) and occurs generally in old forest zones which have declined, during the last centuries, following successive fires (McCoy et al. 1999). There are several unifying features: maquis is evergreen, 1–7 m tall, more or less bushy or with a dense sedge layer, most species are sclerophyllous and it includes some transitional forest forms. Most of the maquis species have a well-developed superficial root system (Jaffré and L'Hullier 2010).

Soil geochemistry

The soil of the study site is a colluvial lateritic soil with following characteristics: coarse sand, 39.4%; fine sand, 22.1%; silt-clay, 37.2%; pH H20, 5.9; pH KCl, 5.6; total C, 42.1 g kg^{-1}; total N, 2.2 g kg^{-1}; total P, 147 mg kg^{-1}; available P (Mehlich), 3 mg kg^{-1}; total Ca, 1.06 g kg^{-1}; total Mg, 5.08 g kg^{-1}; Ca/Mg, 0.207; total Ni, 4.78 g kg^{-1}; DTPA extractable Ni, 91.4 mg kg^{-1}; DTPA extractable Co, 71.0 mg kg^{-1}; DTPA extractable Cr, 0.5 mg kg^{-1}; DTPA extractable Mn, 864.8 mg kg^{-1}.

Sampling, establishment, and growth of trap and single-species cultures

Soil samples were collected from the rhizosphere of four endemic plants: *Codia discolor* (Brongn. & Gris) Guillaumin, *Alphitonia neocaledonica* (Schltr.) Guillaumin, *Dodonaea viscosa* (L.) Jacq. and *Sannantha leratii* (Schltr.) Peter G. Wilson. All these plants species are arbuscular mycorrhizal (Amir et al. 1997; Perrier et al. 2006) and are used for

ecological restoration of New Caledonian degraded mine sites. An area of 200 m^2 was delimited; rhizosphere soil (100 g of soil and roots) of ten plants of each species was sampled. The rhizosphere soil was sampled by first digging a pit of 30-cm diameter and 30-cm depth, close to the plant stems to have access to the roots. Soil was then collected from the border of the pits in order to obtain a sample representing a vertical cross section across the root zones of these plants and then the soil was pooled per plants species. For the four samples of soil (one sample by plant species), 20 pot trap cultures (1 L) were established. Pots were filled with 0.85 kg of an autoclaved substrate consisting of ultramafic soil from the study site. As plant growth in this pure ultramafic soil was very slow, a mixture of 80% ultramafic soil and 20% commercial compost (*v*/*v*) was used. The soil was first autoclaved three times at 120 °C for 1 h, with an interval of 24 h, to eliminate microorganisms. The mycorrhizal inocula (50-g mixture of rhizosphere soils and roots each) were spread as a layer on the surface of the substrate in the pots and covered with a thin layer of the substrate. *Sorghum vulgare* was used as trap plant. Seeds of *S. vulgare* were surface-disinfected in a 1.25% solution of sodium hypochlorite (12° chlorometric) for 15 min, and then rinsed with distilled water in sterilized Petri dishes. The seeds were subsequently sown in sterilized vermiculite (autoclaved for 60 min; at 120 °C) before transfer to experimental pots. Two 2-week-old AMF-free plantlets of *S. vulgare* were removed from the vermiculite and planted in every experimental pot. The trap cultures were kept in a greenhouse (temperature, 21–24 °C; relative humidity, 70%) for 7 months and irrigated manually every 2 days. After 7 months, spores were extracted by wet sieving and sucrose density gradient centrifugation, using a modified method of Daniels and Skipper (1982). For each trap culture, 10 g of soil were wet-sieved, and then 10 mL of the sieved material enriched in spores was well suspended in 20 mL of water in a 50-mL Falcon tube. A 25 mL sucrose solution (70% *v*/*w*) was injected to the bottom of the tube, forming a stepped density gradient that was centrifuged at 900*g* for 3 min. Spores of AMF were collected from the interface of sucrose solution, washed with tap water on a 36-μm sieve for 2 min, and transferred to Petri dishes. Spores were then picked individually under a stereomicroscope and examined morphologically. Then, several spores of each morphotype were surface-sterilized using the procedure of Bécard and Fortin (1988). These steps were carried out aseptically in a laminar flow cabinet. Spores were washed in a 0.05% (*v*/*v*) Tween 20 solution, soaked in a vacutainer tube with 2% (*w*/*v*) chloramine T solution for 10 min, and rinsed three times by centrifugations for 30 s in sterile distilled water. A second treatment with chloramine T followed by rinsing in water was performed in the same manner. Then, spores were washed 10 min in a sterile solution of 200 mg L^{-1} streptomycin and 100 mg L^{-1} gentamycin and rinsed three times by centrifugations for 30 s in sterile distilled water. Spores were used immediately for establishment of single-species cultures, using the "cone-tainer technique" (Koske and Gemma 1997; https://invam.wvu.edu/methods/cultures/single-species-cultures). For this technique, the same substrate used for trap cultures was placed in a 50 mL "cone tainer" (Cooperative Centrale Agricole, Nouméa). As previously described two, 2-week-old, AMF-free plantlets of *S. vulgare* were placed in every "cone-tainer." A total of 100 surface-sterilized spores of each morphotype in 200 μl water were pipetted along the length of the intertwined roots, so they theoretically were in good contact with roots. After two months, the "cone tainers" were transplanted into 450-mL pots filled with the same substrate. Overall, 25 single-species cultures were set up per morphotype and kept in a greenhouse (temperature, 21–24 °C; relative humidity, 70%) for 6 months. They were irrigated manually every 2 days. After 6 months, spores were extracted by wet sieving and sucrose density gradient centrifugation as described previously. Spores from successful, pure single-species cultures were re-inoculated on *S. vulgare* as described previously. After 6 months, spores were extracted and used for morphological and molecular analyses and for root organ culture. The presence of mycorrhizal structures on roots of *S. vulgare* was determined using a clearing and staining protocol for roots (Phillips and Hayman 1970) as modified by Błaszkowski et al. (2006): tissue acidification was performed using 20% hydrochloric acid instead of 1% and trypan blue concentration was 0.1% instead of 0.05%.

Root organ culture

Transformed chicory (*Cichorium intybus* L.) roots were provided by BCCM (Belgian Coordinated Collections of Microorganisms). Routine maintenance of the roots was performed on the modified Strullu-Romand (MSR) medium (Diop et al. 1994) in an inverted position at 28 °C in the dark. The MSR medium contained in milligrams per liter distilled water: $MgSO_4.7H_2O$, 739; KNO_3, 76; KH_2PO_4, 4.1; $Ca(NO_3)_2.4H_2O$, 359; NaFeEDTA, 8; KCl, 65; $MnSO_4.4H_2O$, 2.45; $ZnSO_4.7H_2O$, 0.29; H_3BO_3, 1.86; $CuSO_4.5H_2O$, 0.24; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.035; $Na_2MoO_4.2H_2O$, 0.0024; thiamine, 1; pyridoxine, 0.9; nicotinic acid, 1; calcium penthotenate, 0.9; cyanocobalamine, 0.4; biotine 0.9 × 10–3; sucrose, 6000 and phytagel (SIGMA) 4500. The pH was adjusted to 5.5 before sterilization at 121 °C for 15 min. Fungal inoculum: 500 spores of single-species cultures of each species were extracted and surface-sterilized as previously described. Then, 30–60 spores per Petri plate were spread out on MSR medium. Ten Petri plates were established for each species. Primary mycorrhizal colonization was achieved after 1 week by placing root explants (3 cm) in each Petri plate. Root organ cultures were kept in an inverted position for 6 months at 28 °C in the dark. Every

week in situ observation of different structures: germination, ramification, runner hyphae, branch absorbing structure, and spores of each fungus were performed under a compound microscope (Olympus BX 50). Photographs were taken using a digital camera (Leika DFC 295) on a compound microscope (Olympus BX 50) using Leica Application Suite Version V 4.1 software.

Morphological analyses

Spores from single-species cultures of each new fungus were extracted as previously described. Morphological features of spores and phenotypic and histochemical characters of spore wall layers were determined after examination of at least 100 spores mounted in water, lactic acid, PVLG (Omar et al. 1979), and a mixture of PVLG and Melzer's reagent (1:1, *v*/*v*). For the *Rhizophagus* species, some spores were cleared in a solution of 25% sodium hypochlorite (12° chlorometric) for 5 min, and then rinsed with distilled water in sterilized Petri dishes. The terminology for spore subcellular structures follows Błaszkowski (2012). Spore color was examined under a dissecting microscope on fresh specimens immersed in water. Colors were determined using the INVAM color chart. Photographs were taken using a digital camera (Leica DFC 295) on a compound microscope (Olympus BX 50) equipped with Nomarski differential interference contrast optics and Leica Application Suite Version V 4.1 software. Specimens mounted in PVLG and a (1:1) mixture of PVLG and Melzer's reagent were deposited at the Mycological Herbarium of the National Museum (France, Paris) in MNHN-PC-PC0728837, MNHN-PC-PC0728838, MNHN-PC-PC0728839, MNHN-PC-PC0728840.

Morphological comparisons of *Acaulospora* spp. and *Scutellospora* spp. were performed using species descriptions from the INVAM website (http://fungi.invam.wvu.edu/the-fungi/species-descriptions.html). Exceptions were *A. papillosa*, for which the study of Pereira et al. (2016) was used and *S. spinossima*, *S. pernambucana*, and *S. alterata* for which the original descriptions by Walker et al. (1998), da Silva et al. (2008), and Pontes et al. (2013) were used, respectively.

Molecular analyses

One to three spores of each species from the single-species cultures on *S. vulgare* were crushed using a pipette tip in a 1.5 Eppendorf tube containing 10 μl of ultrapure water, 2 μl was used for polymerase chain reaction (PCR). A DNA fragment of around 1545 bp, covering partial SSU, the whole ITS and the variable D1 and D2 regions of the LSU, was amplified by nested PCR using the AMF-specific primers developed by Krüger et al. (2009). In the first round of PCR, the primers SSUmAf and LSUmAr were used. In the second, nested round of PCR, the primer SSUmCf and LSUmBr were used with 1 μl of the first PCR round product as a template. The PCR mix included 0.4 U of AmpliTaq® 360 DNA polymerase (Applied Biosystems), 1X AmpliTaq® 360 PCR buffer (Applied Biosystems), 0.2 mM of each dNTP, 0.4 μM of each primer and 1 μl of the template in a final volume of 25 μl. The cycling parameters for the first PCR were 3 min at 98 °C followed by 35 cycles of 10 s at 98 °C, 30 s at 60 °C, and 1 min at 72 °C. The program was concluded by a final extension phase of 10 min at 72 °C. The cycling parameters for the second PCR were the same as in the first PCR except for the number of cycles (35) and annealing temperature (63 °C). The PCRs were conducted in triplicates. PCR products were checked on 1% agarose gels. The positive PCR products were cloned into pGEM-T using a pGEM-T easy vector system (Promega) following the manufacturer's instructions. Ligated plasmids were transformed into $CaCl_2$ competent *Escherichia coli* DH5α cells using a heat-shock approach. The transformed bacteria were plated into LB (Luria-Bertani) medium containing ampicillin (50 μg/mL) and grown overnight at 37 °C. A PCR using the universal M13F and M13R primers was performed directly on bacterial colonies to screen for positive clones. Clones that exhibited fragments with the expected size were sequenced on an Applied Biosystems 3730xl capillary sequencer (IRD, Noumea) with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The forward and reverse strands were assembled in ChromasPro (Technelysium Pty Ltd., Australia). The glomeromycotan origin of the sequences was tested by BLAST (Altschul et al. 1997). The new sequence were deposited in the EMBL database under the accession numbers: KY362428, KY362429, KY362430; KY362431, KY362432, KY362433; KY362434, KY362435; KY362436, KY362437, KY362438.

Phylogenetic analyses

A sequence alignment of our data and published glomeromycotan species was obtained using MAFFT 7 (http://mafft.cbrc.jp/alignment/server; Katoh and Standley 2013) using the slow iterative refinement option FF-NS-I (gap opening penalty 1.0, offset value 0.1). Maximum likelihood (ML) analyses of the whole region (SSU-ITS1-5.8S-ITS2-LSU) or of the 28S part were performed using PhyML 3.0 (Guindon et al. 2010) and MEGA 7 software (Kumar et al. 2016) with bootstrap support obtained using 1000 replicates. Phylogenetic trees were viewed and edited using MEGA 7. Bayesian analyses were performed using MrBayes 3.2 (Ronquist and

Huelsenbeck 2003), using the General Time Reversible Model for 10^7 generations and a burnin value of 25%.

Results

Taxonomic analyses

Acaulospora saccata **D. Redecker, Crossay & Cilia, sp. nov. Fig. 1a–j Mycobank MB820531**

Holotype Isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum (France, Paris) MNHN-PC-PC0728838 (slide). This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot of a single-species culture propagated on *Sorghum vulgare* at LIVE in New Caledonia, Nouméa, October 2015, T. Crossay. The previous single-species culture generation originally was set up using 100 spores isolated from rhizospheric soil of a greenhouse pot trap cultures propagated on *Sorghum vulgare* at LIVE in New Caledonia, Noumea, April 2015, T. Crossay. Pot trap cultures had originally been inoculated with rhizospheric soil of *Alphitonia neocaledonica* sampled in an ultramafic *maquis* in New Caledonia, September 2014, T. Crossay. This ultramafic *maquis* is located in south of Grand Terre island of New Caledonia at Plum, (22° 16′ S, 166° 38′ E).

Description Sporocarps unknown. Spores sessile, globose to subglobose, occasionally ovoid, hyaline when young, later to white to pale ochraceous (0/10/50/10), (69–)75(−85) μm in diameter, formed laterally on the sporogenous hypha (Fig. 1a). No sporiferous saccule observed in single-species culture (in vivo). Spore wall with two layers. Layer 1 hyaline, mucilagineous, disappearing during spore maturation, (1.0–)1.2(−1.5) μm thick (Fig. 1f). Layer 2 laminate, pale yellow (0/0/50/10) (4–)5.5(−6.5) μm thick (Fig. 1d–f). Inner wall 1 with one thin, hyaline, semi-flexible layer (1–)1.2(−1.5) μm thick (Fig. 1e–f). Inner wall 2 with two layers, layer 1 hyaline with granular excrescences (or "beads") (0.8–1.6 μm thick), seem to associate with inner face of inner wall 1 (Fig. 1e). Layer 2 in PVLG at least (1.5–)1.8(−3.5) μm thick (Fig. 1e–f), plastic, turning deep beetroot purple (20/80/20/0 to 40/80/20/0) in Melzer's reagent (Fig. 1f), appearing sack-like and thus very difficult to break up by pressure. Germination through spore wall (Fig. 1h), no germination orb was observed.

Etymology From Latin *saccatus*, sac-shaped, referring to the innermost wall of the spore which resembles an elastic bag with folds that is difficult to break.

Habitat Rhizospheric soil in tropical ultramafic *maquis*.

Distribution Known only from the type locality.

Material examined Spores isolated from rhizospheric soil of a greenhouse pot of single- species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay, MNHN-PC-PC0728838.

In vitro morphology Spores formed laterally on the sporogenous hypha and no sporiferous saccules observed (Fig. 1b). Runner hyphae (3.5–)4(−4.5) μm diameter (Fig. 1c–g), often with swellings (Fig. 1c).

Molecular and phylogenetic analysis Phylogenetic analyses of the 18S-ITS1-5.8S-ITS2-28S region firmly places this species in a branch of the genus *Acaulospora*, containing *A. rugosa*, *A. longula*, *A. mellea*, *A. delicata*, *A. dilatata*, *A. morrowiae*, and *A. papillosa* (Fig. 3). Analyses of the whole region (Fig. 3) or of the 28S part (see Supplementary information Fig. S1) using maximum likelihood analyses in MEGA and PhyML clearly separate this species from their closest relatives and from each other.

Notes Of the *Acaulospora* spp. that clustered in the major clade with the basal subclade represented by *A. saccata* (Fig. 3), the new species is morphologically most closely related to *A. delicata*. Only in these two species, the spore wall and inner wall 1 are two- and one-layered, respectively. In the other species, the spore wall comprises three layers, and inner wall 1 consists of two layers. Other features clearly separate *A. saccata* from *A. delicata*. Spores of *A. saccata* are approximately 1.2-fold smaller when globose, and their spore wall is 2-fold thicker. Moreover, layer 2 of inner wall 2 of *A. saccata* is plastic and stains purple in Melzer's reagent, whereas that of *A. delicata* is flexible, does not swell in PVLG (is not plastic), and stains faintly or does not stain at all in this reagent. For more details on the comparison with other species, see Supplementary Text File .

Acaulospora fragilissima **D. Redecker, Crossay & Cilia, sp. nov. Fig. 2a–i Mycobank MB820533**

Holotype Isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Noumea, April 2016, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum

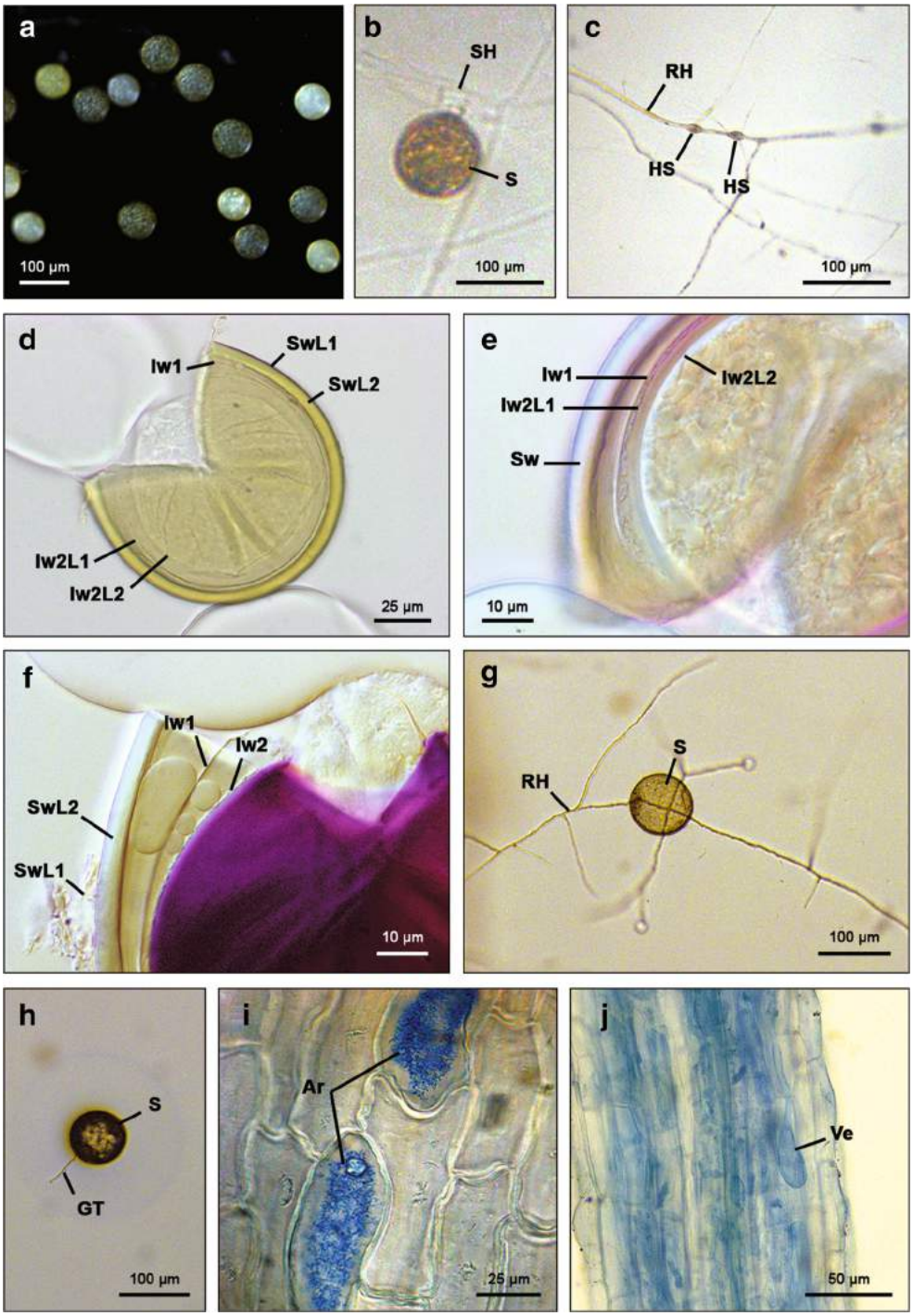

Fig. 1 **a-j** *Acaulospora saccata* spores. **a** Intact spores. **b** Intact spore (S) with sporogenous hyphae (SH) in root organ culture. **c** Runner hyphae (RH) with hyphal swelling (HS) in root organ culture. **d** Spore wall layers 1 and 2 (SwL1, SwL2) and inner walls 1 and 2 (Iw1, Iw2). **e** Inner wall 1 layer 1 (Iw1L1), inner wall 2 layers 1 and 2 (Iw2L1, IW2L2). **f** Spore wall layers 1 and 2 (SwL1, SwL2), inner wall 1 layer 1 (Iw1L1), and inner wall 2 (IW2). **g** Spore (S) and runner hyphae (RH) on root organ culture. **h** Germinal tube (GT) of spore (S). **i**, **j** Mycorrhizal structures of *A. saccata* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: arbuscules (Ar) and vesicles (Ve). **d**, **e** In PVLG. **f** In PVLG + Melzer's reagent

(France, Paris) in MNHN-PC-PC0728839 (slide). This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE in New Caledonia, Noumea, October 2015, T. Crossay. The previous generation of single-species culture originally was inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot trap cultures propagated on *Sorghum vulgare* at LIVE in New Caledonia, Noumea, April 2015, T. Crossay. Pot trap cultures originally had been inoculated with rhizospheric soil of *Alphitonia neocaledonica* sampled in an ultramafic *maquis* in New Caledonia, September 2014, T. Crossay.

Description Sporocarps unknown. Spores sessile, globose to subglobose, occasionally ovoid, creamy white to pale ochraceous (0/10/40/0 to 0/10/70/10), (60–)67(−83) µm in diameter (Fig. 2a). No sporiferous saccule observed in

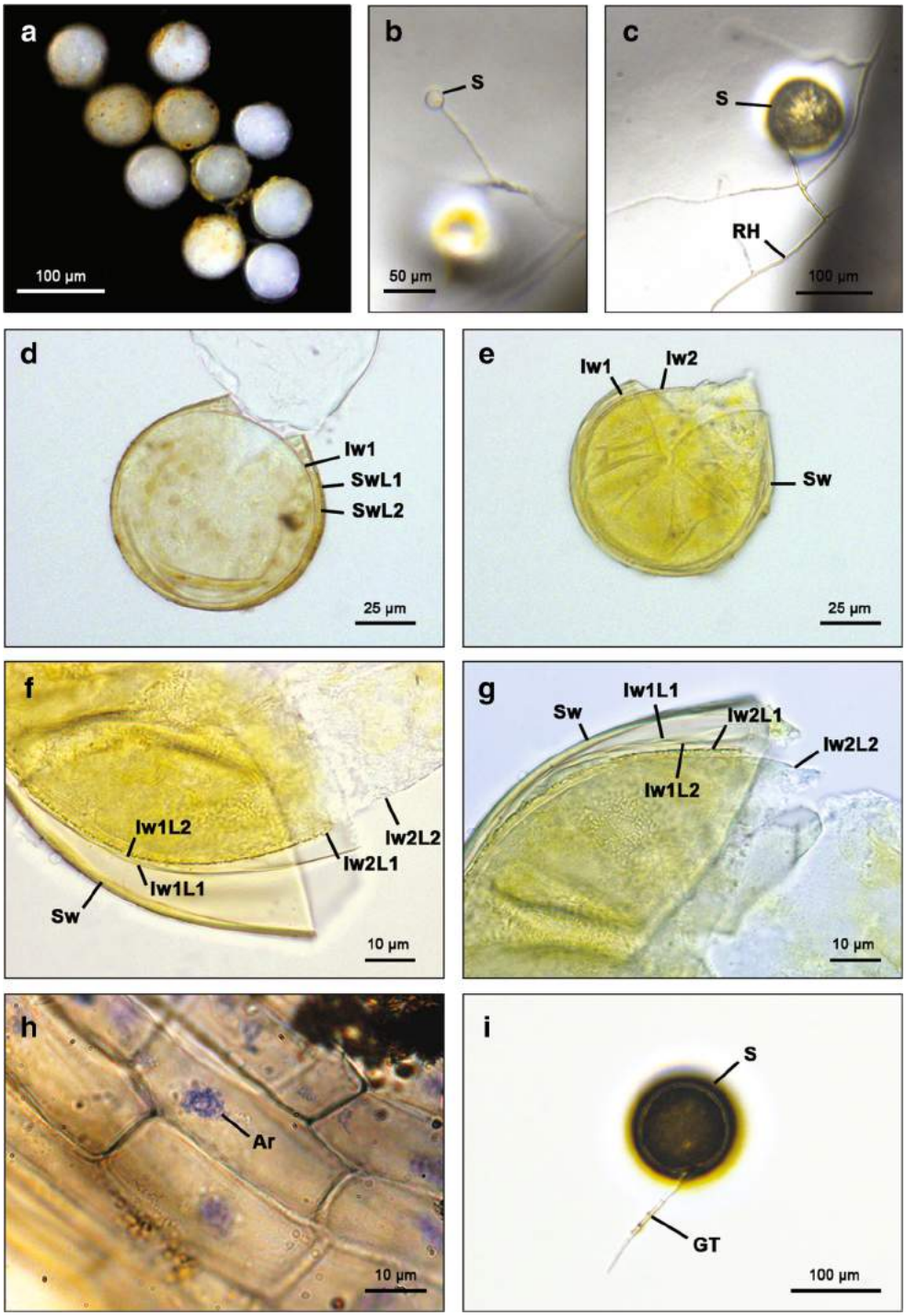

Fig. 2 **a-i** *Acaulospora fragilissima* spores. **a** Intact spores. **b** Intact spore (S) with sporogenous hyphae on root organ culture. **c** Runner hyphae (RH) on root organ culture. **d** Spore wall layers 1 and 2 (SwL1, SwL2) and inner wall 1 (Iw1). **e** Inner walls 1 and 2 (Iw1, Iw2). **f**, **g** Inner wall 1 layers 1 and 2 (Iw1L1, Iw1L2); Inner wall 2 layers 1 and 2 (Iw2L1, Iw2L2). **h** Mycorrhizal structures of *A. fragilissima* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: arbuscules (Ar). **i** Germinal tube (GT) of spore (S). **d**, **g** In PVLG. **e**, **f** In PVLG + Melzer's reagent

single-species culture (in vivo). Spore wall with two layers. Layer 1 hyaline, evanescent, disappearing during spore maturation, (1.0–)1.2(−1.5) µm thick (Fig. 2a–d), often with adhering debris (Fig. 2a). Layer 2 laminate, pale yellow (0/10/40/0) (1.9–)2.8(−3) µm thick (Fig. 2e–f–g). Inner wall 1 very thin, rigid, with two hyaline layers, rarely separating, together (1–)1.2(−1.5) µm thick (Fig. 2f–g). Inner wall 2 composed of two layers (Fig. 2e–g), not staining in Melzer's reagent (Fig. 2f) except for layer 1 which has a light greenish yellow-ochre (10/0/80/0) tint after Melzer's staining. Layer 1 flexible, (0.9–)1(−1.2) µm thick, covered with granular excrescences in PVLG (Fig. 2f–g). Layer 2 thin, hyaline, flexible (0.8–)1(−1.2) µm thick, forming numerous folds, breaking easily by pressure on the cover slip (Fig. 2f–g). Germination through spore wall (Fig. 2i). No germination orb was observed.

Etymology From Latin *fragilissima*, highly fragile, is referring to the spores which break easily and are quickly degraded when stored.

Mycorrhizal structure Forming hyphal coils and arbuscules, staining with Trypan Blue (Fig. 2h).

Habitat Rhizospheric soil in tropical ultramafic *maquis*.

Distribution Known only from the type locality.

Material examined Spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay, MNHN-PC-PC0728839.

In vitro morphology Spores formed terminally on the sporogenous hyphae, no sporiferous saccule observed (Fig. 2b). Runner hyphae (2.5–)3(−3.2) diameter (Fig. 2c).

Molecular and phylogenetic analysis Phylogenetic analyses of the 18S-ITS1-5.8S-ITS2-28S region firmly place this species in a branch of the genus *Acaulospora*, containing *A. rugosa*, *A. longula*, *A. mella*, *A. delicata*, *A. dilatata*, *A. morrowiae*, and *A. papillosa* (Fig. 3). Analyses of the whole region (Fig. 3) or of the 28S part (see Supplementary information Fig. S1) using maximum likelihood analyses in MEGA and PhyML clearly separate this species from their closest relatives and from each other. *A. fragilissima* sp. nov. is well-differentiated by its DNA sequence from other species in the same major clade in the rDNA phylogeny (*A. mellea*, *A. delicata*, *A. dilatata*, *A. morrowiae*, *A.rugosa*, *A. longula*, *A. papillosa*).

Notes *Acaulospora fragilissima* differs from other *Acaulospora* spp. mainly in its molecular phylogeny (Fig. 3). In morphology, the species is most similar to *A. delicata*. These species are differentiated by the following characters: spores of *A. delicata* are clearly larger, differ somewhat in color, being pale yellow with a pale greenish tint, and their inner wall 1 consists of one layer instead of two as in *A. fragilissima*. For more details on the comparison with other species, see Supplementary Text File.

The two closest BLAST hits of *A saccata* and *A. fragilissima* for its partial LSU sequences comprise only environmental sequences from maize roots in China (KF849639, KF849513), none of these closely related in phylogenetic trees (see Supplementary information Fig. S2). Bayesian analyses confirmed the phylogenetic placement of the two species by maximum likelihood (Suppl. Fig. 3).

Scutellospora ovalis D. Redecker, Crossay & Cilia, sp. nov. Fig. 4a–i Mycobank MB820535

Holotype Isolated from rhizospheric soil of a greenhouse pot of a single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Noumea, April 2016, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum (France, Paris) in MNHN-PC-PC0728837 (slide). This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE in New Caledonia, Nouméa, October 2015, T. Crossay. The previous generation of single-species culture originally was inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot trap cultures propagated on *Sorghum vulgare* at LIVE in New Caledonia, Noumea, April 2015, T. Crossay. Pot trap cultures were originally inoculated with rhizospheric soil of *Sannantha leratii* sampled in an ultramafic *maquis* in New Caledonia, September 2014, T. Crossay.

Description Sporocarps unknown. Spores ellipsoid, hyaline when young, yellow ochraceous (0/10/80/0) at maturity, 90–110 × 175–260 μm in diameter, formed on a hyaline bulbous sporogenous cell, 15–20 × 25–30 μm. Subtending hypha hyaline, cylindrical (7–)8(−8.5) μm diameter (Fig. 4a–c). Spore wall with two layers. Layer 1 hyaline, often disappearing as spore ages, (1.2–)1.5(−2) μm thick (Fig. 4g). Layer 2 laminate, pale yellow (0/10/60/0) at maturity, (3.5–)4(−4.5) μm thick (Fig. 4f–h). Inner wall 1 very thin with two layers, layer 1 hyaline, (1–)1.2(−1.5) μm thick, layer 2 hyaline, (1–)1.1(−1.2) μm thick, these two layers are usually adherent and are positioned very close to the spore wall, therefore very difficult to detect (Fig. 4e–f). Inner wall 2 very thick and coriaceous, with two layers, layer 1 semi-flexible, (5–)6(−6.6) μm thick, staining lightly purple in Melzer's reagent (0/20/20/10) (Fig. 4e–f). Layer 2 (1–)1.25(−1.5) μm thick, plastic, forming numerous folds, staining deep beetroot purple in Melzer's (20/80/30/10 to 60/80/30/10) (Fig. 4e–f). Germination shield hyaline, bilobate, 20–25 × 40–45 μm with two germ tubes (Fig. 4d, g). No auxiliary cells observed.

Etymology From Latin *ovalis*, referring to the shape of the spore.

Mycorrhizal structure Forming hyphal coils and arbuscules, staining lightly with Trypan Blue (Fig. 4i).

Habitat Rhizospheric soil in tropical ultramafic *maquis*.

Distribution Known only from the type locality.

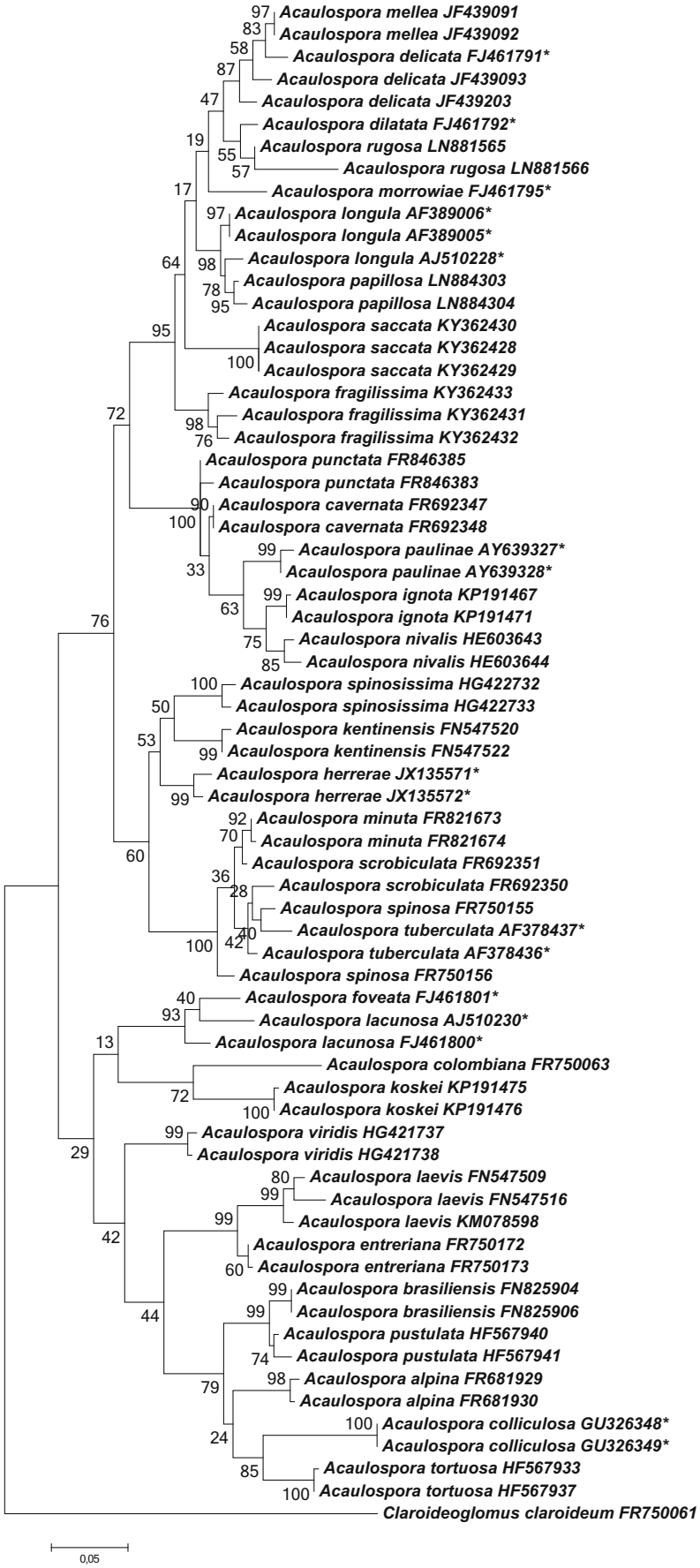

Fig. 3 ML analysis of SSU-ITS-LSU rDNA sequences of *Acaulospora saccata* and *Acaulospora fragilissima* and 31 known species of AMF, including *Claroideoglomus claroideum* as outgroup. Maximum likelihood (ML) phylogenetic tree based on small subunit–internal transcribed spacer–large subunit (SSU-ITS-LSU) rDNA sequences. Sequences labeled with an asterisk (*) represent the LSU gene only. Bootstrap values are given for each branch. The scale bar indicates the number of substitutions per site. Phylogenetic trees were viewed and edited using MEGA 7

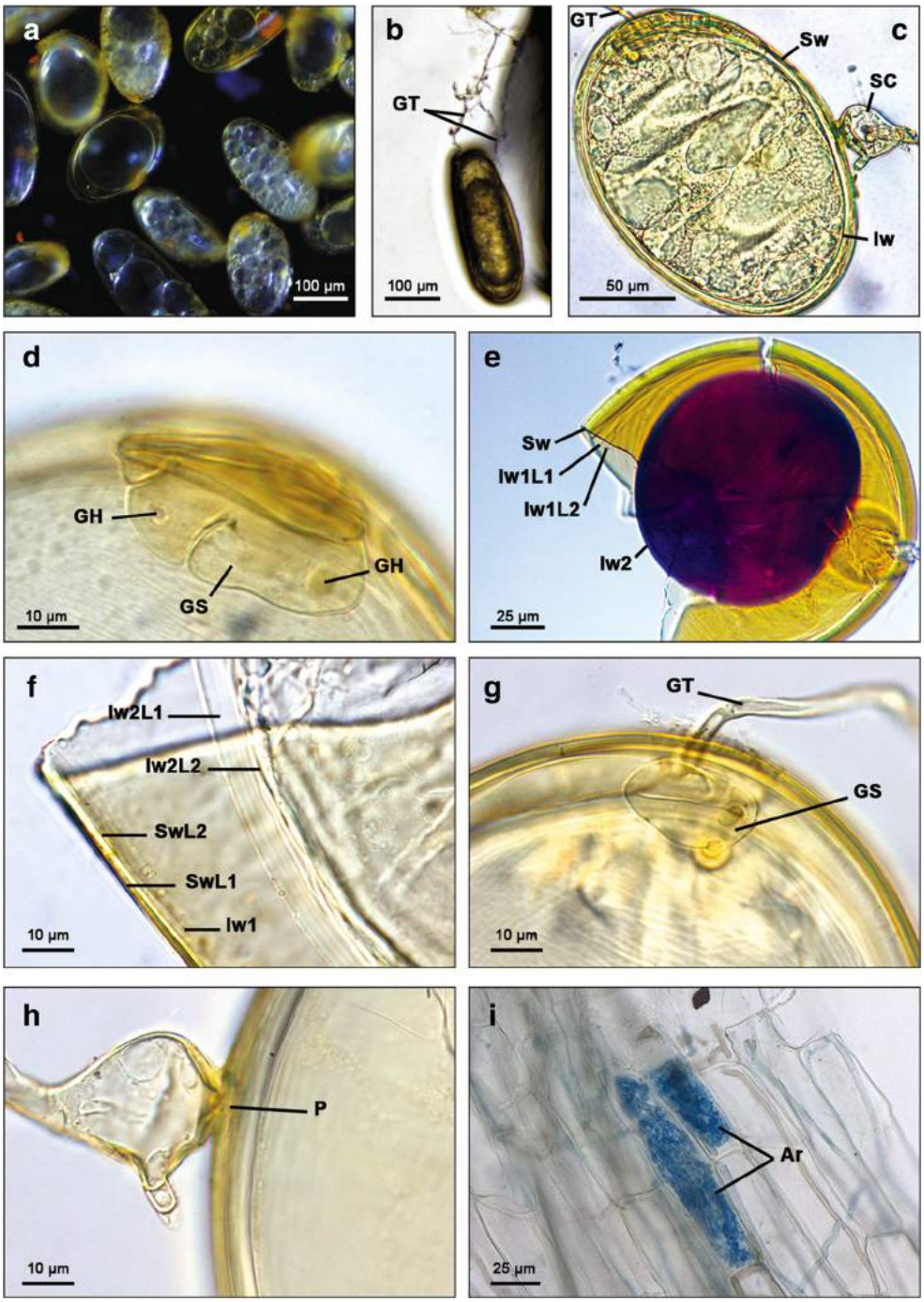

Fig. 4 **a-i** *Scutellospora ovalis* spores. **a** Intact spores. **b** Germinal tube (GT) of spore and ramification of hyphae on root organ culture. **c** Spore wall (Sw), sporogenous cell (sc), germinal tube (GT) and inner wall (Iw). **d** Germination shield (GS) with two germination holes (GH). **e** Spore wall layers 1 and 2 (SwL1, SwL2) and inner wall 1 (Iw1). **f** Spore wall layers 1 and 2 (SwL1, SwL2) and inner wall 1 layers 1 and 2 (IW1L1, IW1L2). **g** Germinal tube (GT) through spore germination shield (GS). **h** Sporogenous cell closed by a plug. **i** Mycorrhizal structures of *Scutellospora ovalis* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: arbuscules (Ar). **c**, **d**, **f**, **g**, **h** In PVLG. **e** In PVLG + Melzer's reagent

Material examined Spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay, MNHN-PC-PC0728837.

In vitro morphology Only germination and ramification (presymbiotic stage) (Fig. 4b) were observed, no sporulation occurred. Germination always with two germ tubes at the same time, through spore germination shield (Fig. 4b). Germinal hyphae (4.5–)5.5(−6) µm diameter (Fig. 4b).

Molecular and phylogenetic analysis Phylogenetic analyses of the 28S rDNA region firmly place this species in a branch of the genus *Scutellospora* (Fig. 5) in the Gigasporaceae, and clearly separate this species from their closest relative, *S. spinosissima* and the remaining species of the genus.

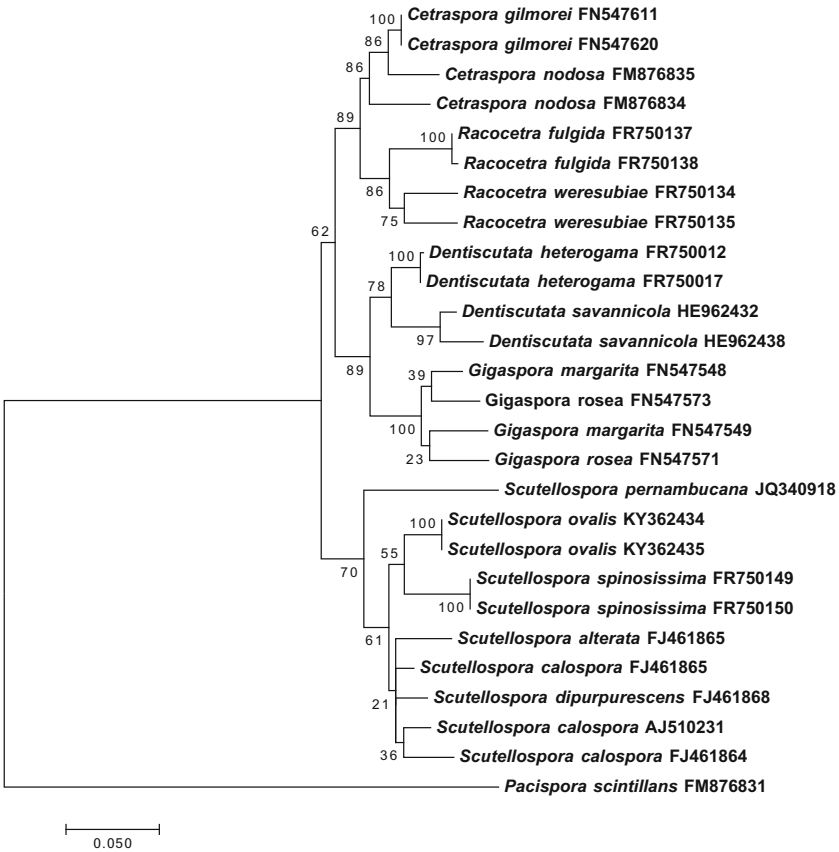

Fig. 5 Maximum likelihood analysis of LSU rDNA sequences of *Scutellospora ovalis* including 12 known species of AMF, including *Pacispora scintillans* as outgroup. Bootstrap values are given for each branch. The scale bar indicates the number of substitutions per site. Phylogenetic trees were viewed and edited with MEGA 7

Notes In the rDNA-based phylogeny, *Scutellospora ovalis* sp. nov. forms a weakly-supported monophyletic group with *S. spinosissima*, a species described from Venezuela (Walker et al. 1998).

Scutellospora dipurpurescens, *S. calospora* and *S. alterata* are grouped in a neighboring loose clade or grade. Interestingly, *S. dipurpurescens*, *S. alterata*, and *S. calospora* share a 21 base pair insertion in the LSU rDNA, which has not been found in other Gigasporaceae. This insertion may constitute a synapomorphy characterizing the clade comprising these three taxa, but a gain in the clade of *Scutellospora* except *S. pernambucana*, followed by a loss in *S. ovalis* and *S. spinosissima* seems also possible.

Scutellospora ovalis differs from other *Scutellospora* spp. by its (i) thicker spore wall layer 2 (mean 4 μm) and (ii) plastic inner wall 2 which is easily extruded from the spore wall. The latter has also been observed in *S. spinossima* (differing from *S. ovalis* by the presence of spines on its spore wall layer 2) but is not possible in *S. calospora* and *S. dipurpurescens*. This feature has not been reported for *S. pernambucana* and *S. alterata*. Overall, spore wall structure of *S. ovalis* is more similar to *S. spinossisima*, with the difference that inner wall 2 of *S. spinossisima* is composed of three layers (vs. two in *S. ovalis*). *Scutellospora alterata* differs from all other *Scutellospora* species by an "evanescent, papillate surface roughening" (Pontes et al. 2013).

The spores of *S. calospora* and *S. dipurpurescens* also exhibit a greenish tint which was not found in *S. ovalis* (see Supplementary information Fig. S4) and was not reported for *S. spinosissima*, *S. pernambucana* and *S. alterata*. For more details on the comparison with other species, see Supplementary Text File.

Rhizophagus neocaledonicus D. Redecker, Crossay & Cilia, sp. nov. Fig. 6a–i Mycobank MB820537

Holotype Isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum (France, Paris) in MNHN-PC-PC0728840 (slide). This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE in New Caledonia, Nouméa, September 2015, T. Crossay. The previous generation of single-species culture originally was inoculated with 100 spores isolated from rhizospheric soil of a greenhouse pot trap cultures propagated on *Sorghum vulgare* at LIVE in New Caledonia, Noumea, March 2015, T. Crossay. Pot trap cultures were originally inoculated with rhizospheric soil of *Alphitonia neocaledonica* sampled in an

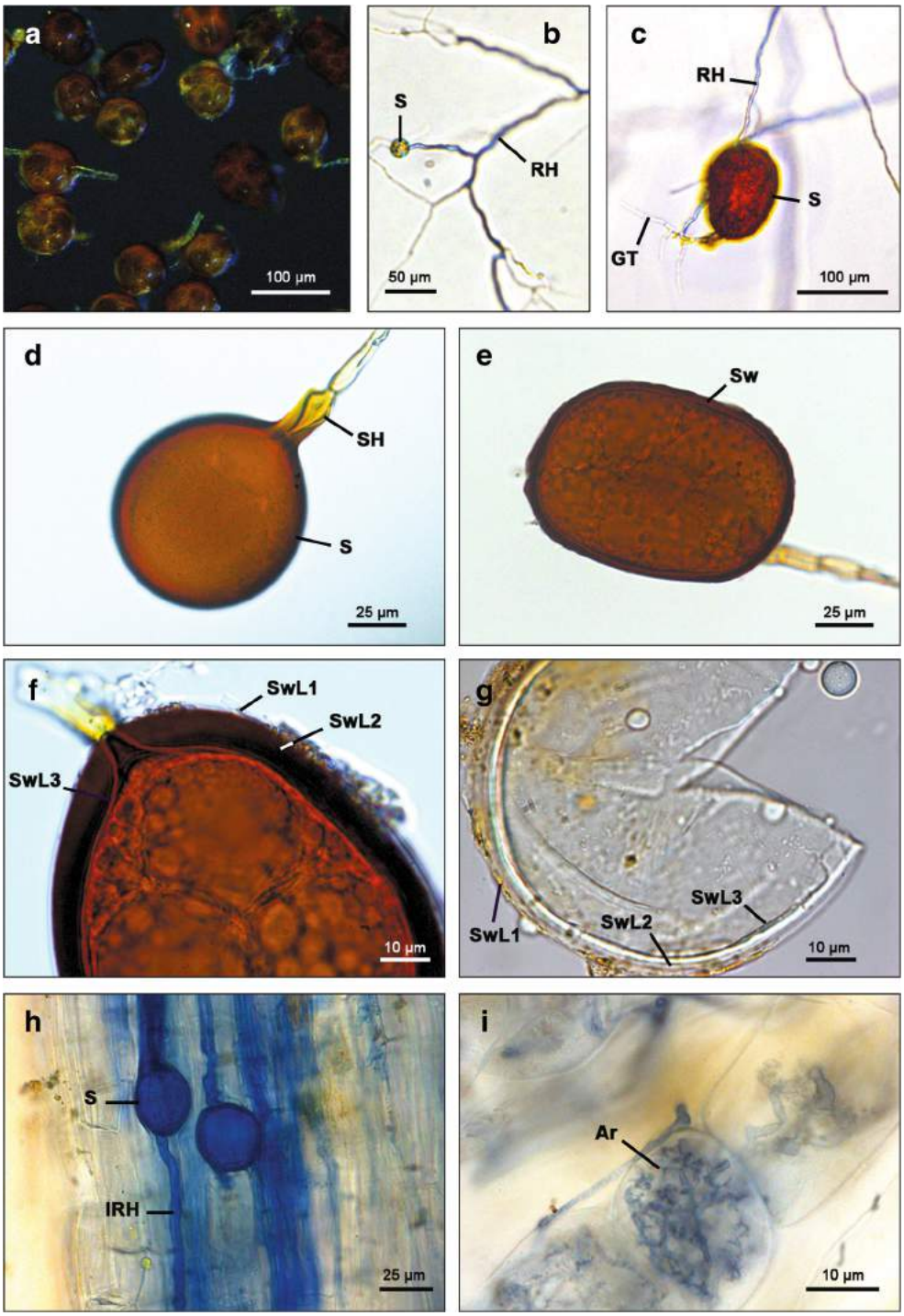

Fig. 6 **a-i** *Rhizophagus neocaledonicus* spores. **a** Intact spores. **b** Intact spore (S) in root organ culture and runner hyphae (RH) on root organ culture. **c** Spore (s), germinal tube (GT), and runner hyphae (RH) on root organ culture. **d** Spore (s) with subtending hyphae (SH). **e** Spore wall (Sw). **f**, **g** Spore wall layers 1, 2, and 3 (SwL1, SwL2, and SwL3). **h**, **i** Mycorrhizal structures of *R. neocaledonicus* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: arbuscules (Ar), spore (S), and intraradical hyphae (IRH). **d**, **f**, **g** In PVLG. **e** In PVLG + Melzer's reagent. G. Spore bleached in a 25% solution of sodium hypochlorite (12° chlorometric)

ultramafic *maquis* in New Caledonia, September 2014, T. Crossay.

Description Sporocarps unknown. Spores mostly globose to subglobose, (61–)75(−83) µm in diameter, sometimes oblong to irregular, 70–82 × 95–113 µm, dark chestnut to coffee brown (40/60/100/0 to 60/80/100/10) (Fig. 6a–e). Spores found singly or in clusters of 2–3. Spore wall with three layers. Layer 1 hyaline, mucilaginous, in mature spores with attached debris, (1.5–)2(−2.5) µm thick, disappearing upon washing the spores during disinfection (Fig. 6f). Layer 2 laminate, dark brown (60/80/100/10), (5.4–)6(−6.5) µm thick (Fig. 6f). Layer 3 fine and tightly attached to layer 2, appearing bright brown (20/60/50/10) inside spores, running down hyphal attachment, (0.8–)1(−1.2) µm thick (Fig. 6f–g). Hyphal attachment cylindrical to constricted (Fig. 6d). Spore occlusion by ingrowth of

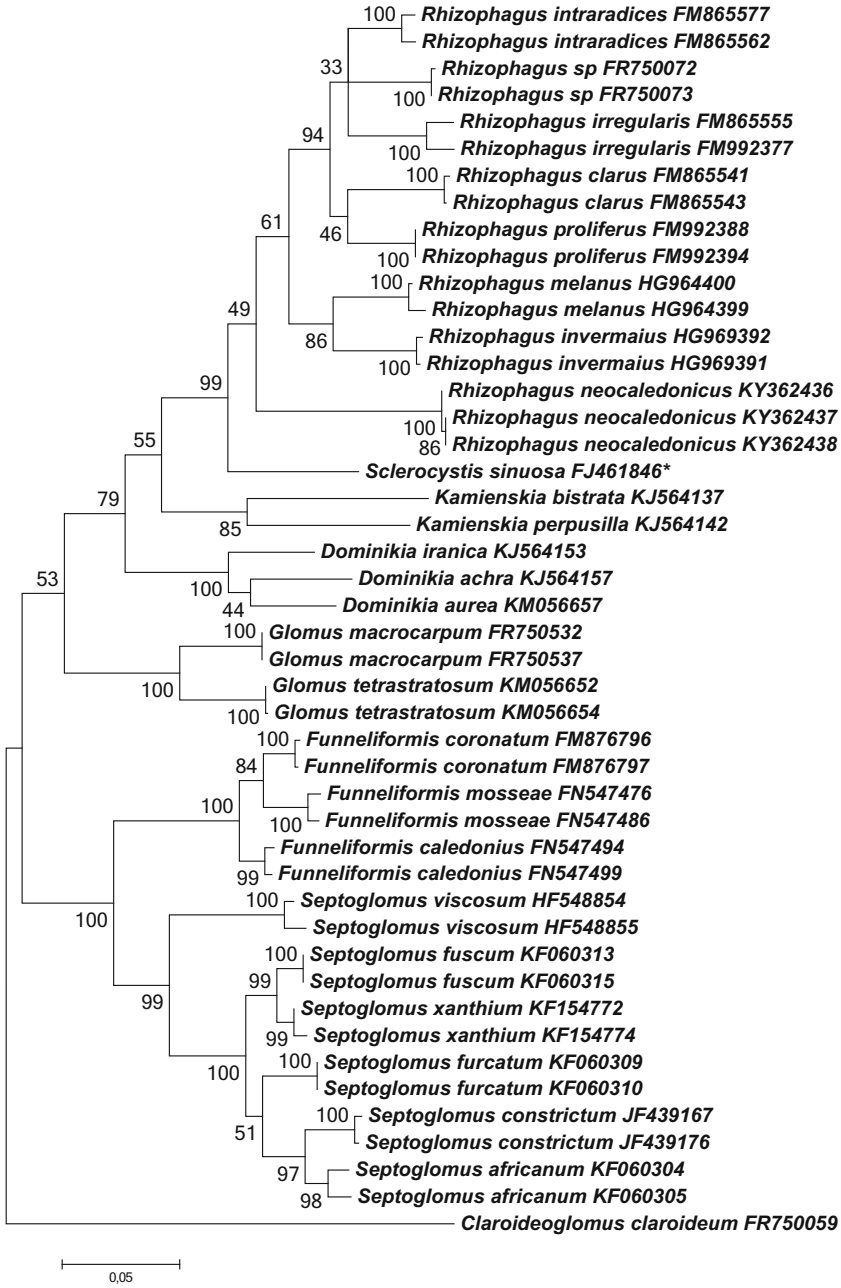

Fig. 7 ML analysis of SSU-ITS-LSU rDNA sequence of *Rhizophagus neocaledonicus* and 24 known species of AMF, including *Claroideoglomus claroideum* as outgroup. Maximum likelihood (ML) phylogenetic tree based on small subunit–internal transcribed spacer–large subunit (SSU-ITS-LSU) rDNA sequence. Sequences labeled with an asterisk (*) represent the LSU gene only. Bootstrap values are given for each branch. The scale bar indicates the number of substitutions per site. Phylogenetic trees were viewed and edited using MEGA 7

spore wall. Spore germination through hyphal attachment (Fig. 6c). Subtending hypha at further distance from spore hyaline, cylindrical (5.5–)6(−6.5) μm diameter (Fig. 6d).

Etymology Indicating the location, New Caledonia, where it was first isolated.

Mycorrhizal structure Forming spores in the roots, vesicles and arbuscules, staining lightly with Trypan Blue (Fig. 4i).

Habitat Rhizospheric soil in tropical ultramafic *maquis*.

Distribution Known only from the type locality.

Material examined Spores isolated from rhizospheric soil of a greenhouse pot of single-species culture propagated on *Sorghum vulgare* at LIVE (Laboratoire Insulaire du Vivant et de l'Environnement) in New Caledonia, Nouméa, April 2016, T. Crossay, MNHN-PC-PC0728840.

In vitro morphology Spores formed intercalarily or terminally (Fig. 6c). In root organ culture, runner hyphae of a diameter of (4–)4.2–(4.5) μm (Fig. 6b, c).

Molecular and phylogenetic analysis Phylogenetic analyses accommodated sequences of *R. neocaledonicus* in a separate clade located basally relative to the clades with sequences of

the other *Rhizophagus* spp. considered (Fig. 7). The closest relatives of the new species were *R. invermaius* and *R. melanus*, which clustered in two sister subclades.

Notes *Rhizophagus neocaledonicus* sp. nov., is clearly distinct by its rDNA sequences. This species is phylogenetically quite distant from well-known *Rhizophagus* species such as *R. irregularis* or *R. clarus*, grouping at the periphery of the genus. Pending additional information on related basal lineages and *Sclerocystis* species (for which few sequences are available) it might later even be incorporated into a new genus. The three closest BLAST hits are environmental sequences from roots of *Panicum virgatum* in a prairie in Kansas (USA, JX276903), *Panicum repens* in a wetland in the Guangdong province of China (KT378084), and a sequence from roots of mycoheterotrophic *Voyria aphylla* from Guadeloupe (HQ857173). None of these sequences is grouping close enough to be considered as conspecific (see Supplementary information Fig. S5).

The only other known species of *Rhizophagus* known to produce dark brown spores are *R. melanus* (Sudová et al. 2015) and *R. invermaius* (Hall 1977). The spore wall of *R. invermaius* is 1.4–1.9-fold thicker and consists of two permanent layers, whereas that of our new species comprises three layers, of which the outermost layer 1 is impermanent. Spores of *R. melanus* are larger and also have a thicker wall. *R. invermaius*, interestingly described in New Zealand (Hall 1977), but also reported from Europe (Oehl et al. 2005) and from South America (Senés-Guerrero & Shulßer 2016).

General discussion

The geographic isolation of the archipelago of New Caledonia and the unusual composition of its soils let it appear possible that AMF show a similarly high degree of endemism as plants. Indeed, several of the AMF we isolated from ultramafic soils were clearly distinct from known species and four of them are described here. Further studies will show whether these fungi are indeed endemic to New Caledonia or whether they are also found in geographically distant locations, as in the case of *Pervetustus simplex*, a fungus we found in the same field site, but which was at the same time found in soils coming from Oman, Greece and Tunisia (Błaszkowski et al. 2017).

In vitro culture of these organisms allowed observations on the pre-symbiotic-stage of the four species described in this article, the formation of spores were also observed for *A. saccata*, *A. fragilissima*, and *R. neocaledonicus*. No sporiferous saccules could be observed in the two *Acaulospora* species, instead spores of *A. fragilissima* were formed terminally on the sporogenous hyphae. Due to the notorious fragility of the sporiferous saccules it is difficult to say whether they were produced in pot culture, in any case they were not observed there. Therefore, this glomoid sporulation may be due to an artifact caused by the in vitro culture conditions or may also mean that spore formation in *Acaulospora* is not necessarily linked to the presence of a sporiferous saccule, thereby putting into question one of the paradigms of AMF taxonomy. *Rhizophagus neocaledonicus*, for instance, forms smaller spores in root organ culture than in pot culture. Spores of these species produced in vitro have not been reintroduced into an in vivo condition on a plant, accordingly the viability of these spores is not yet known and further work is needed to interprete our observations.

These fungi sporulated readily in cultures on *S. vulgare* both in ultramafic soils and in commercial compost, indicating that neither ultramafic soil nor particular metallophyte plants are required for the completion of their life cycle and the production of fungal inoculum.

In summary, our molecular and morphological analyses allow us to conclude that the four AMF species described in this study are new species, which are the first AM fungi newly described from New Caledonia. They have so far been detected only in soils from a single ultramafic area in New Caledonia and further field research is needed to confirm whether they are restricted to this specific environment, and whether the proportion of endemic AM fungi is as high as in plants.

Funding information This work was partly supported by the CNRT "Nickel and its environment," Nouméa, New Caledonia (CSF No. 1PS2013)

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