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Two new species of *Diversispora* (arbuscular mycorrhizal fungi; Glomeromycota) colonizing roots of endemic shrubs on nickel mine tailings in New-Caledonia

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Abstract

Diversispora cerifera and *Diversispora succinacia* are new arbuscular mycorrhizal fungal species, that have been isolated and propagated from spores of rhizospheric soils of native vegetation that had naturally established from seeds on a nickel mine tailings test basin in New-Caledonia. Interestingly, these species were not recorded from ultramafic soils of maquis vegetation endemic to New-Caledonia surrounding the tailings basin. In greenhouse trap and single species cultures, fungi produced numerous spores, which were formed terminally or intercalary on subtending hyphae. Spores of *D. cerifera* are white yellow with a waxy appearance and spores of *D. succinacia* are translucent, amber in color. Spores of the two species are globose to subglobose. Spores of *D. cerifera* 70-100-120 μm in diameter and have three spore wall layers. Spores of *D. succinacia* 60–80 – 110 μm in diameter and have two spore wall layers. *D. cerifera* forms a monophyletic clade next to *D. aurantia* that has a subtending hyphal wall that is continuous with layers 1–3 of the spore wall. Instead, the hyphal wall for *D. cerifera* is continuous only with layers 1 and 2 of the spore wall. Morphologically and phylogenetically, *D. succinacia* is close to *D. sabulosa* and forms a monophyletic clade next to *D. sabulosa* that has smaller spores.

Introduction

With a third of the mainland occupied by ultramafic metalliferous areas, New Caledonia is one of the biggest producers of nickel in the world. The lateritic soils, derived from ultramafic rocks, support a rich and unique sclerophyllous shrubby vegetation: the maquis (Jaffré, 2022). Ultramafic soils harbor a high fraction of the rich endemic flora of this country (Isnard, 2016). Moreover, mycorrhizal studies have shown that many plant groups on ultramafic soils of New-Caledonia are associated with diverse arbuscular mycorrhizal fungi, which facilitate plant growth and adaptation in metal rich substrates (Amir and Ducouso 2010; Lagrange et al. 2011; Crossay et al. 2019; Amir et al. 2022).

Arbuscular mycorrhizal fungi (AMF; Glomeromycota) are microorganisms living in symbiosis with 72–80% of the vascular land plants (Smith and Read 2008; Brundrett 2009; Strullu-Derrien et al. 2016). Despite their ecological and practical importance, the number of described AMF species is still very low compared with other fungal phyla (Ohsowski et al. 2014). The knowledge on AMF diversity has considerably increased during the last two decades, mainly due to the use of DNA-based identification tools, but also to constant progress in morphological identification (da Silva et al. 2006; Krüger et al. 2012; Błaszowski et al. 2015). Many AM fungi are known only from environmental DNA sequences and the number of virtual taxa (i.e., operational taxonomic units or amplicon sequence variants) exceeds the number of AMF species described by traditional morphological analyses of soil-borne spores (Öpik et al. 2013, 2014). It is recognized that targeting unstudied or little-studied environments would result in revealing many novel AMF taxa (Opik et al. 2013; Ohsowski et al. 2014).

Prony Resources hydrometallurgical complex is located on the largest ultramafic mountain range called “large southern massif” in New-Caledonia. Tailings produced by the hydrometallurgical process developed by this company represent a new substrate for ultramafic vegetation establishing naturally

from seeds. This lateritic substrate is a fine material (< 100 microns) of which 60% is composed of ferrous goethite and 10% alunite that naturally occur in laterites which are associated with 15–25% gypsum, and metal hydroxides originating from the neutralization process. Gypsum is rich in calcium and sulfur which are absent from surrounding ultramafic environments and led to the chemical exclusion of many weeds (Jaffré, 2022).

Our morphological and molecular investigation of AMF communities associated with plants growing on these nickel mine tailings revealed the presence of two undescribed AMF species. These species were grown separately in pots after extraction of glomoid spores from the lateritic mine tailings. The aim of the present study was to describe and identify these two new species based on both morphological and molecular analyses.

Material and methods

Study site

The study area is a tailing's storage basin called cell A (166°54'32.96 east, 22°20'28.19 south) established in 2001, in association with a test refinery operation at the Prony Resources New-Caledonia (PRNC) refinery site. Cell A (Fig. 1) is located at an altitude of 130 meter and receives approximately 1895 mm of rain per year. Most of this rain falls between January and March. Rainfall of more than 200 mm in 24 hours is common during this period and is generally associated with cyclonic activity. Temperatures vary from a minimal of 16°C in August to a maximal of 35°C in February.

The test basin was filled with liquid tailings composed of 30% solid material to a depth of 10m in 2002 and progressively dried through natural evaporation and underground drainage piping established to decant water to treatment pond (personal communication with Ph. S. McCoy). By 2007, only a few exotic pioneer grasses, ferns and woody herbs had managed to grow on the tailings: *Imperata cylindrical* (L.) *Raeusch*, *Pennisetum setaceum* (Forssk.) *Chiov*, *Melinis repens* (Willd.) *Zizka*, *Blechnum orientalis* (Thouars) *C. Chr*, *Asplenium* sp. (cf. *australasicum*) (*J. Sm.*) *Hook*, *Blechnum* sp. *petit*, *Lycopodium* sp *L.*, *Pluchea odorata* (L.) *Cass*, *Polygala paniculate* *L.* This vegetation generated an open cover of 10% (Fig. 2. 1). By 2021 exotic vegetation covered 25–50% of the basin (Fig. 2. 2). From 2007 to 2021, native pioneer vegetation progressively colonized the tailing basin, by wind ultramafic maquis dispersed species (*Alphitonia neocaledonica* (Schltr.) *Guillaumin*, *Dodonaea viscosa* *Jacq.*, *Grevillea exul rubiginosa* (*Brongn. & Gris*) *Viro*, *Gymnostoma deplancheanum* (*Miq.*) *L.A.S. Johnson*, *Sannantha leratii* (Schltr.) *Peter G. Wilson*, *Spathoglottis plicata* *Blume*, *Tetraria comosa* (*C.B. Clarke*) *T. Koyama*) which contributed to 10–20% of the abundance of the total vegetation cover that had attained.

Refinery tailing geochemistry

The test tailings is a lateritic substrate remaining after the hydrometallurgical process, added with gypsum and calcareous to neutralize the substrate acidity. It contains metals remaining after the extraction process (iron, magnesium, manganese, chrome, nickel, cobalt), but also calcium oxide and

sulfur, and has a pH of 7.86. The initial mean chemical composition of the tailings in 2001 is : total C, 26.15 g kg⁻¹ ; total N, 1.37 g kg⁻¹ ; total P, 53 mg kg⁻¹ ; total K, 1.81 mg kg⁻¹ ; total S, 5.2g kg⁻¹ ; total Ca, 85 g kg⁻¹ ; total Mg, 7.3 g kg⁻¹ ; Ca/Mg, 11.66; total Ni, 4.12 g kg⁻¹ ; total Fe, 304g kg⁻¹ ; total Cr, 5.86 g kg⁻¹, total Co, 4 g kg⁻¹, total Mn, 8g kg⁻¹, total Al, 17 g kg⁻¹. Chemical analysis of tailings samples taken in 2020 provide another composition: total C, 35.9 g kg⁻¹ ; total N, 1.88 g kg⁻¹ ; total P, 147 mg kg⁻¹ ; total K, 99 mg kg⁻¹ ; total S, 1.9g kg⁻¹ ; total Ca, 13.2 g kg⁻¹ ; total Mg, 11.16 g kg⁻¹ ; Ca/Mg, 1.18; total Ni, 29.5 g kg⁻¹ ; total Fe, 338.4g kg⁻¹ ; total Cr, 8 g kg⁻¹, total Co, 1.9g kg⁻¹, total Mn, 7g kg⁻¹, total Al, 20.3g kg⁻¹.

Sampling, establishment, and growth single-species cultures

Soil samples were collected in tailing's storage basin (cell A) from the rhizosphere of three plants species, two of them are endemic plants of ultramafic maquis of New-Caledonia: *Alphitonia neocaledonica* (Schltr.) Guillaumin and *Sannantha leratii* (Schltr.) Peter G. Wilson, and the last one: *Dodonaea viscosa* Jacq., is native of ultramafic soils of New-Caledonia. These three plants species are all arbuscular mycorrhizal (Amir and Ducouso, 2010, Amir, unpublished) and are used for ecological restoration of New Caledonian degraded mine sites. Five hundred grams of rhizospheric soil and roots of each plant were sampled. The 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. Single-species cultures of the AMF fungus were established directly from spores of soil samples. Spores of soils samples were extracted by wet sieving and sucrose density gradient centrifugation, using a modified method of Daniels and Skipper (1982). For each soil sample, around 10 mL of harvested soil were 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 900g 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 the two morphotypes were surface sterilized using the procedure of Bécard and Fortin (1987). 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 mgL⁻¹ gentamycin and rinsed three times by centrifugations for 30 s in sterile distilled water. One hundred spores of each morphotype were used immediately for establishment of single species cultures as described by Crossay et al. (2018) using *Sorghum vulgare* as the host plant. After six months, spores were extracted and used for morphological and molecular analyses. The presence of mycorrhizae on roots of *S. vulgare* was determined using a clearing and staining protocol for roots (Phillips and Hayman 1970) as modified by Błaszowski (2006):

tissue acidification was performed using 20% hydrochloric acid instead of 1% and trypan blue concentration was 0.1% instead of 0.05%.

Morphological analyses

Spores from single-species cultures 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). The terminology for spore subcellular structures follows Blaszkowski (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-PC0781281 and PC0781282.

All morphological comparisons of *Diversispora* spp. were performed using species descriptions from the INVAM and Blaszkowski. J (<http://www.zor.zut.edu.pl/Glomeromycota/Glomus%20aurantium.html>) websites and with papers of Blaszkowski et al. (2015) and Symanczik et al. (2018).

Molecular analyses

Total genomic DNA of the two new *Diversispora* sp. was extracted from ten single spores. Details of the treatment of the spores prior to polymerase chain reactions (PCRs), the conditions, and primers used in the PCRs to obtain SSU-ITS-LSU sequences were as those described in Crossay et al. (2018).

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 MEGA 7 software with bootstrap support obtained using 1000 replicate. Phylogenetic trees were viewed and edited with MEGA 7 software (Kumar et al. 2016).

Results

Taxonomic analyses

Diversispora cerifera T. Crossay, L. Mai-van'y, H. Amir & S. McCoy, sp. nov.

Holotype Isolated from rhizospheric soil of a greenhouse pot of a single-species culture propagated on *Sorghum vulgare* at laboratory Aura Pacifica in New Caledonia, Noumea, June 2022, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum (France, Paris) in MNHN-PC-PC0781282.

This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of *A. neocaledonica*, *D. viscosa*, *S. leratii*, sampled in a tailing's storage basin naturally vegetated called cell A in New Caledonia, August 2021, T. Crossay.

Description

Sporocarps unknown. Spores globose with one subtending hypha, creamy white to pale ochraceous (0/10/40/0 to 0/10/70/10) with waxy appearance, (70-)100(-120) μm in diameter (Fig. 3. 2). Spores found singly in soil. Spore wall with three layers (Fig. 3. 4). Layer 1, mucilaginous, hyaline, (1.2-)2.5(-3.6) μm thick, usually tightly adherent to the upper surface of layer 2. Layer 2 laminate, permanent, orange-yellow (Figs. 3. 4-5), (3.8-)5.2(-7.5) μm thick. Layer 3, plastic and hyaline, more or less thick with a granular effect (Fig. 3. 4-5). Layer 3 is generally tightly adherent to layer 2. Layers 1, 2 and 3 do not show amyloid or dextrinoid reaction to Melzer's reagent (Fig. 3. 5- 6). Subtending hyphae hyaline, straight, cylindrical, sometimes constricted at spore base; (3.0-)3.5(-4.0) μm wide at spore base (Fig. 3. 6). Wall of subtending hypha hyaline; (1.6-)2.5(-4.3) μm thick at spore base; continuous with layers 1 and 2 of spore wall (Fig. 3. 6). Pore (1.4-)2.4(-3.0) μm in diameter, open or occluded by a straight or curved septum formed by layer 2 of the subtended hyphal wall; septum positioned up to 5.4 μm below the base of the spore. Germination through the spore wall (Fig 3. 3).

Fig. 3: 1-2) Intact *Diversispora cerifera* spores; 3) Germinated spore (S) with runner hyphae (RH); 4-5) Spore wall layers 1, 2, and 3 (SwL1, SwL2, and SwL3); 6) Spore wall layers 1 (SHwL1) and 2 (SHwL2) continuous with wall layers of the subtending hypha at the spore base. 7) Mycorrhizal structures of *D. cerifera* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: intraradical hyphae (IRH). 4) In PVLG. 5-6) In PVLG + Melzer's reagent.

Etymology

From Latin *cerifera* referring to the external color beeswax and waxy spore appearance

Habitat

Iron rich gypsum tailings, generated by hydrometallurgical leaching of ultramafic laterite.

Distribution

Habitat known from only one tailing basin in New Caledonia.

Material examined

Spores isolated from single species cultures in pot with *Sorghum vulgare* in New-Caledonia, Noumea, June 2022.

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 *Diversispora*, containing *D. aurantia* and *D. spurca*, (Fig. 5). Analyses of the whole region of the 18S-ITS1-5.8S-ITS2-28S using maximum likelihood analyses in MEGA clearly separate this species from their closest relatives and from each other. *D. cerifera* sp. nov. is well differentiated by its DNA sequence from other species in the same major clade in the rDNA phylogeny.

Notes Of the *Diversispora* spp. the new species, phylogenetically *D. cerifera* form a monophyletic clade close to *D. aurantia* and *D. spurca* (Fig. 5). Morphologically *D. cerifera* is closer to *D. aurantia*. These two species are more or less similar in spore size and their spore walls have 3 layers. However, one morphological characteristic separates these two species: the hyphae wall is continuous with layers 1-3 of the spore wall for *D. aurantium*, whereas the hyphae wall for *D. cerifera* is continuous with layers 1 and 2 of the spore wall.

Taxonomic analyses

Diversispora succinacia T. Crossay, L. Mai-van'y, H. Amir & S. McCoy, sp. nov.

Holotype Isolated from rhizospheric soil of a greenhouse pot of a single-species culture propagated on *Sorghum vulgare* at laboratory Aura Pacifica in New Caledonia, Noumea, June 2022, T. Crossay. Holotype deposited at Mycological Herbarium of the National Museum (France, Paris) in MNHN-PC-PC0781281. This single-species culture was originally inoculated with 100 spores isolated from rhizospheric soil of *A. neocaledonica*, *D. viscosa* and *S. leratii*, sampled in a tailing's storage basin naturally vegetated called cell A in New Caledonia, August 2021, T. Crossay.

Description

Sporocarps unknown. Spores globose to subglobose with subadjacent hypha, color yellow-orange glossy (0/30/100/10), (60–)80(–110) μm in diameter (Fig. 4. 2). Spores found singly in soil. Spore wall with two layers (Fig. 4. 4-5). Layer 1, permanent, mucilaginous, hyaline, (1–)1.2(–1.4) μm thick, usually floating on the spore wall, sometimes separating from it, especially in vigorously crushed spores (Fig. 4. 5). Layer 2 laminate, permanent, deep yellow to orange yellow, (2.5–)3(3.5) μm (Fig. 4. 5-7). Layers 1 and 2 do not show amyloid or dextrinoid reaction in Melzer's reagent (Fig. 4. 6-7). Subtending hypha hyaline, straight, cylindrical, sometimes constricted at the spore base; (2.7–)3(–3.5) μm wide at the spore base (Fig. 4. 7). Wall of subtending hypha hyaline; (0.8–)0.9(–1) μm thick at the spore base; continuous with spore wall layers 1 and 2 (Fig. 4. 7). Pore (4.0–)5.0(–6.0) μm diam., occluded by domed septum, formed by subtending hyphal wall layer 2; septum positioned up to 3 μm behind the spore base (Fig. 4. 7). Germination through the spore wall (Fig. 4. 3).

Fig. 4: 1-2) Intact *Diversispora succinacia* spores; 3) Germinated spore (S) with runner hyphae (RH); 4-6) Spore wall layers 1 (SwL1) and 2 (SwL2), wall layers of the subtending hypha (SHwL); 7) wall layers 1 of the subtending hypha (SHwL1), wall layers 2 of the subtending hypha (SHwL2), septum of spore (Se); 8-

9) Mycorrhizal structures of *D. succinacia* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: intraradical hyphae (IRH), vesicles (Ve) and spore (S). 4-5) In PVLG. 6-7) In PVLG + Melzer's reagent.

Etymology

From Latin *succinacia* referring to spores external color of amber.

Habitat

Iron rich gypsum tailings, generated by hydrometallurgical leaching of ultramafic laterite.

Distribution

Habitat known from only one tailing basin in New Caledonia.

Material examined

Spores isolated from single species cultures in pot with *Sorghum vulgare* in New-Caledonia, Noumea, June 2022.

Molecular and phylogenetic analysis

Phylogenetic analyses of the 28S rDNA region firmly place this species in a branch of the genus *Diversispora* (Fig. 5) in the Diversisporaceae, and clearly separate this species from their closest relative, *D. sabulosa* and the remaining species of the genus.

Notes Of the *Diversispora* spp. the new species, *D. succinacia* is phylogenetically closely related to *D. sabulosa* but form a monophyletic clade close to *D. sabulosa* (Fig. 5). Morphologically *D. succinacia* is closer to *D. sabulosa*. These two species are not similar in spore size (*D. succinacia* 80 µm diam; *D. sabulosa* 51 µm diam in average), spore walls have 2 layers for each species.

Fig. 5. ML analysis of SSU-ITS-LSU rDNA sequences of *D. succinacia* and *D. cerifera* and 31 known species of AMF, including *Redeckera* (3 species), *Desertispora* (1 species) and *Corymbioglomus* (4 species) as outgroup. Maximum likelihood (ML) phylogenetic tree based on small subunit–internal transcribed spacer–large subunit (SSU-ITS-LSU) rDNA sequences. Sequence labelled with two asterisks (**) represent the partial SSU 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.

Discussion

This study proved that the species *D. succinacia* and *D. cerifera* discovered in the tailing Cell A basin at the Prony Resources refinery in New-Caledonia are new species of the genus *Diversispora*. Phylogenetic analysis clearly identified that *D. succinacia* and *D. cerifera* were not known. Phylogenetic analysis place *D. succinacia* and *D. cerifera* in a distinct clade, nested between a clade with *D. sabulosa*, *D. spurca* and *D.*

aurantium. We have no information on the origin and how these species may have appeared and developed in this basin (Cell A). Nevertheless, some hypotheses are to be explored, which involve an arrival by the winds during cyclonic episodes from surrounding areas. Another possibility is that it arrived at Cell A with anemochore or zoochore seeds from surrounding vegetation composed of endemic species and coastal weeds. It is therefore likely that this new species is also present around the site and more possibly on coastal saline areas where the genetically closest *D. sabulosa* is also found (Symanczik et al. 2018).

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Thomas Crossay, Stephane McCoy, Leslie Maï-van'y and Hamid Amir. The first draft of the manuscript was written by Thomas Crossay, Stephane McCoy, Leslie Maï-van'y and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

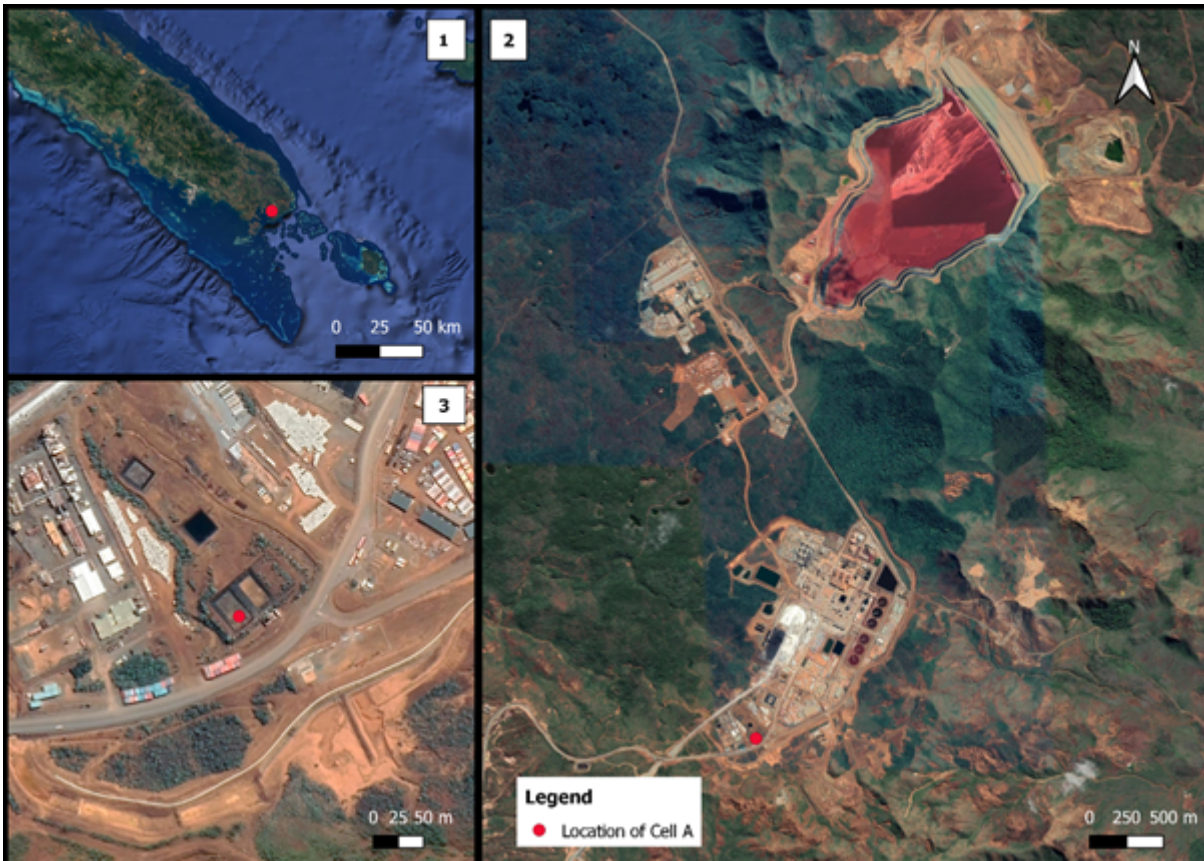


Figure 1

1) Location of Prony Resources refinery in southern New Caledonia, 2) Overview of the southern refinery in Prony, 3) Test basin location (Cell A).



Figure 2

1) Cell basin A in 2007, 2) Cell basin A in 2021 showing natural regeneration.

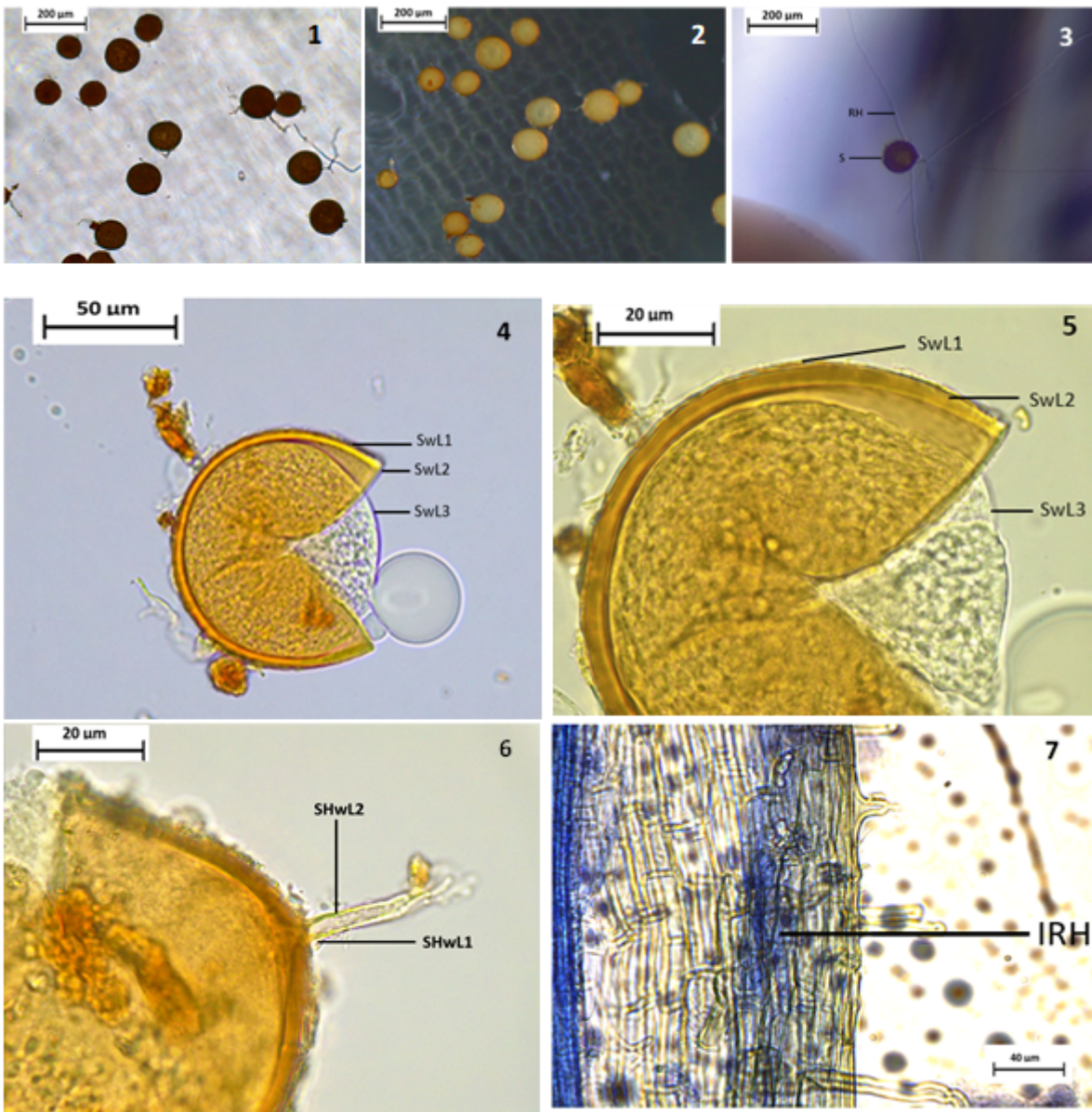


Figure 3

1-2) Intact *Diversispora cerifera* spores; 3) Germinated spore (S) with runner hyphae (RH); 4-5) Spore wall layers 1, 2, and 3 (SwL1, SwL2, and SwL3); 6) Spore wall layers 1 (SHwL1) and 2 (SHwL2) continuous with wall layers of the subtending hypha at the spore base. 7) Mycorrhizal structures of *D. cerifera* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: intraradical hyphae (IRH). 4) In PVLG. 5-6) In PVLG + Melzer's reagent.

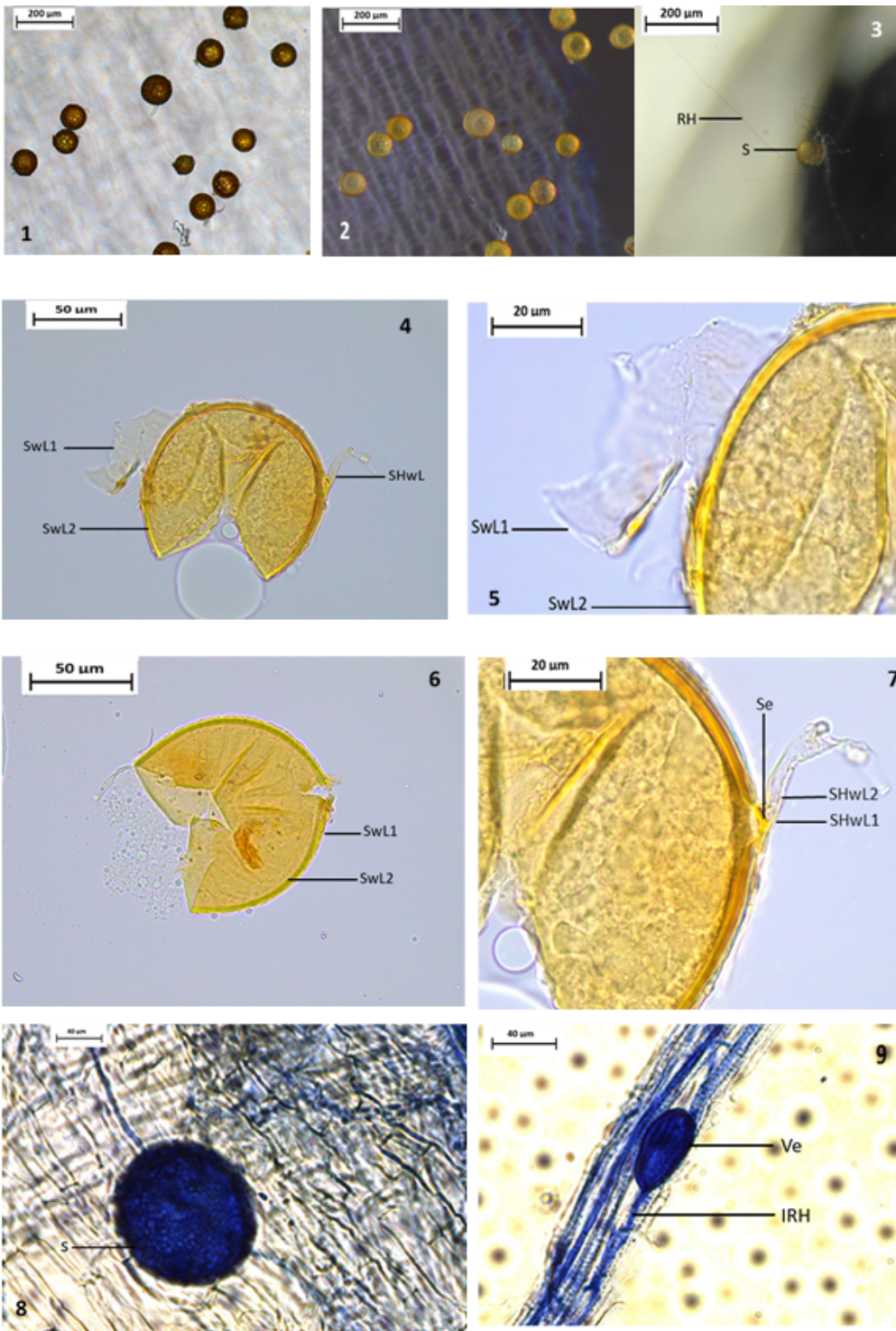


Figure 4

1-2) Intact *Diversispora succinacia* spores; 3) Germinated spore (S) with runner hyphae (RH); 4-6) Spore wall layers 1 (SwL1) and 2 (SwL2), wall layers of the subtending hypha (SHwL); 7) wall layers 1 of the subtending hypha (SHwL1), wall layers 2 of the subtending hypha (SHwL2), septum of spore (Se); 8-9) Mycorrhizal structures of *D. succinacia* in roots of *Sorghum vulgare* stained in 0.1% Trypan blue: intraradical hyphae (IRH), vesicles (Ve) and spore (S). 4-5) In PVLG. 6-7) In PVLG + Melzer's reagent.

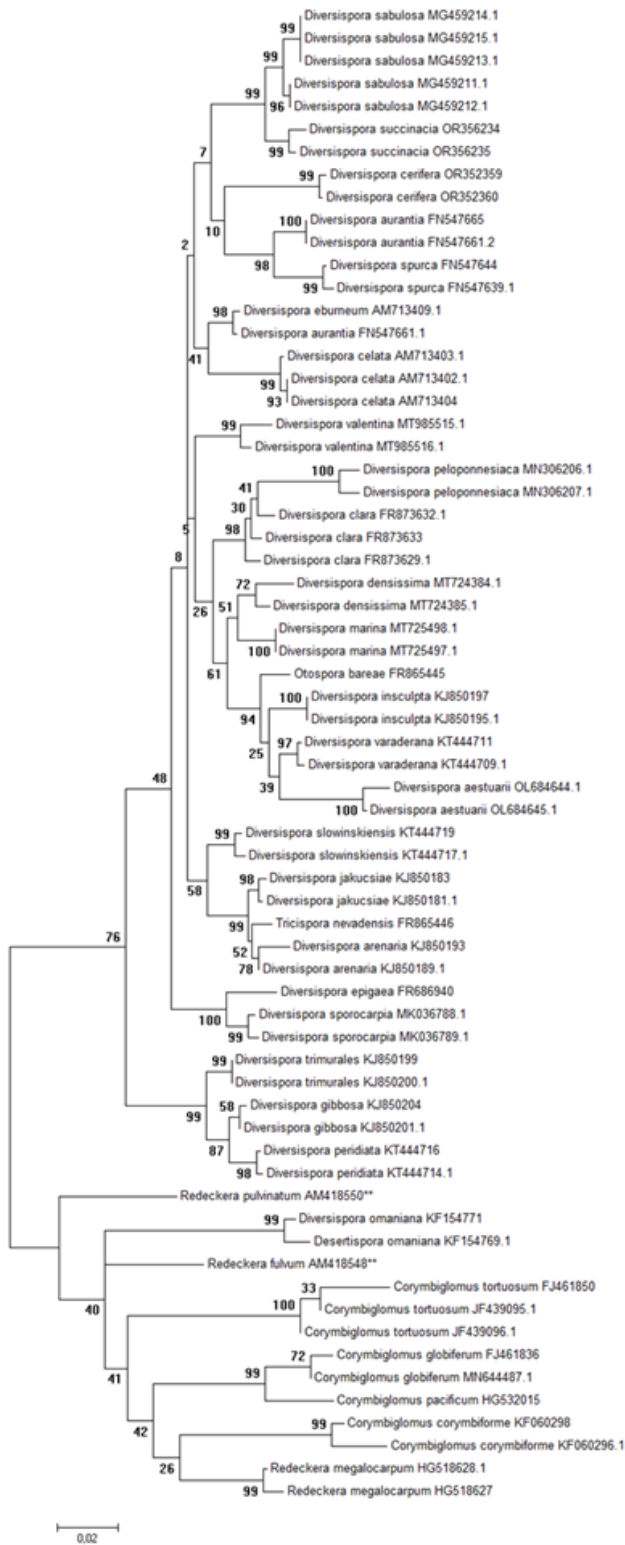


Figure 5

ML analysis of SSU-ITS-LSU rDNA sequences of *D. succinacia* and *D. cerifera* and 31 known species of AMF, including *Redeckera* (3 species), *Desertispora* (1 species) and *Corymbiglomus* (4 species) as outgroup. Maximum likelihood (ML) phylogenetic tree based on small subunit–internal transcribed spacer–large subunit (SSU-ITS-LSU) rDNA sequences. Sequence labelled with two asterisks (**) represent

the partial SSU 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.