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Rhizoglomus cacao, a new species of the Glomeraceae from the rhizosphere of *Theobroma cacao* in Peru, with an updated identification key for all species attributed to *Rhizoglomus*

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With 7 figures

Abstract: A new fungal species was detected in bait cultures of arbuscular mycorrhizal (AM) fungi grown in and on roots of *Zea mais* and *Oryza sativa* as host plants. These plants were initially inoculated with rhizospheric soil substrate derived from a cocoa (*Theobroma cacao*) plantation in the Amazonia lowlands of the province of Lamas, San Martin State, in Peru. The fungus differentiated globose to subglobose spores in the bait culture, singly or in small, relatively loose clusters with up to 30 spores, terminally on pigmented subtending hyphae and have open pores, and thus resemble spores of the genus *Rhizoglomus*. The spores are yellow-white to whitish yellow or creamy yellow, (63–)70–97(–101) × (63–)70–89(–97) in diameter and have three wall layers. In Melzer's reagent, the outer layer stains greyish to pinkish, while the middle and inner layer stain dark purple to almost black. Phylogenetically, the new fungus clusters within *Rhizoglomus* in a separate clade, closest to *R. silesianum*, *R. natalense*, *R. vesiculiferum*, *R. irregulare* and *R. venetianum*. It is here described under the epithet *Rhizoglomus cacao*. An identification key for all species in the genus *Rhizoglomus* is updated in this study.

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Introduction

Cocoa (*Theobroma cacao* L.) is native to tropical rainforests of South America (Motamayor et al. 2002), having its origin in the Western part of the Amazon basin (McElroy et al. 2018). Currently, it is cultivated in multiple tropical countries of Asia, Africa and Central to South America, usually by small farmers holding less than five hectares (DuVal et al. 2017). The main importance of cocoa lays in its grains, which constitutes the raw material for the manufacture of chocolates and other derivates (Mustiga et al. 2019). In Peru, the region with the highest diversity of cocoa is in the Northeast of the country, namely in San Martín State. In this region, cocoa has been cultivated for many years due to its favourable edaphoclimatic conditions, making it a pillar region, that contributes to 38% of the total Peruvian cocoa production (MINAGRI 2019).

Arbuscular mycorrhizal (AM) fungi are obligatory biotrophic and are beneficial symbionts, living in the roots and surrounding soils of approximately 70–80% of all terrestrial plant species worldwide (Brundrett & Tedersoo 2018). Cocoa is not an exception, as it is highly mycothrophic, i.e. highly dependent on AM fungi, which promote cocoa growth in the nurseries and fields (Laycock 1945, Rini et al. 1996, Snoeck et al. 2010).

In the frame of belowground diversity studies in rhizospheric soils of cocoa plantations in Lamas province of San Martín State in Peru, a new species in the Glomeraceae was propagated in AM bait cultures on *Zea mays* and *Oryza sativa* as host plants. By spore morphology, it was attributable to the genus *Rhizoglomus*. The fungus was thoroughly analyzed using concomitant morphological and molecular phylogenetic tools and is hereafter described under the epithet *Rhizoglomus cacao*. Finally, the identification key for the genus *Rhizoglomus*, published by Turrini et al. (2018) was improved and updated in the present paper.

Material and Methods

Study sites, soil sampling: Soil samples (0–30 cm depth) were taken in an agricultural field site planted with cocoa at Pinto Recodo (6°21'0.20" S, 76°36'39.78" W, 688 m a.s.l.) in the Peruvian Amazonia lowlands and adjacent Andean low mountain ranges in the Department San Martín of the province Lamas. The cocoa tree plantation was already six-years old at soil sampling. This area is traditionally used by agroforestry, in which cocoa is grown in mixed cultures with Capirona (*Calycophyllum spruceanum*), Cedro (*Cedrela odorata*) and Paliperro (*Miconia barbeyana Cogniaux*), without any addition of chemical fertilizers and pesticides. Mean annual temperatures are about 25–27 °C, with variation between 17 and 32 °C throughout the year. Mean annual precipitation is approximately 1300 mm.

AM fungal bait cultures: Bait cultures were established in the greenhouse under ambient temperature conditions in cylindrical 5 L pots with 5 kg of substrate. The substrate consisted of a 1:1:1 mixture of coarse river sand, vermiculite and collected rhizosphere soil with root fragments of cocoa. The substrate mixtures were autoclaved at 121 °C for 60 min, 3 weeks before establishment of the bait cultures. At bait culture establishment, the pots were first filled to 75% with the autoclaved substrate. Thereafter 100 g of rhizospheric soils were added to the substrate surface, and seeds of *Zea mays* and *Oriza sativa* were sown together in the pot in order to establish the mycorrhizal association and reproduce spores of the new fungal species together with the native AMF communities. The seeds were surface sterilized before seeding, using sodium hypochlorite (0.5%). Finally, the seeds were covered with the remaining 25% of the autoclaved substrate. The cultures were maintained in the greenhouse of the Universidad Católica Sedes Sapientiae in Nueva Cajamarca (Sector Nuevo Edén, Santa Cruz, cdra. 4 s/n), located in the province of Rioja in San Martín State, for five months, between May and November 2020 with 21, 23 and 25 °C as minimum, mean and maximum air temperatures, respectively. The relative humidity ranged from 55 to 85%. The plants were irrigated every other day, and a Long Anston nutrient solution was added every two weeks, with reduced P contents (60% reduction; Hewitt 1966).

Morphological analyses: The description of the morphological spore characteristics and their subcellular structures are based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske & Tessier 1983), Melzer's reagent, a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994), a mixture of lactic acid to water at 1:1, and water (Spain 1990). Terminology of the spore structure follows Błaszkowski (2012) and Sieverding et al. (2014) for Glomeraceae species. Photographs were taken with a digital camera (Leica DFC 295) on a compound microscope (Leitz Laborlux S), using Leica Application Suite version 4.1 software (Leica Microsystems, GmbH, Bochum, Germany). Specimens mounted in PVLG and a (1:1) mixture of PVLG and Melzer's reagent were deposited at Z+ZT, the joint mycological herbarium of the University of Zurich and the Federal Institute of Technology (ETH) in Zurich, Switzerland).

Molecular analyses: Intact, healthy spores were isolated from the bait culture samples, and cleaned by friction on fine filter paper (Corazon-Guivin et al. 2019a, Corazon-Guivin et al. 2019b, Corazon-Guivin et al. 2019c). Spores were surface-sterilized (Mosse 1962) using a solution of chloramine T (2%), streptomycin (0.02%) and Tween 20 (2–5 drops in 25 mL final volume), for 20 min and rinsed five times in milli-Q water. One independent group of sterile spores, containing 20–30 spores, were selected under a laminar flow hood and individually transferred into Eppendorf PCR tubes. Crude extract was obtained by crushing the spores with a sterile disposable fine-tipped pilon in three µL milli-Q water under the observation at 5x magnification using a Carl Zeiss stereoscope (Corazon-Guivin et al. 2021). Direct PCR of these crude extracts was performed in an automated thermal cycler (Eppendorf Mastercycler nexus, Germany) with a Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions with 0.4 µm concentration of each primer. A two-step PCR was conducted to amplify the ribosomal fragment consisting of partial SSU, ITS1, 5.8S, ITS2 and partial

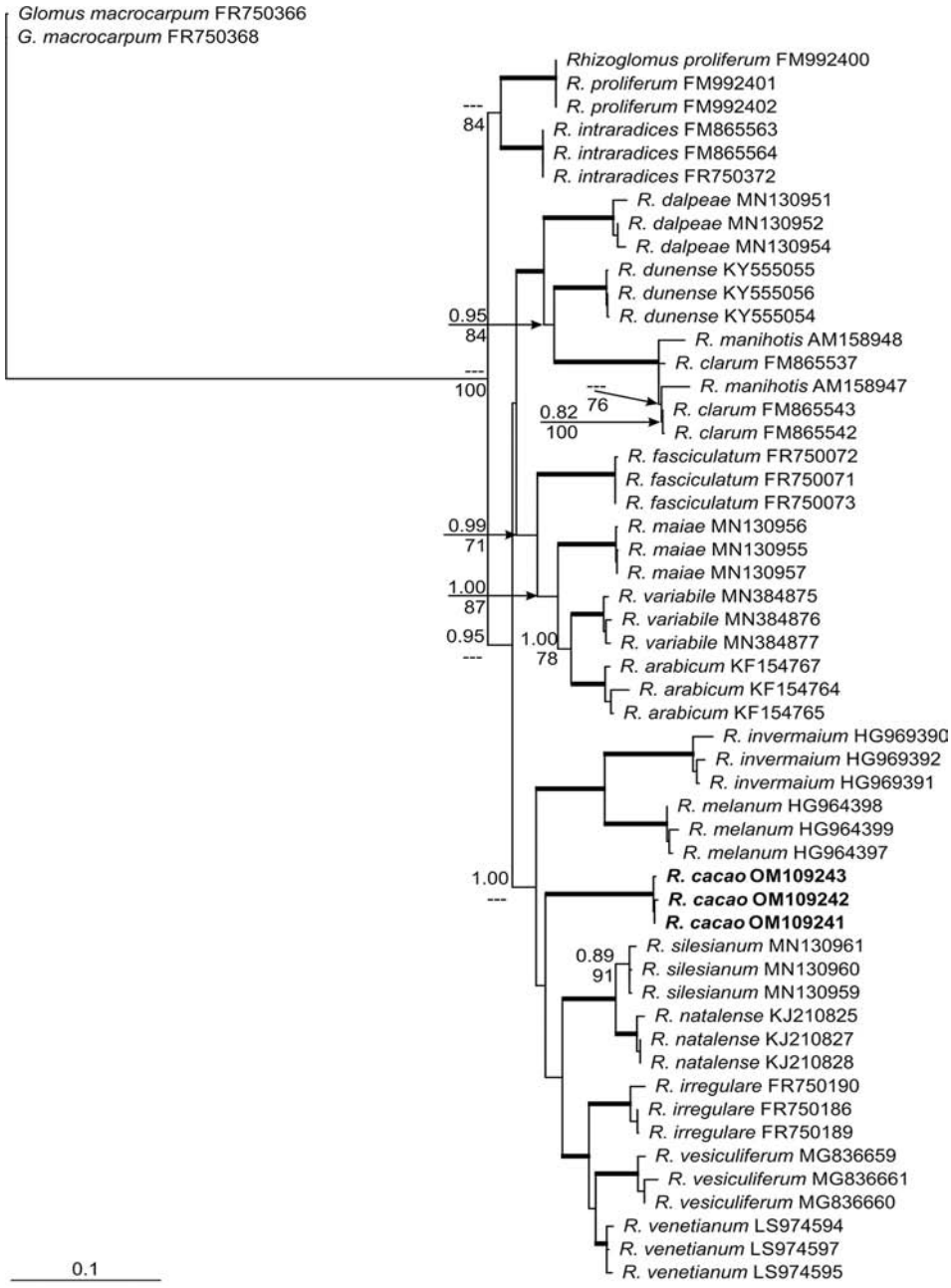
LSU rDNA using the primers SSUmAf/LSUmAr and SSUmCf/LSUmBr, consecutively, according to Krüger et al. (2009). PCR products from the second round of amplifications (~1500 bp) were separated electrophoretically on 1.2% agarose gels, stained with Diamond™ Nucleic Acid Dye (Promega) and viewed by UV illumination. The band of the expected size was excised with a scalpel and isolated from the gel with the GFX™ PCR DNA and Gel Band Purification Kit (Sigma-Aldrich) following the manufacturer's protocol, cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and transformed into One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Recombinant colonies (4) were selected by blue/white screening and the presence of inserts detected by PCR amplification with KOD DNA Polymerase (Sigma-Aldrich) using universal forward and reverse M13 vector primers. After isolation from transformed cells, plasmids were sequenced on both strands with M13F/M13R primers using the BigDye Terminator kit 3.1v (Applied Biosystems). The products were analyzed on an automated DNA sequencer (ABI 3730XL DNA analyzer-Macrogen Inc).

Phylogenetic analyses: The AM fungal sequences (partial SSU, ITS region, and partial LSU rDNA) obtained were aligned with other *Rhizoglomus* sequences from GenBank in ClustalX (Larkin et al. 2007). A fungus called *Glomus macrocarpum* Tul. & C. Tul. by Krüger et al. (2012) was included as outgroup. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian (two runs over 2×10^6 generations, with a sample frequency of 200 and a burnin value of 25 %) and maximum likelihood (1,000 bootstrap) analyses were performed, respectively, in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guidon & Gascuel 2003), launched from Topali 2.5, using the GTR + G model.

Results

Molecular and phylogenetic analyses: The phylogenetic analyses from the partial SSU, ITS region, and partial LSU rDNA sequences placed the new fungus in a separated clade near to *R. silesianum*, *R. natalense*, *R. vesiculiferum*, *R. irregulare*, and *R. venetianum* (Fig. 1). The support values for the clade of the new species were 100% in all analyses. Thus, the new fungal species is described within the genus *Rhizoglomus* under a new epithet. In the BLASTn analysis, the rDNA species sequences with closest match (~ 92%) to the new fungus are from *R. irregulare*. No environmental sequences deposited in the GenBank correspond to the new fungus in the BLASTn analysis.

Fig 1. Phylogenetic tree of the *Rhizoglomus* obtained by analysis from partial SSU, ITS region, and partial LSU rDNA sequences of different *Rhizoglomus* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), respectively. Sequences obtained in this study are in boldface. Only support values of at least 70% are shown. Thick branches represent clades with more than 90 % of support in all analyses. The tree was rooted by *Glomus macrocarpum*.


Glomus macrocarpum FR750366
G. macrocarpum FR750368
Rhizoglomus proliferum FM992400
R. proliferum FM992401
R. proliferum FM992402
R. intraradices FM865563
R. intraradices FM865564
R. intraradices FR750372
R. dalpeae MN130951
R. dalpeae MN130952
R. dalpeae MN130954
R. dunense KY555055
R. dunense KY555056
R. dunense KY555054
R. manihotis AM158948
R. clarum FM865537
R. manihotis AM158947
R. clarum FM865543
R. clarum FM865542
R. fasciculatum FR750072
R. fasciculatum FR750071
R. fasciculatum FR750073
R. maiae MN130956
R. maiae MN130955
R. maiae MN130957
R. variabile MN384875
R. variabile MN384876
R. variabile MN384877
R. arabicum KF154767
R. arabicum KF154764
R. arabicum KF154765
R. invermaium HG969390
R. invermaium HG969392
R. invermaium HG969391
R. melanum HG964398
R. melanum HG964399
R. melanum HG964397
R. cacao OM109243
R. cacao OM109242
R. cacao OM109241
R. silesianum MN130961
R. silesianum MN130960
R. silesianum MN130959
R. natalense KJ210825
R. natalense KJ210827
R. natalense KJ210828
R. irregulare FR750190
R. irregulare FR750186
R. irregulare FR750189
R. vesiculiferum MG836659
R. vesiculiferum MG836661
R. vesiculiferum MG836660
R. venetianum LS974594
R. venetianum LS974597
R. venetianum LS974595
0.1

Morphological analyses and taxonomy

Rhizoglomus cacao Corazon-Guivin, G.A. Silva & Oehl, **sp. nov.**, Figs 2–7

Mycobank MB 843912

Diagnosis: Differs from *Rhizoglomus vesiculiferum* and *R. irregulare* by having a strong, dark purple staining in Melzer's reagent on the two innermost wall layers, and a different spore wall structure, i.e. the second wall layer (SWL2) is structural and laminate, and the adherent innermost layer SWL3 is flexible.

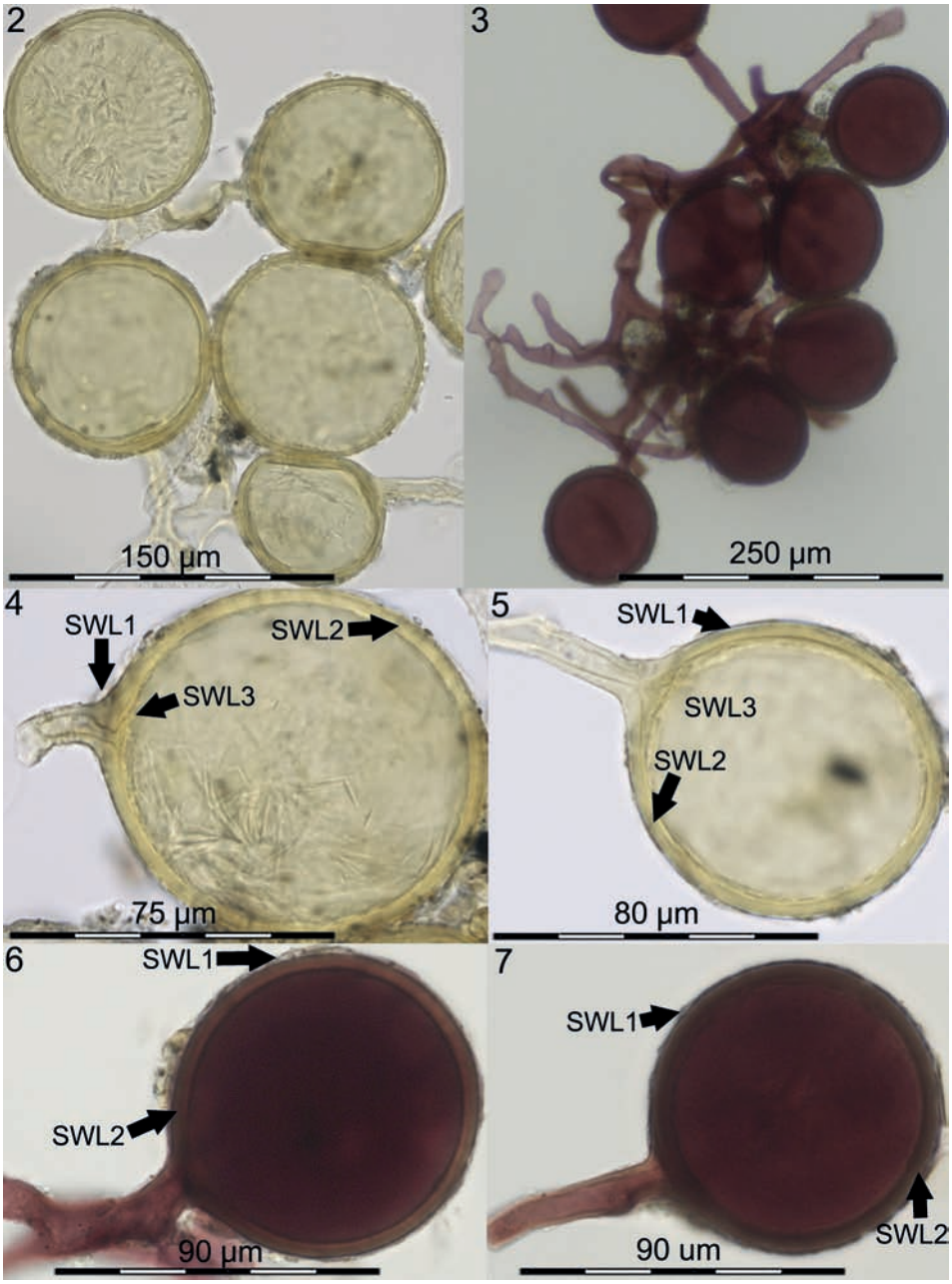
Etymology: *cacao* referring to the tropical cocoa tree, *Theobroma cacao*, from which the fungus was firstly collected.

Holotype: deposited at Z+ZT (accession ZT Myc 66313), derived from a culture established on the host plants of *Zea mays* and *Oryza sativa* in the greenhouse of the Universidad Católica Sedes Sapientiae, Los Olivos, Lima, Peru. Fungal inoculum for the culture derived from a bait culture, inoculated with field soils originating from a cocoa plantation in Pinto Recodo (6°21'0.20" S, 76°36'39.78" W, 688 m a.s.l.), where cocoa is cultured in agroforestry systems together with Capirona (*Calycophyllum spruceanum*), Cedro (*Cedrela odorata*) and Paliperro (*Miconia barbeyana Cogniaux*). Collector was Geomar Vallejos Torres and collection date was 01.05.2019.

Description: Spores formed terminally on subtending hyphae (SH) either singly, in small spore clusters, or, preferably, in loose to compact non-organized spore clusters with 5–30 spores per cluster (Figs. 2–3). Spores are yellow-white to whitish yellow or creamy yellow, globose to subglobose to rarely oblong or rarely irregular, (63–)70–97(–101) × (63–)70–89(–97) µm (Figs. 4–5).

Spore wall has three layers (Figs. 4–7). Outer layer (SWL1) is hyaline to subhyaline, evanescent, 0.7–1.2 µm thick. This layer is often partly degraded and sloughing off that gives a roughening appearance of the spore surface. Second layer (SWL2) is structural, persistent, laminate, yellow-white to whitish yellow or creamy yellow, 2.5–3.7(–4.9) µm thick, expanding up to 4.1–5.5 µm under pressure in lactic acid based mountants. Innermost layer (SWL3) flexible, yellow-white to light yellow, 0.6–1.1 µm thick, usually tightly adherent to SWL2, sometimes separating or showing several folds in crushed spores. In Melzer's reagent, SWL1 stains greyish to pinkish, and SWL2 and SWL3 stain dark purple to almost black (Figs. 6–7).

Subtending hyphae (SH) of spores cylindrical to slightly funnel-shaped, sometimes recurved, 8.0–14.2 µm broad and 15–120 µm long, and without introverted wall thickening toward the spore base (Figs. 2–3). The base generally is open, rarely closed by a septum formed by SWL2 or SWL3 (Figs. 4–7). Such septa can more often be found in some distance from the spore bases within the subtending hyphae (SH), which are 8–15 µm thick, golden yellow to bright yellow brown. SWL1-3 continue in the SH (SHL1-3), which are together 2.0–3.5 µm thick at the spore base. In Melzer's, the SH layers stain as described above for the SW layers, while the mycelial hyphae stain pinkish to purple in Melzer's reagent (Figs. 6–7).



Figs. 2–7. *Rhizoglomus cacao*. **2.** Spore cluster mounted in PVLG. **3.** Spore cluster mounted in PVLG+Melzer's. **4–5.** Spores in PVLG. They are triple-layered (SWL1-3), with hyaline to subhyaline SWL1, yellow-white to whitish yellow or creamy yellow SWL2 and yellow-white to light yellow SWL3. Spore pore at the spore base regularly is open. **6–7.** Spores in PVLG+Melzer's. SWL1 stains greyish to pinkish, and SWL2 and SWL3 stain dark purple to almost black.

Other specimen examined: Isotype also deposited at Z+ZT (ZT Myc 66314).

Distribution: *Rhizoglomus cacao* has so far been found only in trap cultures inoculated with rhizosphere soils and root fragments of cocoa, and in pot cultures with *Zea mays* and *Oriza sativa*. The trap pot culture soil inocula derived from the agroforestry field site in Pinto Recodo (Province of Lamas, Department of San Martín), belonging to the Peruvian Amazonian lowlands and the adjacent low mountain areas that range up to 668 m a.s.l. Soil pH at the site was 5.4 and available P was rather low (10.4 mg P kg^{-1}).

Updated key to the species of the genus *Rhizoglomus*

A key is here updated for the morphological identification of all 22 species belonging to the genus *Rhizoglomus* according to Sieverding et al. (2014), Turrini et al. (2018) and Wijayawardene et al. (2020), Wijayawardene et al. (2022), including the recently described species *R. dalpeae*, *R. maiae*, *R. silesianum* (Błaszkowski et al. 2019), *R. variabile* (Corazon Guivin et al. 2019c), and the new species *R. cacao*. *Rhizoglomus neocaledonicum* was removed from the key, since this species had been transferred in the meantime to the genus *Silvaspora* and does not form spores with open pores at the spore base as typical for *Rhizoglomus* species (Błaszkowski et al. 2021).

1 Species with two spore wall layers: 2

1’ Species with > two spore wall layers:. 4

2 Spores whitish yellow to yellow; species with 1–2 laminae on structural wall layer: 3

2’ Spores light brown to red brown; 50–90 µm, globose to subglobose, formed singly, in small clusters or large, dense sporocarps (up to 15 × 10 × 10 mm); SWL1 hyaline, evanescent, 1–1.5 µm; SWL2 red to dark brown, 3–6 µm, with several laminae . . . . . *R. invermaium* (I.R. Hall) Sieverd., G.A. Silva & Oehl

3 Spores generally < 50 µm; 15–50 µm, whitish yellow to yellow, globose to subglobose, formed singly or in clusters; SWL1 hyaline to light yellow, evanescent, 0.5–1.2 µm; SWL2 whitish yellow to yellow, 0.5–2.0 µm . . . . . *R. microaggregatum* (Koske, Gemma & P.D. Olexia) Sieverd., G.A. Silva & Oehl

3’ Spores generally > 50 µm; 60–110 µm, hyaline to yellow, globose to subglobose, formed in small clusters to dense sporocarps, up to 1.8 × 1.4 × 1.4 mm; SWL1 hyaline, evanescent, 0.5–1.2 µm.; SWL2 yellow to yellow brown, 1.2–2.4 µm, consisting of two laminae that might separate under pressure applied . . . . . *R. aggregatum* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl

4 Species with three spore wall layers:. 5

4’ Species with > three spore wall layers:. 16

5 SWL3 is structural, laminate layer: 6

5’ SWL3 is flexible layer, adherent to structural, laminate layer SWL2:. 9

6 Spores yellowish white to yellow brown: 7

6’ Spores chestnut brown to dark brown; 137–285 µm, globose to subglobose, formed singly or in small clusters; SWL1 is evanescent, hyaline, 0.8–1.2 µm thick; SWL2 is semi-persistent to evanescent, 2.8–5.1 µm thick and shows many fissures in degraded stages. Under pressure on the cover slide single, irregular pieces of SWL2 (about 5–15 × 5–15 µm) may split from the spore surface. SWL3 is dark chestnut brown to black brown, laminate, 7.2–12 µm. . . . . *R. melanum* Sudová, Sýkorová & Oehl

7 Spores ovoid, oblong to often irregular; hyaline to pale yellow, 60–130 × 80–240 µm; they may have deep wall depressions and apical cap-like swellings; SWL1 (0.5–1.5 µm) and SWL2 (0.6–5.0 µm) hyaline and semipermanent; SWL3 hyaline to pale yellow, with inseparable laminae, 1.5–4.4 µm, staining pale orange to deep red in Melzer’s reagent . . . . . *R. irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl

7’ Spores usually globose to subglobose, rarely irregular, without deep wall depressions and apical cap-like swellings 8

8 Spores pastel yellow to light yellow, globose, (69–)90(–108) µm, rarely egg-shaped, 75–83 × 80–91 µm, formed in loose clusters of 4–18 spores (Błaszkowski et al. 2018) or in sporocarps up to 13 × 10 mm (Thaxter 1922, Gerdemann & Trappe 1974), rarely singly in soil; SWL1 finely-laminate, semi-permanent, hyaline, (1.8–)3.1(–4.5) µm thick, usually slightly swelling in PVLG. SWL2 uniform (not divided into visible sublayers), permanent, hyaline, (1.0–)1.3(–2.3) µm, tightly adherent to layer 3, usually difficult to see; SWL3 laminate, permanent, pastel yellow to light yellow, (10.5–)12.0(–13.0) µm; the laminae usually easily separate from each other even in spores slightly crushed in PVLG; SWL1 staining yellowish white to pale yellow, SWL2 yellowish red to reddish white and SWL3 staining dark ruby in Melzer’s reagent . . . . . *R. vesiculiferum* (Thaxt.) Błaszk., Kozłowska, Niezgoda, B.T.Goto & Dalpé

8’ Spores yellow brown to grey brown, often with a greenish tint, 90–135 µm, globose to subglobose, formed singly, in clusters or sporocarps. SWL1 (0.5–1.3 µm thick) and SWL2 (0.8–2.0 µm thick) hyaline and sometimes evanescent (but not regularly, except with in-vitro-grown cultures); SWL3 yellow brown to grey brown, laminate, 3.2–12 µm comprises frequently separating sublayers, which are each 0.5–1 µm thick. SWL1 staining purple in Melzer’s reagent . . . . . *R. intraradices* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl

9 Spores with highly variable spore size. 10

9’ Spores generally either < 90 µm or > 90 µm 11

10 Spores (30)70–185 × (30)65–160 µm, globose to subglobose to rarely oblong or irregular, golden yellow-brown to yellow brown; singly or, preferably, in small spore clusters, usually with 2–50, or more spores per cluster. SWL1 hyaline, evanescent, 0.8–1.5 µm; SWL2, persistent, laminate, golden-yellow to bright yellow brown, 1.6–2.6(3.2) µm; SWL3 flexible, light-yellow to bright yellow, 1.1–2.0 µm, usually tightly adherent to SWL3, sometimes separating or showing a few folds in crushed spores. In Melzer’s reagent, SWL2 staining pinkish purple to purple. SH cylindrical to slightly funnel-shaped, sometimes recurved, (6)9.0–15.5(18) µm broad and 12–200 µm long. Base generally not closed by a septum, but open. Such septa observable in 8–25 µm distance from the spore bases, SH golden yellow to bright yellow brown. SH layers in total 2.5–3.6 µm thick usually tapering to 0.5–1.5 µm towards the mycelia hyphae . . . . . *R. variabile* Corazon-Guivin, G.A. Silva & Oehl

10’ Spores colorless, hyaline, 39–125 µm, globose to subglobose, formed singly in soils; SWL1 semi-permanent, smooth or slightly roughened, 1.0–5.0 µm; SWL2 finely laminate, 4.0–8.8 µm; SWL3 uniform, to laminate, when up to 2.0 µm, (semi-)flexible; SWL1 stains pinkish white to dark red in Melzer’s, while SWL3 turns pale yellow . . . . . *R. dunense* Błaszk. & Kozłowska

11 Spores generally < 100 µm 12

11’ Spores generally > 100 µm 14

12 Spores hyaline, yellow-white to whitish yellow or creamy yellow to creamy 13

12’ Spores 50–70 µm, yellow to yellow brown, globose to subglobose, formed singly, in clusters or sporocarps, 1.0 × 0.6 × 0.5 mm; three layered wall up to 12 µm; SWL1 hyaline, 0.8–2.0 µm, evanescent; SWL2 light brown, laminate, 4.0–8.0 µm; SWL3 hyaline, 1.5–2.0 µm. . . . . *R. antarcticum* (Cabello) Sieverd., G.A. Silva & Oehl

13 All layers of the spores and subtending hyphae stain purple black to black in Melzer’s; spores hyaline to creamy, 70–90 µm, globose to subglobose, formed singly, in clusters or large sporocarps, SWL1 hyaline, 0.5–1.2 µm, evanescent; SWL2 laminate, 2.0–7.5 µm, persistent; SWL3 (semi-) flexible, 0.5–1.4 µm . . . . . *R. fasciculatum* (Thaxt.) Sieverd., G.A. Silva & Oehl

13’ In Melzer’s, SWL1 stains greyish to pinkish, and SWL2 and SWL3 stain dark purple to almost black. Spores yellow-white to whitish yellow or creamy yellow, globose to subglobose to rarely oblong or rarely irregular, (63–)70–97(–101) × (63–)70–89(–97) µm. SWL1 hyaline, evanescent, 0.7–1.2 µm; SWL2 persistent, laminate, yellow-white to whitish yellow or creamy yellow, 2.5–3.7(–4.9) µm, expanding up to 4.1–5.5 µm under pressure in lactic acid based mountants; SWL3 flexible, yellow-white to light yellow, 0.6–1.1 µm thick, usually tightly adherent to SWL2, sometimes separating or showing several folds in crushed spores . . . . . *R. cacao* Corazon-Guivin, G.A. Silva & Oehl

14 Spores globose to subglobose; (86–)100(–121) µm; rarely ovoid; 78–84 × 98–120 µm, orange to light brown; formed in soil in clusters with 3 to > 50 spores, rarely singly, and frequently inside roots; SWL1 unit, permanent, semi-flexible, hyaline to orange, (1.0–)1.4(–2.5) µm, tightly adherent to SWL2; SWL2 laminate, permanent, semi-flexible, orange to light brown, (4.8–)6.4(–10.0) µm, consisting of very thin, <0.5 µm, laminae, tightly adherent to each other, not separating even in vigorously crushed spores; SWL3 unit, permanent, flexible to semi-flexible, hyaline, (0.8–)1.0 (–1.8) µm, usually easily separating from SWL 2 in crushed spores, tightly adherent to layer 2 even in vigorously crushed spores. Only SWL2 stains brownish red to brownish violet in Melzer’s. SH orange to light brown; straight or recurved, cylindrical, rarely slightly constricted at the spore base, (14.3–)17.2(–23.0) µm wide at the spore base . . . . . *R. dalpeae* Błaszk., Piątek, Yorou, Zubek, Jobim, Niezgoda & B.T. Goto

14’. Spore generally > 120 µm 15

15 Spores hyaline (to white), becoming, whitish yellow with age, 120–290 µm, globose to subglobose, formed singly or in loose clusters; SWL1 1.2–2.3 µm, evanescent to semipersistent, staining purple in Melzer’s, SWL2 5–20 µm laminate, persistent; SWL3 2.0–9.0 µm, often becoming yellow with age . . . . . *R. clarum* (T.H. Nicolson & N.C. Schenck) Sieverd., G.A. Silva & Oehl

15’ Spores light yellow to brownish yellow, 145–450 µm, globose to subglobose, formed singly or in loose clusters; SWL1 hyaline, evanescent, 2.0–5.0 µm, staining light purple in Melzer’s; SWL2 hyaline to light yellow, persistent, 10–16 µm; SWL3 yellow to yellow brown, flexible, consisting of 1–2 laminae, 0.5–2.0 µm . . . . . *R. manihotis* (R.H. Howeler, Sieverd. & N.C. Schenck) Sieverd., G.A. Silva & Oehl

16 SWL4 is structural, laminate layer. 17

16’ SWL4 is flexible layer, adherent to structural, laminate layer SWL3 19

17 Spores generally < 75 µm; 40–75 µm, hyaline to yellowish white or subhyaline, globose to subglobose, formed in clusters; spore wall with four layers, 3.3–5.8 µm in total, SWL1 and SWL2 2.2–3.8 µm in total; SWL3 of irregular thickness, 0.7–2.0 µm, finely laminate, and SWL4 is 0.5–1.0 µm, also finely laminate; SH hyaline to very pale yellow, 5–12 µm wide, Pore 2.0–3.7 µm wide at spore base, straight for 8–10 µm. . . . . *R. proliferum* (Dalpé & Declerck) Sieverd., G.A. Silva & Oehl

17’ Spores generally > 75 µm 18

18 Hyaline outer spore wall layers difficult to differentiate; spores pale yellow to brownish yellow, 110–172 µm, globose to subglobose, formed singly or in small clusters (2–5 spores). SWL1 hyaline, mucilaginous, 0.8–2.5 µm, reddish in Melzer’s, SWL2 hyaline, rigid, 1.6–2.8 µm, SWL3 hyaline to pale yellow, semi-flexible, easily separated from SWL2, 1.5–2.0 µm, non-reactive to Melzer’s reagent; SWL4 yellow to brownish yellow, laminate, 2.6–3.8 µm, staining dark red in Melzer’s . . . . . *R. custos* (C. Cano & Dalpé) Sieverd., G.A. Silva & Oehl

18’ Spores pale yellow to grayish yellow, globose to subglobose, (65–)111(–138) µm, rarely ovoid; 77–80 × 83–96 µm; formed in soil in loose to compact clusters with a few to hundreds of spores, rarely singly, and frequently inside roots. SWL1 unit, semi-permanent, flexible, hyaline, (1.0–)1.2(–1.8) µm; always quickly swelling and forming a halo, when mounted in PVLG, separating from SWL2 by 6.5–15.8 µm; deteriorating slowly with age, rarely completely sloughed off even in older spores, frequently incorporating soil debris: SWL2 unit, permanent, semi-flexible, hyaline, (1.5–)1.9(–2.5) µm. SWL3 unit, permanent, semi-flexible, hyaline, (1.3–)1.6(–2.0) µm, sometimes slightly separating from SWL2. SWL 4 laminate, permanent, semi-flexible, pale yellow to grayish yellow, (2.5–)3.1(–4.3) µm; sublayers easily separating from each other in crushed spores; SWL4 usually strongly separated from SWL3 in crushed spores. In Melzer’s, SWL1-4 stain pale yellow, pale red to pastel red, grayish red to brownish red, and light orange to reddish brown, respectively. SH pale yellow to grayish yellow; straight or recurved, cylindrical, rarely slightly funnel-shaped or slightly constricted at the spore base; (10.6–)11.8(–13.4) µm. SH wall pale yellow to grayish yellow, (4.6–)5.0(–5.5) µm thick; consisting of four layers continuous with SWL1-4. Pore (2.1–)2.5 (–2.7) µm wide at the spore base, open . . . . . *R. silesianum* Magurno, Niezgoda, Malicka, Jobim, B.T. Goto & Błaszk.

19 Spores generally < 90 µm 20

19’ Spores in average > 90 µm 21

20 Spores pastel yellow to light brown; globose to subglobose; (63–)72(–82) µm; rarely ovoid; 57–68 × 68–89 µm, formed in an epigeous, compact cluster without a peridium. Cluster light yellow to grayish brown, irregular, 12.4 × 6.5 mm, containing hundreds of randomly distributed glo-

moid spores. SWL1 evanescent, semi-permanent, hyaline, (0.8–)1.3(–2.0) µm, usually highly deteriorated, rarely completely sloughed off. SWL2 unit, semi-flexible, permanent, hyaline to yellowish white, (1.0–)1.4(–2.0) µm, tightly adherent to SWL3; SWL3 laminate, semi-flexible, permanent, smooth, pastel yellow to light brown, (4.8–)13.7(–22.3) µm, consisting of very thin, <0.5 µm, laminae, usually tightly adherent to each other; sometimes SWL3 splits into two to three groups of laminae in vigorously crushed spores. SWL4 unit, flexible to semi-flexible, permanent, hyaline, 1.0–1.3 µm, usually tightly adherent to SWL3, sometimes separating. In Melzer's, SWL1 & 2 non-reactive and SWL3 stain light brown to violet brown and SWL4 pastel pink to grayish rose SH pale yellow to grayish yellow; straight or recurved, cylindrical, rarely slightly funnel-shaped or slightly constricted at the spore base; (6.8–)9.6(–11.8) µm wide at the spore base. SH wall pale yellow to grayish yellow; (2.3–)3.7(–4.8) µm thick at the spore base; consisting of three layers continuous with SWL1-3. Pore (1.5–)2.9(–4.3) µm wide and open at the spore base; the channel connecting the lumen of the subtending hypha with the interior of spores frequently closed by a septum continuous with SWL4. Septum usually positioned at half the thickness of SWL3 . . . . . *R. maiae* Jobim, Błaszk., Niezgoda & B.T.Goto

20' Pigmented inner wall layer separating into several layers/laminae under pressure; spores, pale yellow to grayish yellow, globose to subglobose, (33–)65(–105) mm; sometimes ovoid to irregular, 30–85 × 50–125 µm, formed in clusters up to 0.8 × 1.0 mm; SW difficult to interpret; SWL1 hyaline, evanescent, 0.6–2.5 µm, SWL2 readily separating in 2–4 laminae, which might be counted as SWL2–5, in total up to 5.5 µm thick . . . . . *R. arabicum* (Błaszk., Symanczik & Al-Yahya'ei) Sieverd., G.A. Silva & Oehl

21 Spores pastel yellow to light yellow, 75–133 µm, globose to subglobose, formed single or in loose clusters up to 1.2 mm in diam; SWL1 hyaline, semi-permanent, 1.0–5.3 µm; SWL2 hyaline, permanent and unit, 0.8–1.5 µm; SWL3 laminate, pastel yellow to light yellow, 6.3–14.0 µm, consisting of laminae up to 0.8–1.0 µm, frequently easily separating from each other in crushed spores; SWL4 hyaline and flexible, 0.8–2.0 µm. SWL1 and SWL3 stain reddish white to grayish red and brownish violet to violet brown in Melzer's reagent, respectively. . . . . *R. natalense* (Błaszk., Chwat & B.T. Goto) Sieverd., G.A. Silva & Oehl

21' Spores golden-yellow to bright yellow brown, globose to subglobose, 75–145 × 72–140 µm, formed singly, in small spore clusters, or, preferably, in loose to compact sporocarps up to 2.5 × 2.0 × 2.0 mm. SWL1, hyaline, evanescent, 0.6–1.3 µm; SWL2, hyaline, evanescent, 0.8–1.4 µm; SWL3 structural, persistent, laminate, golden-yellow to bright yellow brown, 2.0–3.5 µm, expanding up to 7.5 µm under pressure in lactic acid based mountants; SWL4 flexible, light-yellow to bright yellow, 0.6–1.4 µm, usually tightly adherent to SWL3, sometimes separating or showing several folds in crushed spores. In Melzer's, SWL1 and SWL2 stain pinkish, while SWL3 stain purple . . . . . *R. venetianum* Oehl, Turrini, & Giovann.

Note on *Rhizoglomus* versus *Rhizophagus*

Since 2015, all publications on new fungal species, phylogenetically belonging to the genus *Rhizoglomus* (e.g. Sudová et al. 2015, Turrini et al. 2018, Błaszkowski et al. 2021) were published as *Rhizoglomus* species. Thus, we have decided to place the new species in *Rhizoglomus* instead of *Rhizophagus*, also due to the fact that it is impossible to know if or where *Rhizophagus populinus* P.A. Dang., the type species of *Rhizophagus* P.A.

Dang., clusters in Glomeromycota. The second fungal species described in earlier times as a *Rhizophagus* species, *R. tenuis* Greenall (Greenall 1963), was recently attributed to the phylum Mucoromycota instead of the Glomeromycota due to phylogenetic analyses (Walker et al. 2018). The morphological taxonomy for the Glomeromycota species is based in the subcellular structures of the spore, and practically there is nothing about the spore of *R. populinus* in the original description. Furthermore, the author of *Rhizophagus* (Dangeard 1896) has not designated or deposited type specimens, but just illustrations. There were no holotype, no isotype and no paratype established for *P. populinus*. There are no sequences for *R. populinus*, thus it is not possible to place *R. populinus* in any clade of the Mucoromyceta by phylogenetic analyses. Considering the above mentioned, at the best, *Rhizophagus* would be a genus "incertae sedis" in the kingdom fungi, in the Mucoromyceta, or, when considering the arbuscular mycorrhizal structures drawn by Dangeard (1896), in the Glomeromycota. Dangeard (1896) decribed *Rhizophagus* as a 'deadly' root pathogen and Saccardo & Trotter (1912) placed *Rhizophagus* into the Peronosporaceae, based on the description and some of the drawings that were published by Dangeard in 1900. Hence, the genus *Rhizophagus* and *R. populinus* are members of the Peronosporales.

Note on *Glomus macrocarpum*

'Glomus macrocarpum' was selected by us as the outgroup species of our phylogenetic analyses. It is the type species of the family Glomeraceae and also of the whole phylum Glomeromycota. However, it has never been shown by any researcher that the spore morphology of the isolate analyzed on molecular phylogenetic bases, really corresponds to the isolate originally described by Tulasne & Tulasne (1844). The spore morphology of the sequenced isolate was never presented, illustrated or discussed. Furthermore, the type (Tulasne & Tulasne 1844) and the lectotype (Berch & Fortin 1983) of this species were isolated in France, however the sequences related to *G. macrocarpum* are from a fungus isolated in the UK. Whether the sequences of the British isolate belong to the species described about 165 years earlier, this is a mystery and more than doubtful. It is another open research question, which should be resolved in the future.

Discussion

Rhizoglomus cacao can be distinguished from all other *Rhizoglomus* species by the combination of spore size and staining reaction of the three spore wall layers in Melzer's reagent. Morphologically, it is most similar to *R. fasciculatum*, which forms creamy spores, whose three spore wall layers stain all purple black (Walker & Koske 1987, Turrini et al. 2018), while spores of the new fungus are yellow-white to whitish yellow and only the two innermost wall layers stain deep purple to purple black. All other *Rhizoglomus* species with a similar spore wall structure (evanescent SWL1, structural SWL2 and flexible

SWL3) have significantly larger spores and do not intensively stain dark purple to purple black on both the structural and the innermost flexible layer (*R. antarcticum*, *R. clarum* and *R. manihotis* (Nicolson & Schenck 1979, Schenck et al. 1984, Cabello et al. 1994, Sieverding et al. 2014, Turrini et al. 2018).

Phylogenetically, *R. cacao* is closest to *R. silesianum*, *R. natalense*, *R. vesiculiferum*, *R. irregulare* and *R. venetianum*, of which *R. vesiculiferum* and *R. irregulare* form also three spore wall layers, but with a different structure (innermost SWL3 is structural, while SWL1 and SWL2 are hyaline and evanescent or permanent and unit; Błaszkowski et al. 2018, Turrini et al. 2018). The other three species, *R. silesianum*, *R. natalense* and *R. venetianum*, all form four wall layers (see identification key above).

So far, the new fungus *R. cacao* was only found in cocoa, and no environmental sequences deposited in the GenBank correspond to the new fungus in the BLASTn analysis. Because the spores resemble those of *R. fasciculatum*, it is likely that in some older collections the new species was morphologically mis-identified. Hence, it is not yet possible to make a clear statement about the distribution of *R. cacao*. Most probably, it might have a larger distribution than in the Neotropical region of San Martin in Peru.

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