Journal of Applied Botany and Food Quality 92, 250 - 257 (2019), DOI:10.5073/JABFQ.2019.092.035

[1]Laboratorio de Biología y Genética Molecular, Universidad Nacional de San Martín, Morales, Peru
[2]Instituto Nacional de Innovación Agraria (INIA), Dirección General de Recursos Genéticos y Biotecnología, La Molina - Lima, Peru
[3]Departamento de Micologia, CB, Universidade Federal de Pernambuco, Cidade Universitária, Recife, Brazil
[4]Agroscope, Competence Division for Plants and Plant Products, Ecotoxicology, Wädenswil, Switzerland

Acaulospora aspera, a new fungal species in the Glomeromycetes from rhizosphere soils of the inka nut (*Plukenetia volubilis* L.) in Peru

Mike Anderson Corazon-Guivin[1*], Agustin Cerna-Mendoza[1], Juan Carlos Guerrero-Abad[1,2], Adela Vallejos-Tapullima[1], Gladstone Alves da Silva[3], Fritz Oehl[4*]

(Submitted: July 1, 2019; Accepted: September 8, 2019)

Summary

A new fungal species of the Glomeromycetes, *Acaulospora aspera*, was isolated from the rhizosphere of the inka nut (*Plukenetia volubilis*) in San Martín State of Peru (Western Amazonia) and propagated in bait cultures on *Sorghum* spp., *Brachiaria brizantha*, *Medicago sativa* and *P. volubilis* as host plants. The fungus forms brownish yellow to yellow brown spores, (120-)135-195 × (120-)130-187 µm in diameter. The surface of the structural spore wall layer is crowded with small depressions, 0.4-0.7 µm in diameter, up to 0.8 µm deep, and only 1.1-1.8 apart, giving the spore surface a rough, washboard-like appearance, especially when the outermost, evanescent wall layer has disappeared. Phylogenetically, the new species is close to *A. spinosissima, A. excavata* and to other morphologically more similar species such as *A. spinosa* and *A. tuberculata*, which form spiny or tuberculate projections on the outermost, semi-persistent spore wall layer, or *A. herrerae*, *A. kentinensis*, *A. scrobiculata* and *A. minuta*, which on the structural spore wall layer all have more pronunced pits than *A. aspera*. In this study, also the name of *A. spinosissima* was validated, as it had been preliminary declared invalid because of a typing error in the diagnosis section of its original description.

Key words: *Acaulospora spinosissima*, *Acaulosporaceae*, arbuscular mycorrhizal fungi, biodiversity, Glomeromycetes, phylogeny.

Introduction

Arbuscular mycorrhizal (AM) fungi are beneficial soil fungi that deliver a couple of important ecosystem services, such plant growth promotion and improved water infiltration and soil aggregation (Sieverding, 1991). During the last decades, the knowledge about the taxonomy, diversity and biogeography of arbuscular mycorrhizal (AM) fungi has considerably been increased (e.g. Janos and Trappe, 1982; Schenck et al., 1984; Sieverding et al., 2014; Błaszkowski et al., 2015; Pontes et al., 2017; Turrini et al., 2018). This is in particular true for species of the family Acaulosporaceae (Furrazola et al., 2013; Palenzuela et al., 2013, 2014, 2015; Pereira et al., 2015). While in 1974 only two *Acaulospora* species were known (Gerdemann and Trappe, 1974) that raised to 15 species in 1990 (Schenck and Pérez, 1990), an increasing number of *Acaulospora* spp. could be included in the continuously developing morphological identification keys (Oehl et al., 2006, 2012; Lin et al., 2019). At time, already > 50 species are attributed to the Acaulosporaceae (Lin et al., 2019).

Especially some tropical areas are considered as hot-spots for Acaulosporaceae (Sieverding, 1991; Pagano et al., 2013; Pereira et al., 2015; Bonfim et al., 2016; Jobim et al., 2018; Marinho et al., 2018). In San Martín State, located in the transition of the last mountain ridges in the Peruvian Andes towards the Amazonian lowlands, a few new AM fungal species have been detected recently, such as two new species of the Glomeraceae, *Funneliglomus sanmartinensis* and *Microkamienskia peruviana* (Corazon-Guivin et al., 2019a, b). Remarkably, these were isolated in rhizosphere soils of the inka nut (*Plukenetia volubilis* L.) that usually is grown there in mixed culture systems together with several annual crops, such as *Zea mays, Phaseolus vulgaris, Arachis hypogaea,* seeded between the rows of the main crop inka nut. The inka nut is native to the Peruvian Amazon and has gained increasing agronomic and economic attention in Peru and other tropical countries due to its elevated contents of unsaturated fatty acids (omega 3, 6, and 9; Chirinos et al., 2013; Srichamnong et al., 2018; Wang et al., 2018). The inka nut plant, its cultivation and the fruits/nuts are presented in the Figs. 1-12.

During diversity studies in inka nut plantations of the Peruvian Amazonian lowlands and adjacent low level mountain ranges of San Martin State in Peru, a further new glomeromycotean fungus was detected morphologically resembling to *Acaulospora laevis*, *Acaulospora spinosissima* and *Acaulospora herrerae* in spore color and sizes (Gerdemann and Trappe, 1974; Furrazola et al., 2013; Oehl et al., 2014). The objective of the present study was to analyze thoroughly the spores of the new fungus both morphologically and phylogenetically and to describe it under the new epithet *Acaulospora aspera*. Finally, we validated the name of the fungus *Acaulospora spinosissima*, for which in the original description a typing error in the accession number of the holotype slide had been made in the diagnosis section (Oehl et al., 2014).

Material and methods

Study sites and Soil sampling

Soil samples (0-30 cm depth) were repeatedly taken in agricultural field site with inka nut at Palmiche (06°20´02.40´´S, 076°36´00.00´´W; 858 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 site is a traditional agroforestry site, where the inka nut is grown in a mixed culture with maize, beans, peanuts and other annual field crops without addition of chemical fertilizers and pesticides. Mean annual temperatures in the study area are about 25-27 °C, with variation between 18 and 32 °C throughout the year. Mean annual precipitation is approximately 1300 mm.

AM fungal pot cultures

Bait cultures were established in the greenhouse under ambient temperature conditions, in cylindrical 3 L pots with 3 kg of substrate. The substrate consisted of a 1:1 mixture of collected field soil samples and coarse river sand. The substrate mixtures were autoclaved

* Corresponding author

Figs. 1-12 ***Plukenetia volubilis*****: 1.** Inka nut plantation at the type location in Palmiche (province Lamas, San Martín, Peru). The annual agricultural crops maize, peanuts and beans planted in the inter-rows had already been harvested at sampling time. **2-3.** Inka nut plants grown at the University of San Martín in Tarapoto. The inka nut plantations in San Martín State last for 4-5 years in the same field and can be replaced by new plantations or other field crops. **4-6.** Inka nut capsule fruits in different ripening stages. **7-9.** Inka nut plants in different development stages, grown in the greenhouse of the University San Martín in Tarapoto. Inka nut is a semi-woody, climbing plant and need support for vertical growth at early stages, either abiotically or by living plants. **10.** Decapsulation of a seed. **11.** Dark brown, field collected seeds, each 1-2 cm in diam. **12.** Toasted almonds of the inka nut, photographed at a local market in Lamas.

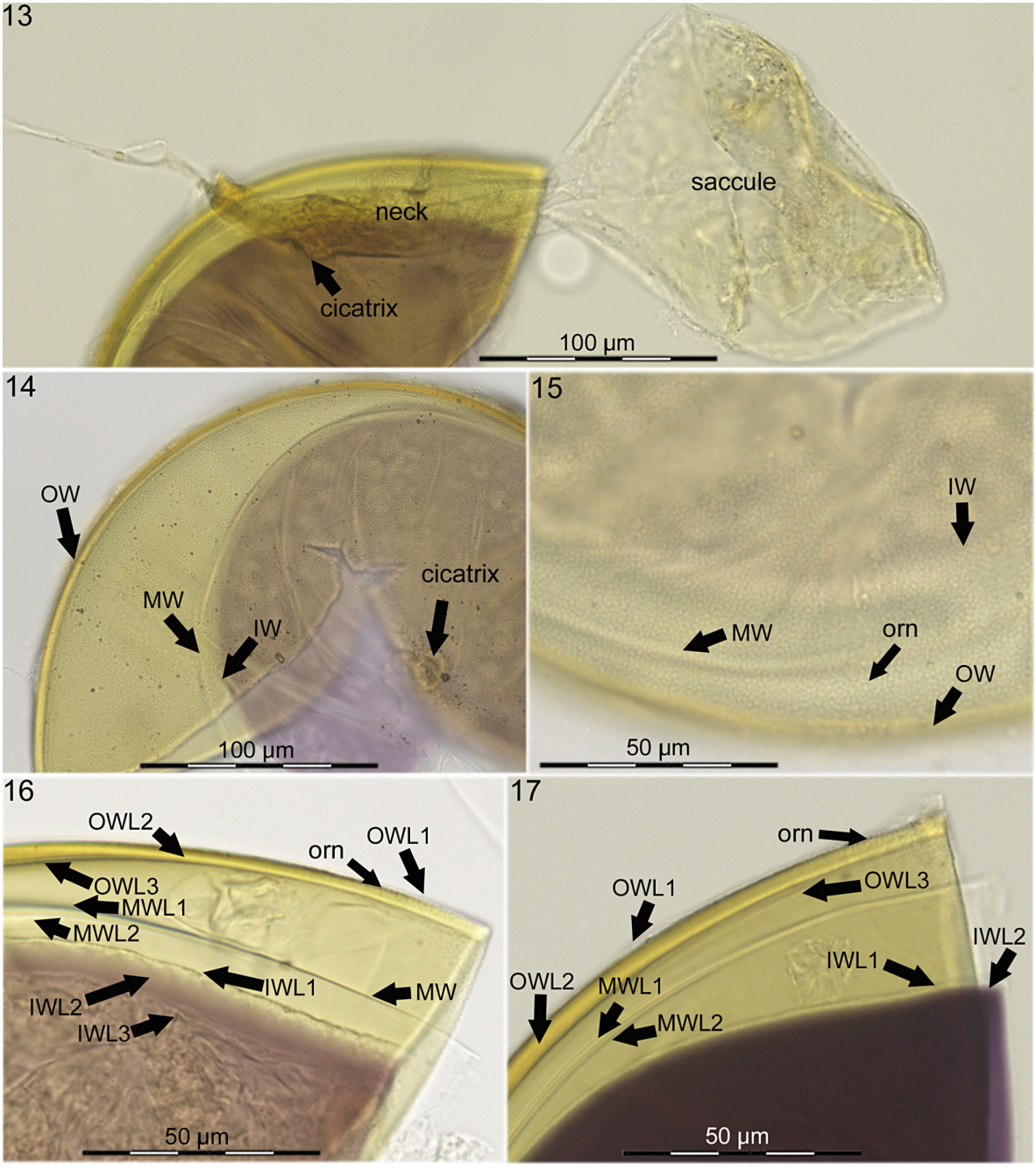
at 121 °C for 60 min, three weeks before establishment of the bait cultures. At bait culture establishment, the pots were first filled to 75% with the autoclaved substrate. Thereafter 100 g of rhizospheric soils were added to the substrate surface, and five seeds either of *Sorghum vulgaris* L., alfalfa (*Medicago sativa* L.), bread grass (*Brachiaria brizantha* (Hochst. ex A. Rich.) R.Webster) and inka nut (*Plukenetia volubilis* L.) were seeded 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 Facultad de Ciencias Agrárias, Universidad Nacional de San Martín-Tarapoto for eight months, with 21.4 °C, 29 °C and 38.2 ºC as minimum, mean and maximum temperatures, respectively. The relative humidity was from 48 to 74% between April and November 2018. The pots were irrigated every other day and fertilized with a Long Anston nutrient solution with reduced P contents (60% reduction; Hewitt, 1966) every two weeks. So far, the new fungal species has not reproduced spores in single species cultures.

Morphological analyses

AM fungal spores were obtained from the field soil and bait culture samples by a wet sieving, density-gradient, centrifugation and decanting process as described by SIEVERDING (1991). The spore morphological characteristics and their subcellular structures were described from specimens mounted in: (i) polyvinyl alcohol-lactic acid-glycerol (PVLG); (ii) a mixture (1:1) of PVLG and Melzer's reagent; (iii) a mixture of lactic acid and water (1:1); (iv) Melzer's reagent; and (v) water. The spore structure terminology follows OEHL et al. (2012) and FURRAZOLA et al. (2013) for species with acaulosporoid spore formation. Photographs (Figs. 13-17) were taken with a Leica DFC 290 digital camera on a Leitz Laborlux S compound microscope using the Leica Application Suite Version V 2.5.0 R1 software. Specimens mounted in PVLG and PVLG+Melzer's reagent were deposited at Z+ZT (ETH Zurich, Switzerland).

Molecular analyses

Intact, healthy spores were isolated from field soil and bait culture samples, and superficially cleaned of soil particles by friction on



Figs. 13-17 ***Acaulospora aspera*****: 13.** Crushed spore formed laterally on a collapsing sporiferous saccule. **14.** Crushed spore stained in Melzer's reagent and showing three walls: OW, MW & IW. Spore surface ornamentation, and also the spore base visible, leaving a permanent cicatrix. **15.** The asper, 'washboard-like' ornamentation (orn) on the outer surface of the pigmented, structural spore wall layer, in planar view. **16-17.** Segments of crushed spores with triple layered OW (OWL1-3), bi-layered MW (MWL1-2) and triple layered IW (IWL1-3), in cross view. The washboard-like ornamentation is on the surface of the pigmented, structural layer (OWL2), thus a persistent structure, and consists of innumerous small and shallow pits. IWL1 with beaded wall, as typical for Acaulosporaceae species; IWL2 staining purple to dark purple, when exposed to Melzer's reagent.

cellulose filter paper (WHATMAN, Grade 50; CORAZON-GUIVIN et al., 2019a). 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. Three independent sterile spores were selected under a laminar flow hood and individually transferred into Eppendorf PCR tubes. Crude extract was obtained by crushing two individual spores with a sterile disposable micropestle in 23 µL milli-Q water, as described by PALENZUELA et al. (2013). 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 (09) 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 phylogeny was reconstructed by independent analyses of the ITS region and the partial SSU, 5.8S, and partial LSU rDNA data sequences. The AM fungal sequences obtained were aligned with other Acaulosporaceae sequences from GenBank in ClustalX (LARKIN et al., 2007), generating two data sets (alignments). Two separate trees were constructed covering the ITS region of the rDNA (first data set) and the partial SSU, 5.8S and partial LSU rDNA (second data set), since some species related to *A. aspera* (e.g. *A. excavata*) have previously not been fully sequenced on the whole DNA fragment suggested by KRÜGER et al. (2009) as barcode to Glomeromycota. *Gigaspora margarita* W.N. Becker & I.R. Hall 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 5×10^6 generations with a sample frequency of 500 and a burn-in value of 25%) and maximum likelihood (1000 bootstrap) analyses were performed in MrBayes 3.1.2 (RONQUIST and HUELSENBECK, 2003) and PhyML (GUINDON and GASCUEL, 2003), respectively, launched from Topali 2.5, using the GTR (for the first data set) and GTR + G (for the second data set) models.

Results

Validation of *Acaulospora spinosissima*

A typing error of the Holotype accession number occurred in the diagnosis of the original description of *A. spinosissima*, made the species name invalid according to the article 40 of the International Code of Nomenclature for algae, fungi, and plants (ICN, 'Melbourne Code'). Thus, we are here validating the species name *Acaulospora spinosissima*, giving unequivocally the correct Holotype: ZT Myc 52168, which is identical with the number given in the holotype section of the original description (OEHL et al. 2014).

Acaulospora spinosissima Oehl, Palenz., I. C. Sánchez, Tchabi, Hount. & G. A. Silva, sp. nov.
Mycobank MB 831729
For diagnosis see Sydowia 66(1): 34 (2014).

Holotypus: ZT Myc 52168 (deposited at Z + ZT – common mycological herbarium of the University and ETH of Zurich, Switzerland).

Acaulospora aspera Corazon-Guivin, Oehl & G.A. Silva, sp. nov. Figs. 13-17.
Mycobank MB 831730

Diagnosis: Differing from *A. spinosissima* by small, irregular washboard-like depressions of OWL2 instead of small spines on the outer spore surface, and from *A. herrerae* by smaller depressions, which do not form a reticulum on the outer wall layer surface, giving *A. aspera* an asper, washboard-like appearance.

Etymology: Latin, *aspera*, (= rough) referring to the rough, washboard-like surface of the structural spore wall layer.

Holotypus: Accession ZT Myc 60145, deposited at Z+ZT, specimen derived from a bait culture established on the host plant inka nut (*Plukenetia volubilis*) in the greenhouse of the Molecular Biology and Genetics Laboratory, Faculty of Agricultural Sciences, National University of San Martín-Tarapoto, Peru. Fungal inoculum for the culture originated from an inka nut plantation in Palmiche (06°20´02.40´´S, 076°36´00.00´´W; 858 m a.s.l. Collector was Mike Anderson Corazon Guivin and collection date was 25.03.2018. Isotypes (ZT Myc 60156) were also deposited at Z+ZT. Living cultures of the fungus are currently maintained at the Universidad Nacional de San Martín-Tarapoto.

Description: Sporiferous saccules are hyaline and singly formed at the end of mycelial hyphae. The saccule termini are globose to subglobose (120-190 × 110-180 µm), with 2-3 wall layers that are in total 2.4-4.2 µm thick. The saccule necks are 25-55 µm broad at the saccule termini, about 15-30 µm at the point of spore formation, and taper to 12-25 µm in 100-250 µm distance from the spore towards the mycelium. The saccule usually collapses after the spore wall has formed and usually is detached from mature spores.

Spores form laterally on the neck of sporiferous saccules in 80-240 µm distance from the saccule termini. They are globose to subglobose, brownish yellow to yellow brown, (120-)135-195 × (120-)130-187 µm in diameter and have three walls.

Outer wall consists of three layers (OWL1-OWL3). Outer layer (OWL1) is hyaline to subhyaline, 1.6-3.1 µm thick, evanescent. Second layer (OWL2) is brownish yellow to yellow brown, persistent, laminated, 2.6-4.9 µm thick. The surface of this structural layer is crowded with small, irregular depressions, 0.4-0.7 µm in diameter, up to 0.8 µm deep, and only 1.1-1.8 apart, giving the spore surface a rough, washboard-like appearance, especially when OWL1 has completely sloughed off. The inner layer of the outer wall (OWL3) is concolorous with OWL2, about 0.6-1.1 µm thick and often difficult to observe. None of the OW layers stains in Melzer's reagent.

Middle wall is hyaline, bi-layered and thin; 1.7-2.9 µm thick in total. Both layers (MWL1 and MWL2) are semi-flexible, tightly adherent

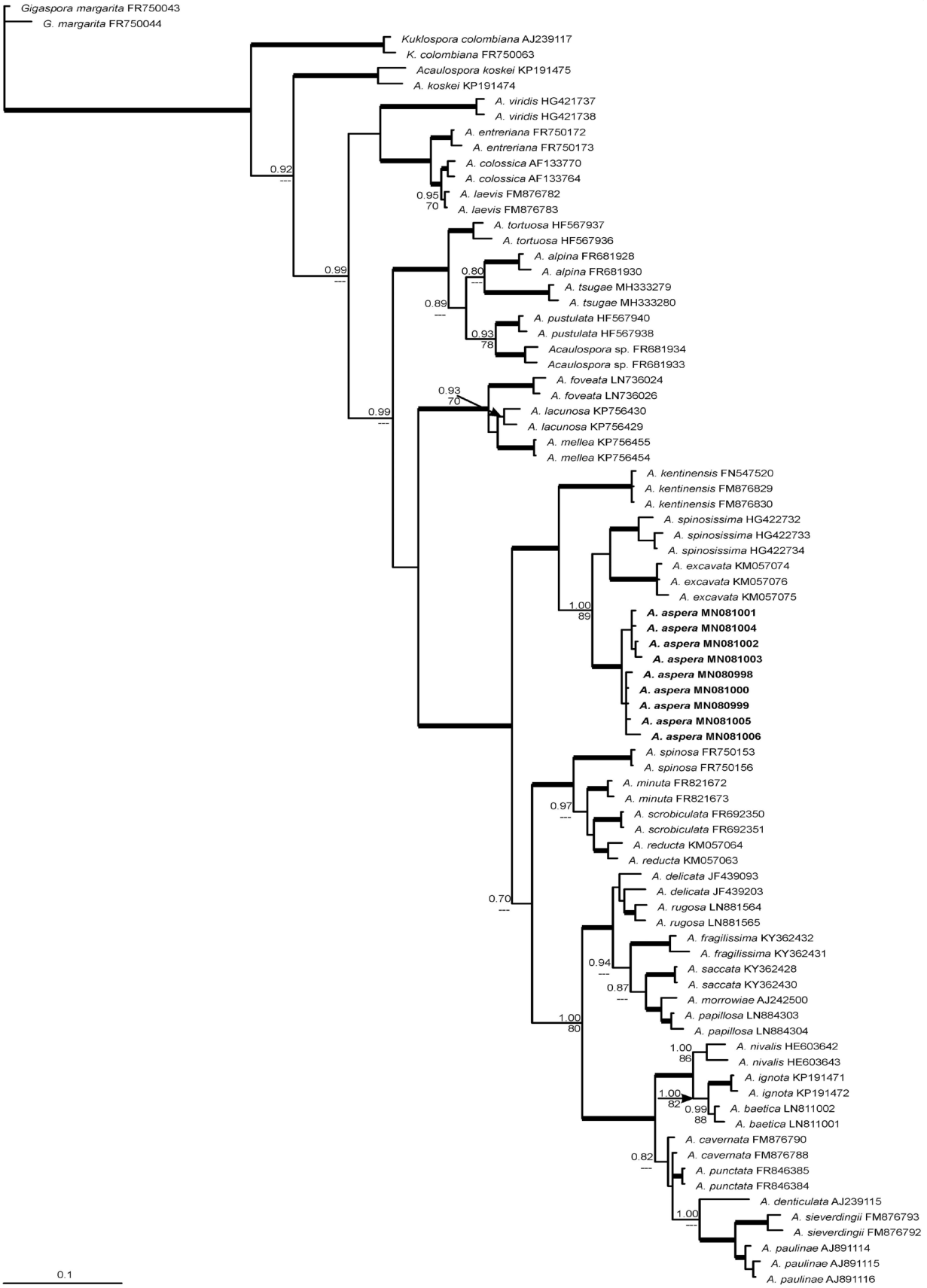


Fig. 18: Phylogenetic tree of the Acaulosporaceae obtained by analysis from sequences of the ITS region of the rDNA from different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), respectively. 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 *Gigaspora margarita*.

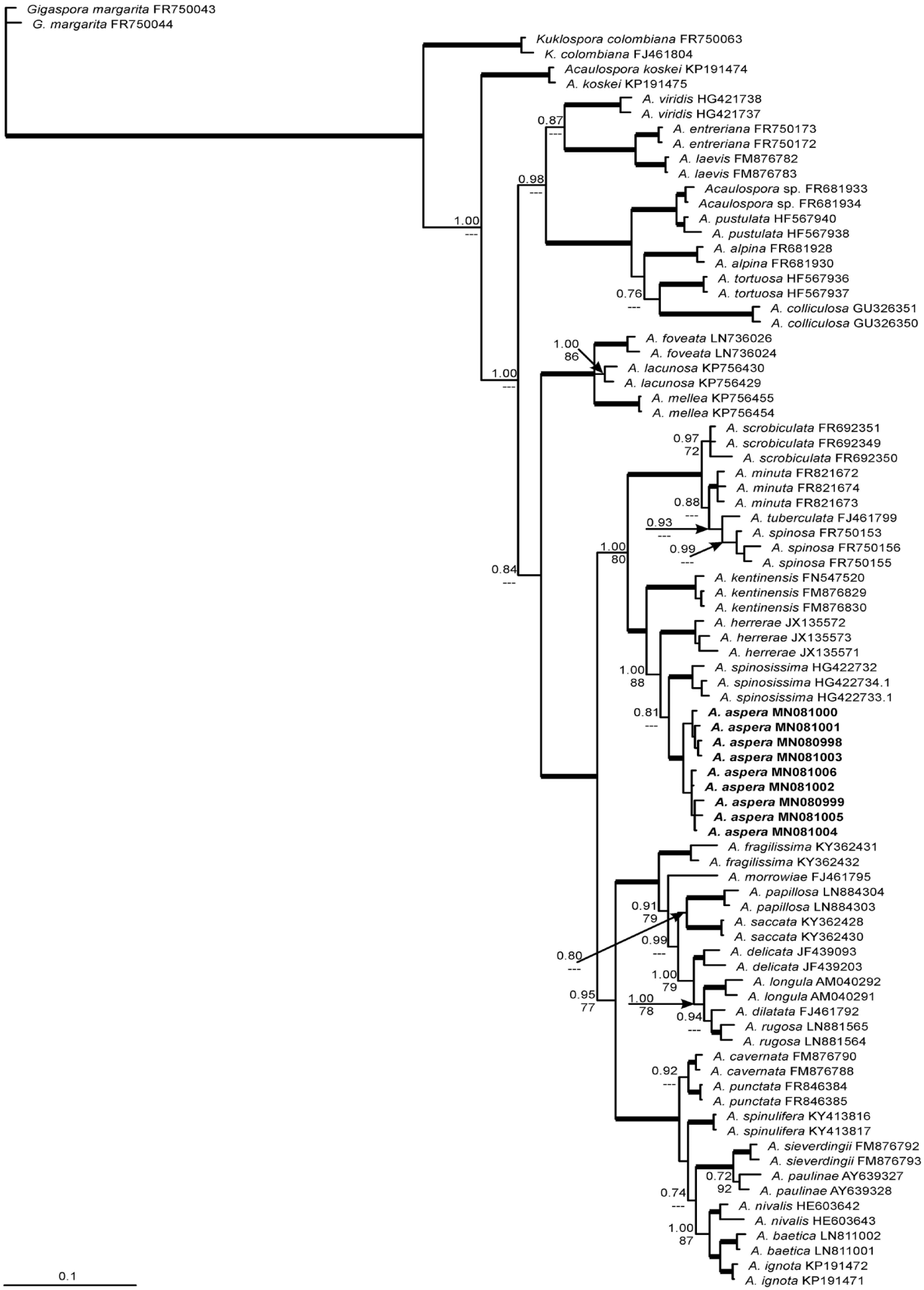


Fig. 19: Phylogenetic tree of the Acaulosporaceae obtained by analysis of partial SSU, 5.8S, and partial LSU rDNA sequences from different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (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 *Gigaspora margarita*.

to each other and thus often appear as being only one wall layer. None of the MW layers stains in Melzer's reagent.

Inner wall is hyaline, with two to three layers (IWL1-IWL3). The IWL1 is about 1.3-2.8 µm thick with a 'beaded', granular structure. IWL2 is 2.2-4.5 µm thick and regularly stains pinkish purple to dark purple in Melzer's reagent. IWL3 is 0.6-1.2 µm and usually very difficult to detect since it is closely adherent to IWL2.
Cicatrix remains after detachment of the connecting hypha, 9-15 (-22) µm wide. The pore is closed by inner laminae of OWL2 and by OWL3.

Mycorrhiza formation: not known from single spore culture, as the new fungus was isolated from rhizosphere soil of inka nut and from bait cultures.

Molecular analyses: The phylogenetic analyses from the ITS region and partial SSU, 5.8S, and partial LSU rDNA sequences placed the new fungus in a separated clade near to *A. spinosissima, A. excavata, A. herrerae* and *A. kentinensis* (Figs. 18 and 19). The clade for the new species was supported by, at least, values of 99% in all analyses. In the BLASTn analyses, the LSU rDNA sequences with closest match (97%) to the new fungus are from *A. spinosissima*. Considering the ITS region of the rDNA, the most related species to *A. aspera* were *A. excavata* and *A. spinosissima* with 94% and 93% of maximum identity, respectively. No environmental sequences deposited in the GenBank correspond to the new fungus in the BLASTn analysis.

Distribution: So far, the fungus was found in one agroforestry site (one agroforestry site, an inka nut plantation of Palmiche in the Province of Lamas, Department of San Martín. Soil texture: sandy clay loam, soil pH at the site was 7.8, and available P was 12.1 mg P kg^{-1}.

Discussion

The new AM fungus *A. aspera* can easily be distinguished from all other species in the Acaulosporaceae through morphological and molecular analyses. Morphologically, the fungus is most similar to *A. spinosissima*, *A. herrerae* and *A. laevis*, which have either spines on the subhyaline evanescent to semi-persistent spore wall layer *(A. spinosissima*) or a reticulate-pitted spore ornamentation structural spore wall layer (*A. herrerae*) or a smooth outer spore wall (*A. laevis*; Gerdemann and Trappe, 1974; Furrazola et al., 2013; Oehl et al., 2014). Moreover, the washboard-like ornamentation of *A. aspera* is more subtle than of the pitted or spiny ornamentation in any the other known *Acaulospora* species such as *A. kentinensis*, *A. minuta*, *A. punctata*, *A. scrobiculata*, *A. spinosa*, *A. tuberculata*, *A. spinulifera* or *A. ignota* (Trappe, 1977; Wu et al., 1995; Oehl et al., 2011; Błaszkowski et al., 2015; Pontes et al., 2017). Phylogenetically, *A. aspera* forms a well separated clade and is clearly distinct from all either morphologically similar or less similar *Acaulospora* species.
Acaulospora aspera is already the third new glomeromycotean fungus described from the rhizosphere of inka nut. This might be not surprising as these are, to our knowledge, only a few reports on AM fungal diversity from the Peruvian Amazon and adjacent montane areas (e.g. Ruíz et al., 2011; Rojas-Mega et al., 2014). Remarkably, all our new fungal species were found in mixed cropping plantations of the inka nut without fertilization. We assume that not only inka nut, but also the accompanying plants (e.g. maize, cassava, beans, peanuts and tomatoes) might be highly dependent on AM fungi in such low-input situations.

Acknowledgements
The authors thank all the members of the Laboratorio de Biología y Genética Molecular for collaborating in the publication of this article and to the farmer of the town of Palmiche (Lamas) for providing us with the facilities for the collection of soil sample.

Funding information: The study was financially supported by the Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica, (FONDECYT, Peru) for the financing granted within the framework of the project with SUBVENTION AGREEMENT N° 187-2015-FONDECYT. Gladstone Alves da Silva thanks to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the Fellowship granted (Proc. 312186/2016-9).

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ORCID
Mike Anderson Corazon-Guivin https://orcid.org/0000-0001-6027-4255
Agustin Cerna-Mendoza https://orcid.org/0000-0002-4827-9966
Juan Carlos Guerrero-Abad https://orcid.org/0000-0002-7285-9506
Gladstone Silva https://orcid.org/0000-0002-5652-7800
Fritz Oehl https://orcid.org/0000-0003-1492-7985

Adresses of the corresponding authors:
Mike Anderson Corazon-Guivin, Laboratorio de Biología y Genética Molecular, Universidad Nacional de San Martín, Jr. Amorarca N° 315, Morales, Peru
E-mail: macorazong@unsm.edu.pe

Fritz Oehl, Agroscope, Competence Division for Plants and Plant Products, Ecotoxicology, Müller-Thurgau-Strasse 29, CH-8820 Wädenswil ZH, Switzerland
E-mail: fritz.oehl@agroscope.admin.ch

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