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Acaulospora flava, a new arbuscular mycorrhizal fungus from *Coffea arabica* and *Plukenetia volubilis* plantations at the sources of the Amazon river in Peru

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Summary

A new arbuscular mycorrhizal fungus, *Acaulospora flava*, was found in coffee (*Coffea arabica*) and inka nut (*Plukenetia volubilis*) plantations in the Amazonia region of San Martín State in Peru. The fungus was propagated in bait cultures on *Sorghum vulgare*, *Brachiaria brizantha* and *Medicago sativa* as host plants. It differentiates typical acaulosporoid spores laterally on sporiferous saccule necks. The spores are light yellow, bright yellow to yellow brown, (95-)105-160 × (95-)100-150 µm in diameter and have smooth spore surfaces. Phylogenetically, *A. flava* clusters in a well-separated clade, nearest to *A. kentinensis*, followed by *A. herrerae*, *A. spinosissima*, *A. excavata* and *A. aspera*, of which remarkably *A. spinosissima*, *A. excavata* and *A. aspera* had also been found in inka nut plantations of San Martín State during the last years.

Here, we report also *A. herrerae* and *A. fragilissima* as fungal symbionts within the rhizosphere of coffee and the inka nut. The later two fungi had so far been recorded by concomitant morphological and molecular analyses only from tropical islands, *A. herrerae* from Cuba in the Golf of Mexico and *A. fragilissima* from New Caledonia in the southwest Pacific Ocean close to Australia. In this study, the ITS region of *A. herrerae* was analyzed for the first time and deposited in the public databases.

In total, we already recovered fourteen *Acaulospora* species from coffee and inka nut plantations in San Martín State of Peru, suggesting that *Acaulospora* species are frequent and beneficial symbionts in coffee and inka nut roots in San Martín State of Peru.

Key words: agroforestry, farming systems, Glomeromycota, Acaulosporaceae, soil biodiversity.

Introduction

In the family Acaulosporaceae, there are several major clades of *Acaulospora* species, which have either smooth spores or spores with pitted or projected surface ornamentations. Major clades of *Acaulospora* species with smooth to roughened surfaces are for instance the species of the *A. laevis* and *A. longula* complexes (Lin et al., 2019), while species of the *A. scrobiculata/spinosa* complex and the *A. cavernata* and *A. sieverdingii* complexes are regularly pitted or have projections on the spore surface (Oehl et al., 2011; Błaszkowski et al., 2015; Corazon-Guivin et al., 2019a; Lin et al., 2019). Other major clades appear to habour both, species with smooth or roughened surfaces and also species with permanent pitted or projected surface ornaments, such as the *A. alpina* and *A. foveata* clades (Oehl et al., 2006; Lin et al., 2019).

In the rhizosphere of the inka nut, several *Acaulospora* species had already been found with spore surface ornamentations, for instance *A. aspera* (Corazon-Guivin et al., 2019a). In our most recent survey from coffee and inka nut plantations in San Martín State of Peru, we found spores and obtained sequences of three other *Acaulospora* species. So far, two of these species had not yet been reported from continental America or other continents by concomitant morphological and molecular analyses, but only from two tropical islands, *A. herrerae* from Cuba, and *A. fragilissima* from New Caledonia (Furrazola et al., 2013; Crossay et al., 2018). The third fungal species is new to science and is presented hereafter under the epithet *A. flava*. Additionally we present spore morphological illustrations and molecular phylogenies of the two other species.

Material and methods

Study sites, soil sampling

Between January and April 2019, soil samples (0-30 cm depth) were repeatedly taken in an agricultural field site located in Barranquita (6°10'20.91"S; 76°53'47.71"W; 321 m.a.s.l.) in a coffee plantation, and in Chazuta (6°33'53.98"S; 76°6'38.60"W; 298 m.a.s.l.) and in Santa Rosa de Huayali (6°44'32.12"S; 76°9'11.51"W; 740 m.a.s.l.) in a inka nut plantation. The first site is located in the province Moyobamba and the two other sites in the province San Martín. Both provinces belong to the Department (State) San Martín in the transition zone of Peruvian Amazonia lowlands and adjacent Andean low mountain ranges. These sites are traditional agroforestry sites, where the inka nut is grown in mixed cultures together with maize, banana, and other field crops, while coffee is grown with forest species. Both, inka nut and coffee have been grown without addition of chemical fertilizers and pesticides. Soil pH was 6.4 in Barranquita and in Chazuta and 5.4 in Santa Rosa de Huayali, while available P ('Olsen-P') was 11.6 mg P kg^{-1}, in Barranquita, 16.3 mg P kg^{-1}, in Chazuta and 12.0 mg P kg^{-1} in Santa Rosa de Huayali. In the province of Moyobamba, the mean annual temperatures are about 18-28 °C, with variation between 16 and 33 °C throughout the year, mean annual precipitation is approximately 2021 mm. In the province of San Martín the mean annual temperatures are about 23-33 °C, with variation between 19 and 37 °C throughout the year, mean annual precipitation is approximately 1380 mm.

AM fungal bait cultures

Bait cultures were established in the greenhouse under ambient temperature conditions, in cylindrical 3 L pots with 3 kg of substrate, using field soil samples of the three field sites described above. Three bait cultures were established per field site. The substrate consisted of a 2:1:1 mixture of field-collected soil samples, vermiculite and coarse

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river sand. The substrate mixtures were autoclaved at 121 °C for 60 minutes, three weeks before establishment of the bait cultures. At inoculation and 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., *Medicago sativa* L. and *Brachiaria brizantha* (A. Rich.) Stapf were seeded in order to establish the mycorrhizal association and reproduce spores of the new fungal species together with the complete 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 Agrarias, Universidad Nacional de San Martín-Tarapoto for eight months, with 21.4 °C ± 2.0, 29.0 ± 3.0 °C and 36.0 ± 2.0 ºC as minimum, mean and maximum temperatures, respectively. The relative humidity was from 46 to 75% between May and December 2019. The pots were irrigated every other day and fertilized with a Long Ashton nutrient solution every two weeks, with reduced P contents (60% reduction; = 20 µg P mL^{-1}; Hewitt, 1966).

Morphological analyses

Spores of the new fungus were found in the bait cultures from Barranquita and Chazuta, while spores of two other *Acaulospora* species were found in Barranquita or Santa Rosa de Huayali, respectively. Single spores of each fungus were separated from their bait culture samples by a wet sieving process as described by Sieverding (1991). The described morphological spore characteristics and their subcellular structures are based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske and 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 in water (Spain, 1990). The terminology of the spore structure basically is that presented in Błaszkowski (2012) and Oehl et al. (2012) for species with acaulosporoid spore formation. Photographs were taken with a digital camera (Leika DFC 295) on a compound microscope (Leitz Laborlux S), using Leica Application Suite Version V 4.1 software. Specimens mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1) were deposited at Z+ZT (ETH 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., 2019b). 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. For the new species two independent groups of sterile spores (from Barranquita and Chazuta, respectively), containing each 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 5× magnification using a stereoscope (Carl Zeiss). For the two other species, each 20-30 spores were also provided from their isolation sites and processed accordingly. 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. The amplified DNA was 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 TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and transformed into One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). For the new species thirteen recombinant colonies (6 colonies for Barranquita and 7 colonies for Chazuta), and for the two other species five and six recombinant colonies, respectively, 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 sequence data. 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). *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 3×10^6 generations, with a sample frequency of 300 and a burnin value of 25%) and maximum likelihood (1,000 bootstrap) analyses were performed, respectively, in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and PhyML (Guindon and Gascuel, 2003), launched from Topali 2.5, using the GTR + G model.

Results

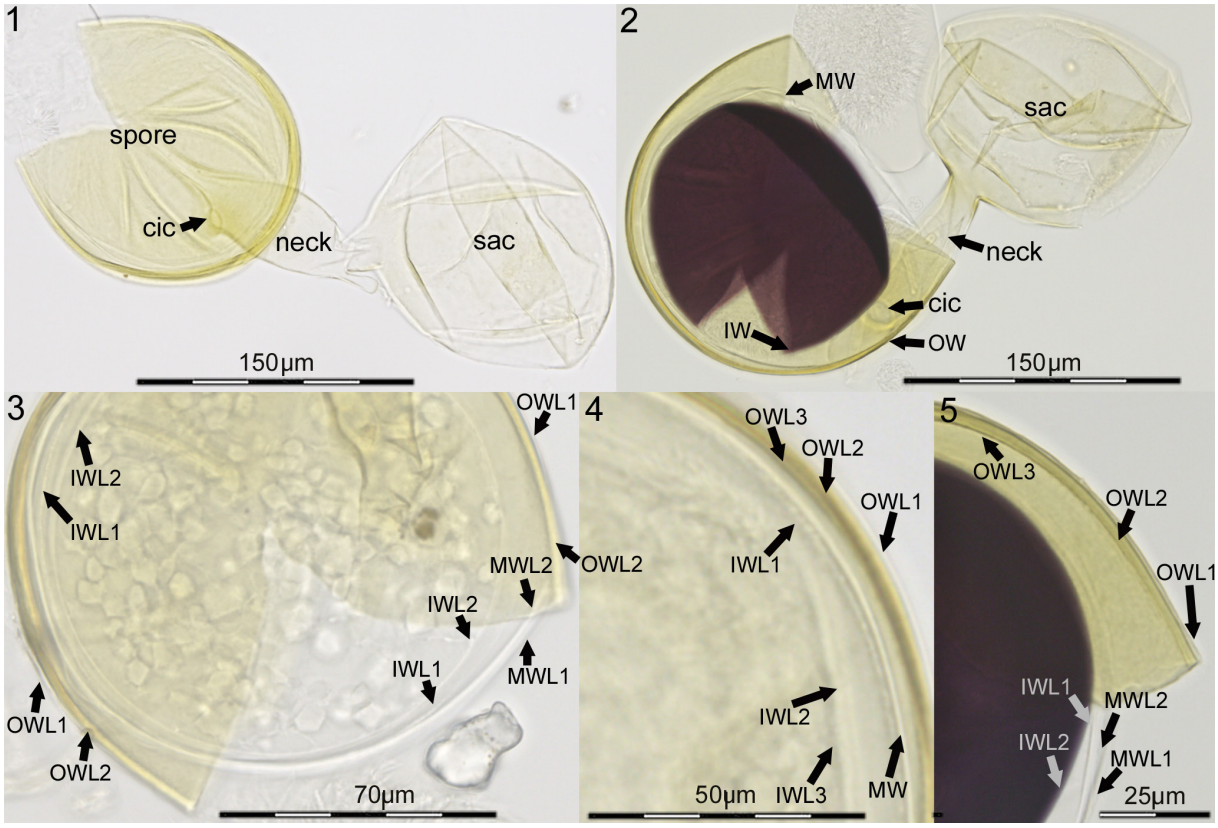
Taxonomy

Acaulospora flava Corazon-Guivin, G.A. Silva & Oehl **sp. nov.**
Figs. 1-5
MycoBank MB 839773

Diagnosis: Differing from *A. mellea* by larger spores, lighter-colored spores and a generally thinner structural outer wall layer (OWL2), from *A. laevis* by smaller spores and thinner spore walls, and from *A. dilatata*, *A. kentinensis*, *A. herrerae*, *A. spinosissima*, *A. excavata* and *A. aspera* by smooth spore surfaces.

Etymology: Latin, *flava*, (= yellow to yellow brown) referring to the spore color of the new species.

Holotypus: Accession ZT Myc 64714, 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 Chazuta (6°33'53.98"S 76°6'38.60"W, 298 m.a.s.l.). Collector was Mike Anderson Corazon Guivin and collection date was 25.04.2019. Isotypes (ZT Myc 64715) were also deposited at Z+ZT. Living cultures of the fungus are currently established at the Universidad



Figs. 1-5: *Acaulospora flava*. **1-2.** Spores in PVLG and PVLG + Melzer's reagent with three walls (OW, MW & IW) and multiple layers (OWL1-3; MWL1-2; IWL1-3) formed laterally on the neck of sporiferous saccules, and forming a single permanent cicatrix on the outer wall. **3-5**. Spore segments of crushed spores in PVLG or PVLG + Melzer's. OWL1 smooth, evanescent and hyaline, OWL2 permanent, structural and bright yellow to yellow-brown, OWL3 thin and difficult to observe as closely adherent to OWL2. MWL1-2 hyaline, flexible and often not easily separated by pressure on the cover slide. IWL1 generally only 0.7-1.1 µm thin, and thus, its beaded ornamentation is only observed, when clearly separated from IWL2. IWL2 stains purple to dark purple in Melzer's. IWL3 difficult to observe as only 0.6–1.1 µm thick and usually closely adherent to IWL2.

Nacional de San Martín-Tarapoto. Paratypes derived from a coffee plantation in Barranquita (6°10'20.91"S 76°53'47.71"W, 321 m.a.s.l.).

Description: Sporiferous saccules are hyaline and singly formed at the end of mycelial hyphae. The saccule termini are globose to subglobose, 100-150 × 98-125 µm, with 2-3 wall layers that are in total 2.4-4.2 µm thick. The saccule necks are 20-50 µm broad at the saccule termini, about 20-30 µm at the point of spore formation, and taper to 11-18 µm in 30-90 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 50-120 µm distance from the saccule termini. They are globose to subglobose, (rarely light yellow), bright yellow to yellow brown, (95-)105-160 × (95-)100-150 µm in diameter and have three walls.

Outer wall consists of three layers (OWL1–OWL3). Outer layer (OWL1) is hyaline to subhyaline, 0.8-1.2 µm thick, evanescent. Second layer (OWL2) is brownish yellow to yellow brown, persistent, laminated, 2.6-4.9 µm thick. 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; 1.4-2.1 µm thick in total. Both layers (MWL1 and MWL2) are semi-flexible, tightly adherent 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 0.7-1.1(-1.5) µm thick with a 'beaded', granular structure, which usually is difficult to observe, since IWL1 is rather thin and generally adherent to IWL2. IWL2 is 1.8-2.8 µm thick and regularly stains pinkish purple to dark purple in Melzer's reagent. IWL3 is 0.6-1.3 μm and usually very difficult to detect since it is closely adherent to IWL2.
Cicatrix remains after detachment of the connecting hypha, 8-15 (-20) µm wide. The pore is closed by inner laminae of OWL2 and by OWL3.

Molecular analyses: The phylogenetic analyses from the ITS region and partial SSU, 5.8S, and partial LSU rDNA sequences placed *A. flava* in a separate clade near to *A. kentinensis* (Figs. 6 and 7). The clade for the new species was supported by 75% and 76% bootstrap values (ML analyses) and 0.88 and 0.94 Bayesian posterior probabilities for the ITS and SSU-5.8S-LSU data set, respectively. In the BLASTn analyses, the sequences with closest match (96%) to the new fungus are from *A. kentinensis*. One environmental sequence, related to *A. flava* (99.5% of identity), was found in roots from pigeon pea (*Cajanus cajan* (L.) Millsp.) in Yuanmou County, southwest China (Li et al., 2009).

Distribution of *A. flava* and other Acaulosporaceae species in San Martín State of Peru: So far, the new fungus was found in a coffee plantation in Barranquita and in an inka nut plantation, both located in San Martín State of Peru. In Barranquita, it was found together with *A. herrerae* (Figs. 6-9; ZT Myc 64716), which so far

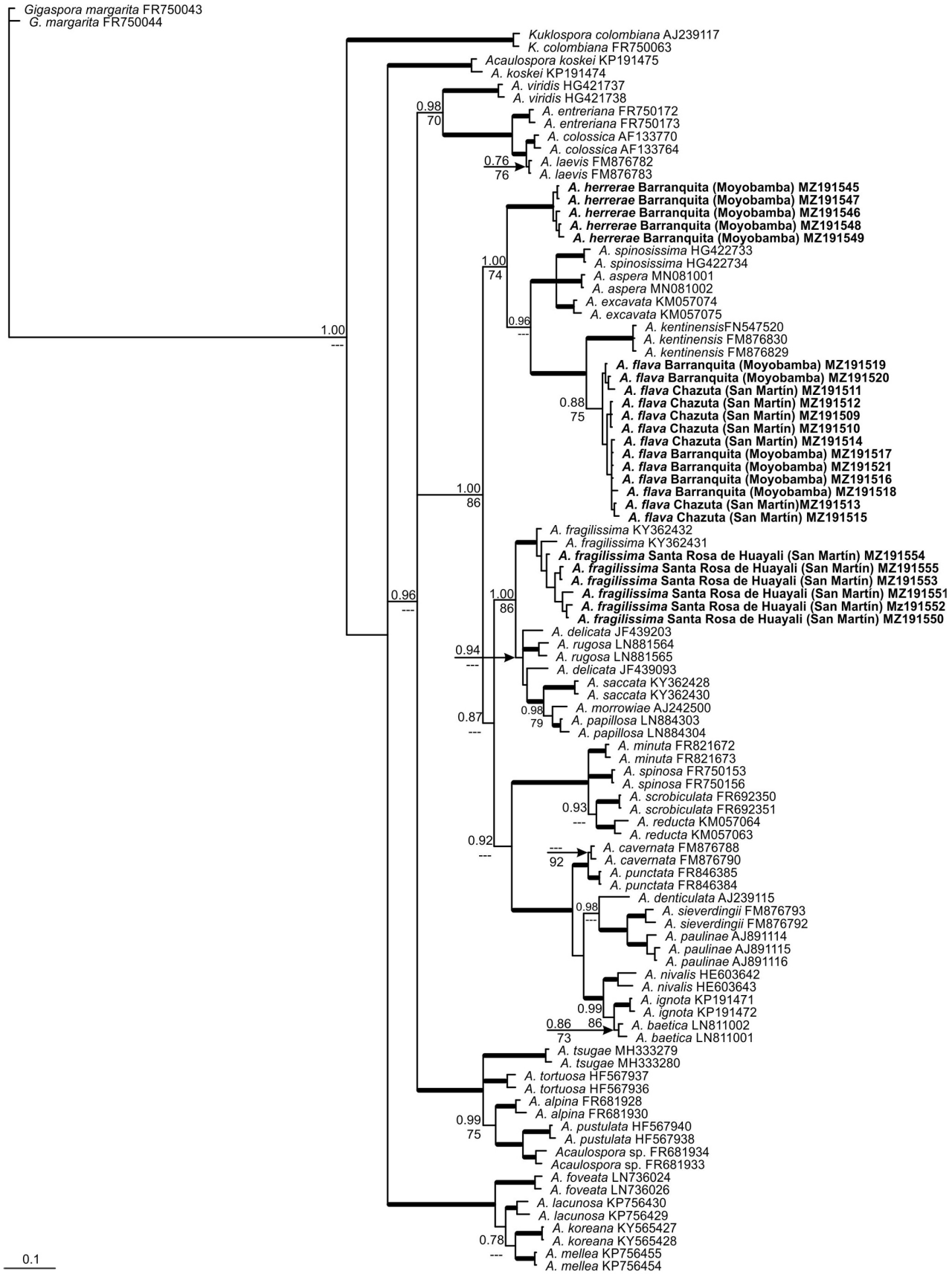


Fig. 6: Phylogenetic tree of the Acaulosporaceae obtained by analysis from sequences of the ITS region of the rDNA from different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), respectively. 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*.

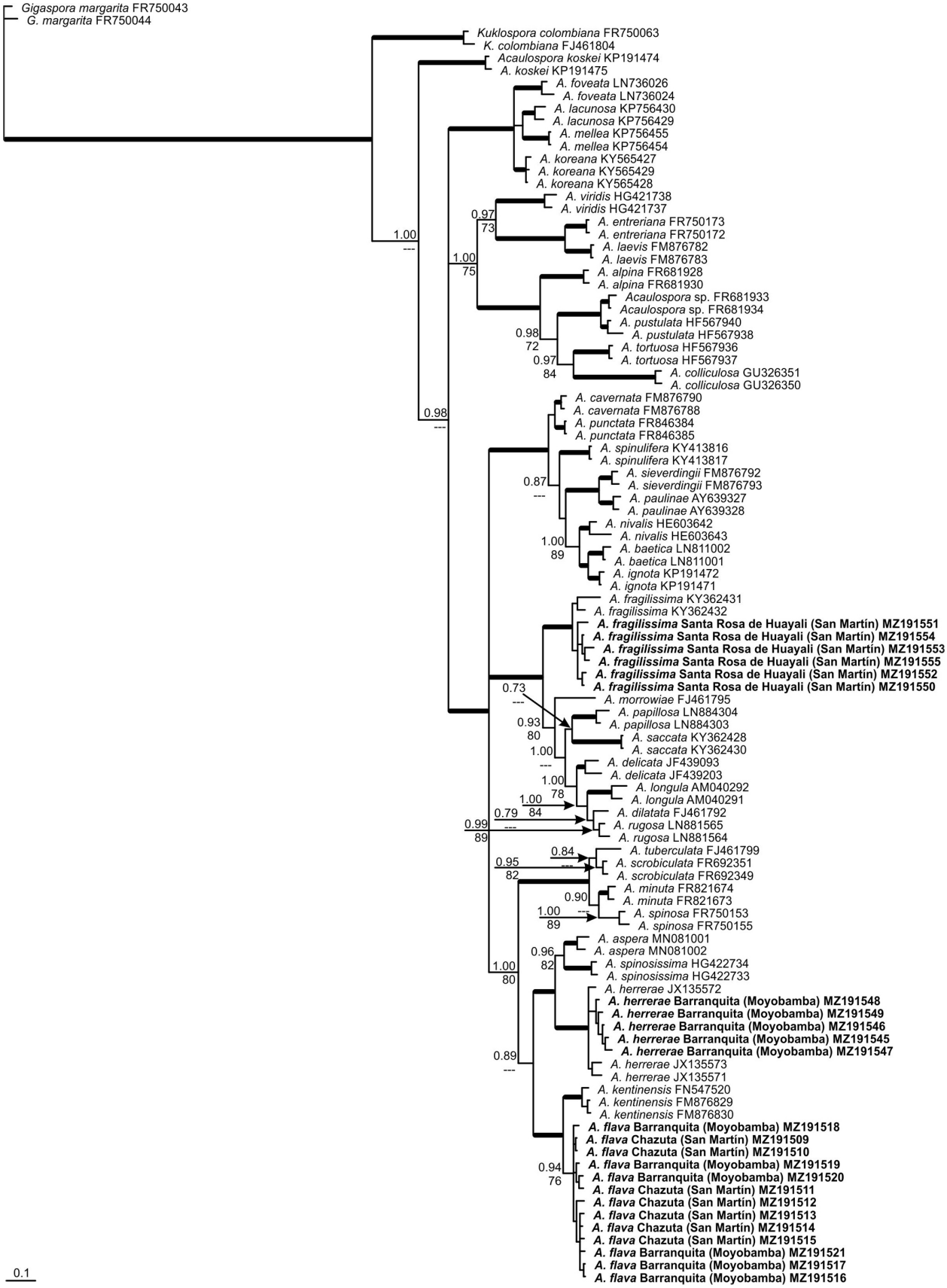
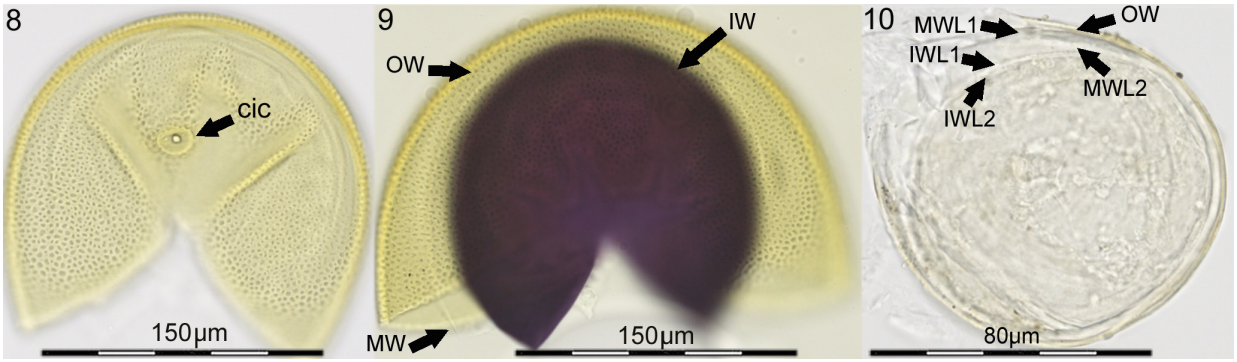


Fig. 7: Phylogenetic tree of the Acaulosporaceae obtained by analysis of partial SSU, 5.8S, and partial LSU rDNA sequences from different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), 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*.



Figs. 8-9: *Acaulospora herrerae*. Crushed spores in PVLG and PVLG + Melzer's reagent. Spores showing three walls (OW, MW, IW) and typical reticulate surface ornamentation. One permanent cicatrix (cic) is recognized, as a remnant of the spore formation laterally on the neck of a sporiferous saccule, which has already detached from the spores. **Fig. 10.** *Acaulospora fragilissima*. Spore showing three, rather thin and fragile walls (OW, MW, IW) and multiple layers (MWL1-2, IWL1-2). From the outer wall, only OWL2 is well visible, while of OWL1 only a few remnants are left.

has not yet been found in concomitant morphological and molecular phylogenetic studies outside of Cuba (Furrazola et al., 2013). In this study, we also present molecular phylogenetic data (Figs. 6 and 7) and a morphological illustration for *A. fragilissima* (Fig. 10, ZT Myc 64717), showing for this species the first record outside of New Caledonia (Crossay et al., 2018).

In total, already 14 *Acaulospora* species were found in San Martín State either in coffee or inka nut plantations. There are several species with close phylogenetic relationship to *A. flava* (*A. kentinensis*, *A. spinosissima*, *A. herrerae*, *A. excavata* and *A. aspera*), but also species of the *A. foveata* complex (*A. lacunosa* and *A. mellea*), species of the *A. scrobiculata/spinosa* complex (*A. scrobiculata*, *A. rehmii*, *A. spinosa*), species of the *A. longula* complex (*A. morrowiae* and *A. fragilissima*), *A. laevis* of the *A. laevis/entreriana* complex, and finally also *A. elegans*, for which phylogenetic data are not yet available. *Acaulospora flava* is the second *Acaulospora* species, originally described from Peru, after *A. aspera* (Corazon-Guivin et al., 2019a).

Discussion

Acaulospora flava can be distinguished from all other species in the Acaulosporaceae by spore size, color and spore wall structure. The morphologically most similar species to *A. flava* are *A. laevis, A. dilatata* and *A. mellea* (Lin et al., 2019). *Acaulospora laevis* generally forms larger spores (> 150 µm; Gerdemann and Trappe, 1974; Lin et al., 2019), while *A. mellea* (Schenck et al., 1984) regularly forms smaller spores ((72-)95-105(-126) µm) than *A. flava*, and *A. dilatata*, which is most similar to *A. flava* in spore size (78-130 µm), has minute pits on the roughened outer spore surface (Morton, 1986). Interestingly, all these *Acaulospora* species phylogenetically cluster in different major clades: *A. laevis* is together with *A. colossica*, *A. entreriana* and *A. viridis, A. mellea* clusters with *A. foveata*, *A. koreana* and *A. lacunosa*, while *A. dilatata* groups within the *A. longula* complex closest to *A. rugosa* and *A. longula*, and finally *A. flava* is phylogenetically closest to *A. kentinensis* within the large *A. kentinensis/A. excavata* complex. Also remarkably, within the *A. kentinensis/A. excavata* complex, *A. flava* is the first of nine species without spore surface ornamentation, neither having pits or projections, but smooth spore surfaces. In this sense, the spore alone of *A. flava* is morphological most similar to *Kuklospora colombiana* (synonym *A. colombiana*), the most ancestral species known in the Acaulosporaceae (Figs. 6 and 7), which forms its spores within the neck of the sporiferous saccules, but not laterally on the neck of the saccules, as observed for *A. flava*.

The new fungus is here described from coffee or inka nut plantations of two provinces in San Martin State of Peru, isolated from the rhizosphere of coffee and the inka nut. A much wider distribution can be assumed for *A. flava*, as we were able to attribute ecological sequences from the public data bases to the new fungus, originally obtained from roots of pigeon pea in Yuanmou County, southwest China (Li et al., 2009).

Within this study we found two further *Acaulospora* species in the coffee and inka nut plantations, *A. fragilissima* and *A. herrerae*. *Acaulospora herrerae* was found several times in tropical Brazil by spore morphology (e.g. Winagraski et al., 2019), but by concomitant spore morphology and molecular phylogeny these two fungi have been found hitherto only from two tropical islands, *A. fragilissima* from New Caledonia and *A. herrerae* from Cuba. To our opinion, *A. fragilissima* can easily be overlooked by spore morphology, or may remain unidentified due to its fragile features, especially from field soil samples, while *A. herrerae* might have been formerly confused with *A. scrobiculata* due to their relative close similarity (Trappe, 1977; Furrazola et al., 2013). Our results are quite important, suggesting and confirming that also these species, *A. fragilissima* and *A. herrerae*, have a much wider distribution in tropical areas than known so far.

In total, we already recovered fourteen *Acaulospora* species from coffee and inka nut plantations in San Martín State of Peru, belonging to different phylogenetic major clades. Two of these *Acaulospora* species, *A. aspera* and *A. flava* were so far undesribed species, both belonging to the *A. kentinensis/A. excavata* complex. From this complex, several other species were found in coffee and inka nut plantations in Peru, showing that this species complex is frequent and species-diverse in San Martín State. Given the fact that so many *Acaulospora* species were found in a rather short time of 3-5 years, a much higher diversity of these taxa can be expected in coffee and inka nut plantations in Peru. Importantly, the data do not only suggest that *Acaulospora* species might be frequent, but also beneficial symbionts in coffee and inka nut plantations of San Martín State in Peru, since significant plant growth promotion was already shown for several *Acaulospora* species from other tropical environments, for instance for white yam (*Dioscorea rotundata* Poir.) in Western Africa (Tchabi et al., 2010).

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Conflicts of interest

No potential conflict of interest was reported by the authors.

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