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# *Acaulospora minuta*, a new arbuscular mycorrhizal fungal species from sub-Saharan savannas of West Africa

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## Summary

A new arbuscular mycorrhizal (AM) fungal species of the genus *Acaulospora* (Glomeromycota) was frequently recovered from both undisturbed and cultivated agro-ecosystems of sub-Saharan West Africa, namely in Benin. It abundantly reproduced spores in trap cultures using *Sorghum bicolor*, *Dioscorea cayenensis* and *Dioscorea rotundata* in the glasshouse, and pure, monosporic cultures were readily established on *Hieracium pilosella* and *Sorghum bicolor*. It forms bright yellow-orange to orange-brown spores, (150-)175-230 in diameter, that have minute pits that are 0.5-1.2(-1.8) µm in diameter, 0.5-1.1 µm deep, and 1.0-2.5 µm apart. The species superficially resembles *Acaulospora scrobiculata*, which forms subhyaline to olive creamy spores that have larger, more irregular and deeper pits. *Acaulospora minuta* was one of the most frequent AM fungi collected during a study in the Guinea and Sudan Savannas in Benin. It was frequently recovered from yam (*Dioscorea* spp.) fields, cultivated during the first year following savanna clearance, but was not recovered from fields later in the crop rotation cycle from either traditional or intensive agricultural ecosystems. It was also not recovered from long-term (> 7 years) regenerating savanna, under fallow following cultivation, indicating the vulnerability of this fungus to mechanical and/or biological disturbance, even under traditional West African low-input agro-ecosystems.

## Introduction

The propagation of *Acaulospora* species in pure cultures is regularly frustrating and difficult, with monosporic cultures not recommended due to the unknown duration of their spore dormancy and uncertain germination capacity. Conversely, following the generation of supposedly single species cultures using multiple spore inoculation, DNA extraction, amplification and sequencing procedures have revealed numerous sequences casting doubt on the purity of the cultures, either due to inadvertent inoculation of mixed species or due to contamination during their subsequent maintenance. Consequently, doubts regarding the reliability of *Acaulospora* species sequences have been expressed, particularly with respect to species which are difficult to identify, such as *A. longula* Spain & N.C. Schenck, *A. morrowiae* Spain & N.C. Schenck, *A. mellea* Spain & N.C. Schenck, *A. rugosa* J.B. Morton, *A. dilatata* J.B. Morton and *Kuklospora colombiana* Spain & N.C. Schenck (SCHENCK et al., 1984; MORTON, 1986).

In order to determine the effects of native arbuscular mycorrhizal (AM) fungi on the growth of white (*Dioscorea rotundata* Lam.) and yellow yam (*D. cayenensis* Poir) about 900 monosporic cultures of several *Glomus*, *Sclerocystis*, *Acaulospora* and *Kuklospora* species were initiated (TCHABI et al., 2010). Although the overall success rate of the culturing was low (approximately 10%), several isolates of *Sclerocystis*, *Acaulospora* and *Kuklospora* species that have traditionally proved difficult to cultivate were generated, maintained and screened on the desired host plants (TCHABI et al., 2010), beside *G. mosseae, G. etunicatum* and other easily cultured *Glomus* species. One of the species that was cultured during the study was an undescribed species, referred to as *Acaulospora* sp. WAA1 and *Acaulospora* sp. WAAc1 in TCHABI et al. (2008, 2009a), respectively, or as WA-*A.WA1-3* (TCHABI et al., 2010). Thus, the objective of the current study was to analyze the morphological and molecular characteristics of this particular species towards a full and accurate description under a new epithet, and to compare the species both morphologically and phylogenetically with related *Acaulospora* species.

## Materials and methods

### Study area and sites

Study sites comprised 27 natural, fallow, and cultivated agro-ecosystems, located within the Sudan (SU), Northern Guinea (NG), and Southern Guinea (SG) savanna ecological zones of Benin, sub-Saharan West Africa. The climate follows a gradient from SG through NG to SU savannas of decreasing annual rainfall and increasingly longer dry seasons from 5 to 8 months (TCHABI et al., 2008). The SG has two wet and two dry seasons per annum, while NG and SU are monomodal. The vegetation consists of trees, shrubs and grasses with tree and shrub prominence decreasing from south to north (e.g. ADJAKIDJE, 1984; ADJANOHOUN, 1989; TCHABI et al., 2008). The soils are predominantly ferruginous Ferralsols. The selected sites were either natural savannas or yam fields established in the first year after (forest) savanna clearance, mixed cropping systems, groundnut (*Arachis hypogaea* L.) and intensively managed cotton (*Gossypium hirsutum* L.) fields. Sites located in long-term fallows (≥7 years old) were also included to compare species present in undisturbed sub-Saharan savannas with those present in restored fallows and under varying levels of cropping intensification and soil disturbance.

### Soil sampling and culturing of AM fungi

Soils were sampled as described in TCHABI et al. (2009a) towards the end of the 2004 wet season in September/October and during the subsequent dry season in February 2005. Soil pH, organic carbon, and available phosphorus were determined using standard methods

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(TCHABI et al., 2008, 2009b). The spore material used during the study originated directly from field samples. Extensive attempts were made to propagate the AM fungal species present in the field samples through 'bait' cultures using various hosts (*Brachiaria humidicola* (Rendle) Schweick., *Stylosanthes guianensis* (Aubl.) Sw., *Sorghum bicolor* (L.) Moench, *Dioscorea cayenensis* Lam., *D. rotundata* Poir). Several bait culture systems were established (TCHABI et al., 2008, 2009a) inoculating 5-10% field soils to autoclaved substrate (Terragreen: Quartz sand mixture; 3:1 [wt/wt]). The AM fungal communities were cultivated for between 8-24 months and the host plants periodically analyzed for mycorrhizal infection and AMF spore formation. In total, the bait culturing resulted in the establishment and reproduction of 45 AM fungal species (TCHABI et al., 2009a), and the species described below abundantly produced spores in 19 bait culture pots inoculated with soils collected during the dry season.

When monosporal cultures were established immediately following spore isolation from actively growing trap cultures, an AMF symbiosis between the new fungus and the host plants selected could not be established. However, when monosporal cultures were established from spores isolated from bait culture samples that had been air-dried and stored for 3 months before inoculation (TCHABI et al., 2009a), AM symbiosis establishment and new AMF spore formation succeeded in 19 monosporal isolates, with 11 isolates deriving from site 'ns3' (at 08°19.661'N; 001°51.340'E), and 8 isolates deriving from site 'ns1' at (07°45.739'N; 002° 27.519'E).

### Morphological analyses

Spores were extracted from field soils, bait cultures and the 19 monosporal pure cultures by wet sieving and sucrose centrifugation (BRUNDRETT et al., 1994) before being mounted in PVLG, PVLG + Melzer's reagent (BRUNDRETT et al., 1994), and water (SPAIN, 1990). Terminology used in the species description follows SPAIN et al. (2006), OEHL et al. (2006) and PALENZUELA et al. (2008, 2011) for species with spore formation laterally on the neck of sporiferous saccules. Photographs in Fig. 1-10 were taken with a digital camera (Leika DFC 290) on a compound microscope (Leitz Laborlux S) using Leica Application Suite Version V 2.5.0 R1 software. Specimen mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at Z+ZT (mycological herbarium at ETH Zurich, Switzerland) and URM (mycological herbarium of the Federal University of Pernambuco in Recife, Brazil) herbaria.

### Molecular analyses

Crude extracts from five spores were obtained by crushing surface-sterilized spores with a sterile disposable micropestle in 40 µL milli-Q water, as described by FERROL et al. (2004). The spores derived from two monosporal pure cultures (isolates FO366 and FO378) isolated from the type location. Spores were sterilized with chloramine T (2%) and streptomycin (0.02%) (MOSSE, 1962). PCRs were performed in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, California) with a pureTaq Ready-To-Go PCR Bead (Amersham Biosciences Europe GmbH, Germany) 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 were separated electrophoretically on 1.2% agarose gels, stained with Gel Red™ (Biotium Inc., Hayward, CA, U.S.A.) and viewed by UV illumination. The band of the expected size was excised with a scalpel and isolated from the gel with the QIAEX II Gel Extraction kit (QIAGEN, USA) following the manufacturer's protocol, cloned into the pCR2.1 vector (Invitrogen, Carlsbard, California), and transformed into one shot TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells, cloned DNA fragments were sequenced with vector primers (WHITE et al., 1990) in both directions by Taq polymerase cycle sequencing on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST programs (ALTSCHUL et al., 1990). The new sequences were deposited in the EMBL database under the accession numbers FR821672-FR821675 and FR869690-FR869691 for the isolates FO366 and FO378, respectively.

Phylogenetic analyses: The AM fungal sequences (comprising rDNA ITS1, 5.8S subunit and ITS2) were aligned in ClustalX (LARKIN et al., 2007) and edited with the BioEdit program (Hall, 1999) to obtain a final alignment. *Glomus etunicatum* W.N. Becker & Gerd. was included as an outgroup. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (MILNE et al., 2004). Bayesian (two runs over 1 x $10^6$ generations with a burn in value of 2500) and maximum likelihood (1000 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. Neighbor-joining (established with the model cited above) and maximum parsimony analyses were performed using PAUP*4b10 (SWOFFORD, 2003) with 1000 bootstrap replications.

## Results

### Latin diagnosis

***Acaulospora minuta*** Oehl, Tchabi, Hount., Palenz., I.C. Sánchez & G.A. Silva sp. nov. (**Fig. 1-10**)

MycoBank MB 561834

Sacculus sporiferus hyalinus aut pallido-luteus, globosus vel subglobosus, (150-)170-225 µm in diametro et formationi sporae praecedens. Sporae singulae lateraliter formatae ad hypham in 30-80(-100) µm distantia ad sacculum terminalem, obscuro-aurantiae vel aurantio-fulvae, globosae, (150-)175-230 µm in diametro, vel subglobosae vel ellipsoideae (165-225 x 170-235 µm). Sporae tunicis tribus: tunica exterior, media et interior. Tunica exterior in totum 4.2-7.5(-8.5) µm crassa, stratis tribus: stratum exterius hyalinum, tenue et evanescens; stratum medium laminatum, obscuro-aurantium vel aurantio-fulvum, depressionibus regularibus 0.5-1.2(-1.8) µm in diametro, 0.5-1.1(-1.6) µm profundis, in interiorem strati huius insculptis et in distancia 1.0-2.5-(3.5) µm; stratum interius flavum vel fulvum, subtile. Tunica media tenuis stratis duobus et tunica interior stratis tribus, uterque tunicae hyalinae et flexibiles. Tunica interior 2.5-4.0 µm in totum; solo stratum medium tunicae interioris pallide colorans reagente Melzeri. Typus #46-4601: Z+ZT Myc 3343.

### Holotype

Specimens of a monosporic pure culture (isolate FO366; collection date December 2006) established from AMF communities propagated from a sub-Saharan (Southern Guinea) savanna (08°20'N; 01°51'E) in Benin (West Africa) on *H. pilosella*, and deposited at Z+ZT (Z+ZT Myc 3343). Isotypes isolated from the same pot culture deposited at Z+ZT (Z+ZT Myc 3344). Paratypes isolated from other monosporic pure cultures (e.g. FO378) obtained from the same site deposited at (Z+ZT Myc 3345, 3346 & 3347) and URM (82547). Paratypes obtained from other pure cultures on *H. pilosella* derived from another sub-Saharan (Southern Guinea) savanna (07°46'N; 02°28'E) in Benin, deposited at Z+ZT (Z+ZT Myc 3348 & 3347) and URM (82548). Paratypes isolated from trap cultures and field sites derived

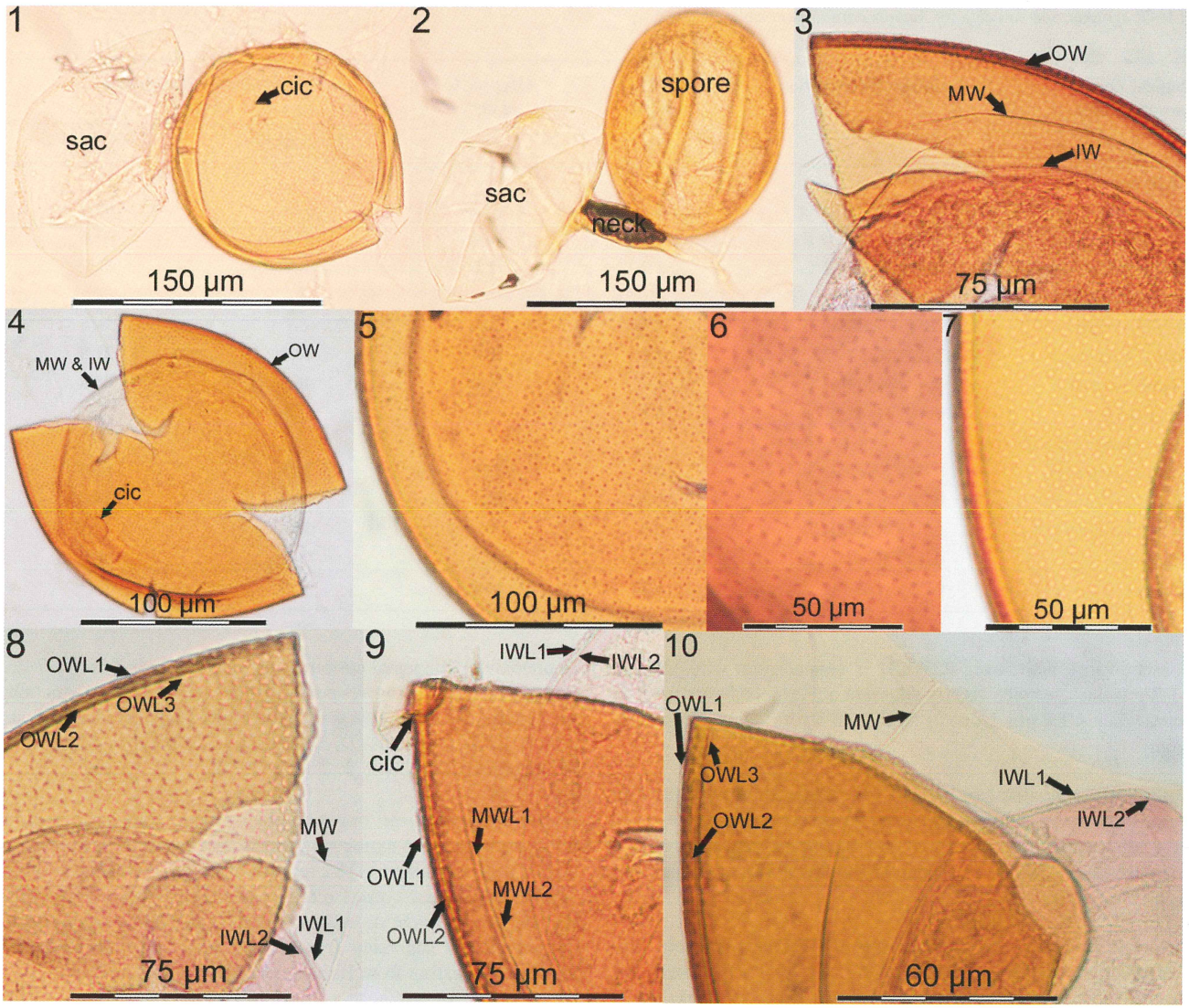


**Fig. 1-10:** *Acaulospora minuta*: 1. Spore and sporiferous saccule (sac) attached. 2. Spore formed laterally on the neck of a sporiferous saccule leaving a cicatrix on the spore base (cic). 3. Fragment of a crushed spore with outer, middle and inner wall (OW, MW and IW). 4. Crushed spore with characteristic minute surface ornamentation. 5-7. Minute surface ornamentation on OW in different augmentation and planar view. 8-10. Wall structure in crushed spores mounted in PVLG + Melzer's reagent: outer wall layers (OWL1-3), bi-layered middle wall mw (MWL1-2) and bi- to triple-layered inner wall (only IWL1-2 visible); OW in cross view. The granular 'beaded' ornamentation on IWL1 is regularly visible; the second layer of the inner wall (IWL2) stains pinkish to light purple in Melzer's reagent.

from a total of nine sites (see Tab. 1) with natural Guinea savanna vegetation (seven sites) or from sites under yam cultivation within the first year following savanna clearance.

## Description

**Sporiferous saccules** are hyaline, globose to subglobose, (150-) 170-225 µm in diameter, with a mono- to bi-layered wall that is 2.4-4.8 µm thick in total (Fig. 1-2); saccules formed terminally or intercalary on mycelial hyphae. The saccules usually collapse following spore wall formation and usually become detached from mature spores in soil samples and older pot cultures.

**Spores** form laterally on the neck of sporiferous saccules at 30-80 (-100) µm distance from the globose saccule terminus (Fig. 2). The spores are yellow-orange when young becoming dark orange to dark orange-brown at maturity, and globose, (150-)175-230 µm in diameter, to subglobose to rarely ellipsoid, 170-225 x 165-235 µm. They have three walls (an outer, middle and inner wall (OW, MW, IW; Fig. 3-4) and have a characteristic shallow, minutely pitted ornamentation on the outer spore surface (Fig. 4-7).

**Outer spore wall** consists of three layers (OWL1, OWL2 and OWL3) which, combined, is 4.2-7.5(-8.5) µm thick (Fig. 8-10). Outer layer (OWL1) is hyaline, unit, 0.9-2.0 µm thick, evanescent and thus, usually absent in mature spores. Second layer (OWL2) is dark yellow-orange to dark orange-brown, laminate, 2.5-5.3 µm thick and have shallow, minute pit-like depressions. Pits are regular to slightly irregular in uncrushed spores, 0.5-1.2(-1.8) µm in diameter (Fig. 5-7) and regularly inconspicuous, since they are just 0.5-1.1 µm deep, exceptionally up to 1.6 µm deep (Fig. 8-10). The distance between the pits is 1.0-2.5-(3.5) µm. In crushed spores the round pits may become oblong to oblong-curved depending on the pressure and pressure direction applied on the cover slides.

**Tab. 1:** Geographic and soil data for *Acaulospora minuta* isolation sites

| Ecological Zone Sampling sites | Geographic location | pH ($H_20$) | Organic C g $kg^{-1}$ | Available phosphorus mg $kg^{-1}$ | |
|---|---|---|---|---|---|
| | | | | (Na-Acetate) | (Citrate) |
| Sudan Savanna | | | | | |
| Natural Savanna 1 | 10°56'N; 01°32'E | 6.1 | 13.9 | 47.6 | 69.9 |
| Natural Savanna 2 | 10°17'N; 01°20'E | 6.3 | 10.4 | 11.8 | 17.5 |
| Yam field 1 | 10°08'N; 01°51'E | 5.9 | 11.6 | 3.9 | 8.7 |
| Northern Guinea Savanna | | | | | |
| Natural Savanna 3 | 08°43'N; 02°40'E | 6.6 | 9.3 | 8.7 | 8.7 |
| Natural Savanna 4 | 09°11'N; 02°12'E | 6.5 | 28.4 | 13.1 | 21.8 |
| Natural Savanna 5 | 09°03'N; 02°04'E | 6.7 | 36.0 | 46.3 | 65.5 |
| Yam field 2 | 08°55'N;02°33'E | 5.8 | 11.0 | 7.0 | 8.7 |
| Southern Guinea Savanna | | | | | |
| Natural Savanna 6 | 07°46'N; 02°28'E | 6.7 | 24.9 | 52.8 | 83.0 |
| Natural Savanna 7 | 07°57'N; 02°26'E | 7.2 | 44.1 | 27.3 | 37.6 |
| Natural Savanna 8 | 08°20'N; 01°51'E | 6.5 | 20.3 | 28.8 | 34.9 |
| Yam field 3 | 07°49'N; 02°15'E | 6.1 | 9.3 | 8.7 | 8.7 |
| Yam field 4 | 07°55'N; 02°11'E | 6.7 | 16.8 | 10.9 | 13.1 |

The inner spore wall layer (OWL3) is concolorous with OWL2, 0.8-1.2 µm thick, usually tightly adherent and especially difficult to observe when < 1.0 µm. None of these wall layers stains in Melzer's reagent.

**Middle wall** is hyaline, bi-layered and rather thin; in total 1.2-2.0 µm; both layers (MWL1 and MWL2) are semi-flexible (Fig. 9), tightly adherent to each other and consequently often appears as a single wall layer (Fig. 8, 10). None of the layers reacts to Melzer's reagent.

**Inner wall** is hyaline, with three layers (IWL1-3) that are 2.5-4.0 µm thick in total (Fig. 8-10). The IWL1 is 1.0-1.5 µm thick, and has a 'beaded', granular structure; IWL2 is 1.1-2.0 µm thick; IWL3 is ~ 0.5 µm thick and quite difficult to detect due its close adherence to IWL2. Only IWL2 shows a light, pale pink reaction to Melzer's reagent (Fig. 6), which is usually visible in crushed spores only and not observed in all specimens.

**Cicatrix** remains after detachment of the connecting hypha (Fig. 4, 9) and is 15-25 µm wide. The pore is closed by some of the inner laminae of OWL2 and by OWL3.

**Etymology:** Latin: *minuta*, referring to the small, shallow pits on the outer surface of the second layer of the outer wall.

**Specimen examined:** BENIN. Holotype and isotypes deposited at Z+ZT, deriving from a pure culture of the fungus: Paratypes from additional pure cultures also obtained from the type location and paratypes from pure cultures obtained from a second location in the Southern Guinea savanna of Benin (see above) deposited at Z+ZT and at URM.

**Commentary:** The new fungus has, to date, been recorded only from Benin, sub-Saharan West Africa, in seven natural savanna sites and in the rhizosphere of yam (*Dioscorea* spp.) at four yam production sites cultivated in the first year after savanna clearance (Tab. 1). During sampling, it was not detected in any field cultivated by any other of the traditional crops in any of the subsequent crop rotations following yam production (Tchabi et al., 2008, 2009a), nor in intensively managed cotton fields or in the long-term (> 7 years) fallow sites, following several years of agricultural production. In the greenhouse, *A. minuta* was successfully propagated on a series of native and exotic plants (*D. cayenensis*, *D. rotundata*, *S. bicolor* and *H. pilosella*) (Tchabi et al., 2009a, 2010).

**Molecular analyses:** For the phylogenetic analyses, sequences obtained from DNA extracts of spores, and comprising the ITS1, the 5.8S rDNA subunit and ITS2 regions of the ribosomal gene of about 550 bp length were considered. Phylogenetic analyses firmly placed the four sequences of *A. minuta* into the genus *Acaulospora* adjacent to, but clearly separated from sequences of two *A. scrobiculata* isolates originating from Benin and Brazil (Oehl et al., 2011).

## Discussion

This new AM fungus can readily be distinguished from all other AM fungi in the Acaulosporaceae due to the spore formation laterally on sporiferous saccules and also by spore size, color and the diagnostic minute pits on the outer spore surface. *Acaulospora* spp. with the most similar spore characteristics include *A. scrobiculata* (Trappe, 1977) and *A. sieverdingii* Oehl et al., 2011 (Oehl et al., 2011). However, *A. scrobiculata* forms subhyaline to olive creamy spores that have larger, more irregular and deeper pits than those of *A. minuta*, while *A. sieverdingii* forms substantially smaller, hyaline to subhyaline spores, with an ornamentation that is also more irregular than that of *A. minuta*.

Other *Acaulospora* spp. with similar pitted ornamentation on the spore surface or similar spore size or spore color are *A. alpina* Oehl et al., *A. cavernata* Błaszk., *A. excavata* Ingleby and C. Walker, and *A. foveata* Trappe and Janos (Oehl et al., 2006; Błaszkowski et al., 1989; Ingleby et al., 1994; Janos and Trappe, 1982). However, the yellow to orange yellow spores of *A. alpina* are substantially smaller and have deeper, conical pits, while the pits of creamy-spored *A. cavernata*, yellow-spored *A. excavata* and (light reddish-)brown-spored *A. foveata* are at least twice to four to eight times larger and two to four times deeper than the pits of the new fungus (Oehl et al., 2006, 2011). The molecular phylogenetic

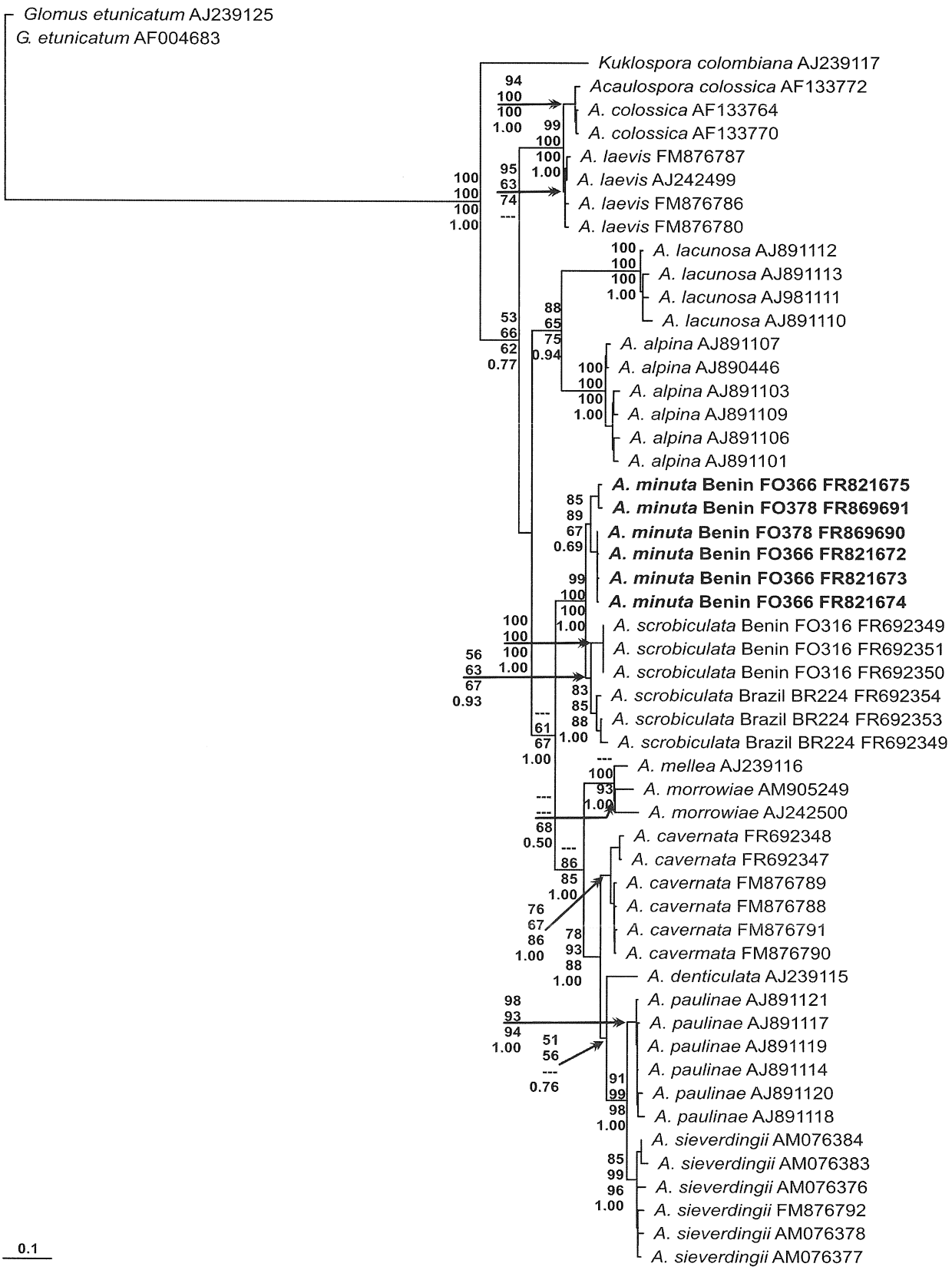


**Fig. 11:** Phylogenetic tree of the Acaulosporaceae obtained by Maximum Likelihood analysis from ITS1, 5.8S rDNA and ITS2 sequences of different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses, respectively. The tree was rooted by *Glomus etunicatum*. Sequences of *A. minuta* are shown in boldface. Only topologies with bootstrap values of at least 50% are shown. (Consistency Index = 0.64; Retention Index = 0.87). Note: *Kuklospora colombiana* (sensu Sieverding and Oehl 2006) is the former *Entrophospora colombiana* (SCHENCK et al., 1984). The *A. cavernata* isolate had originally been deposited in the European Bank of Glomeromycota under the accession number BEG33 as *A. scrobiculata* (OEHL et al., 2011).

fungus in a separate clade adjacent to two isolates identified as *A. scrobiculata* (OEHL et al., 2011).

Remarkably, while *A. scrobiculata* has a wide tropical distribution across the globe (e.g. SOUZA et al., 2011; PÉREZ-CAMACHO and OEHL, unpublished results from Bolivia) and has been frequently recovered from traditional and even intensively cultivated agro-ecosystems in the sub-Saharan savannas of Benin, *A. minuta* was not detected from sites in any of the agro-ecosystems under study after the first year of savanna or forest clearance (TCHABI et al., 2008). As with *Racocetra beninensis* (TCHABI et al., 2009b), *A. minuta* was neither recovered from soil samples taken from sites following 7 years of regenerative fallows, in transition to secondary forests. This finding indicates the vulnerability of both fungi to ecosystem disturbance, and their sensitivity even to traditional West African low-input agro-ecosystems.

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