



***Acaulospora viridis*, a new species in the Glomeromycetes from two mountain ranges in Andalucía (Spain)**

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With 14 figures and 1 table

Abstract: A new *Acaulospora* species was found in two adjacent mountain ranges in Andalucía (southern Spain), i.e. in Sierra Nevada National Park at 1270–3200 m asl around roots of *Artemisia umbelliformis*, *Sorbus hybrida*, *Laserpitium longiradium* among others, and in a shrubland and grassland at 1855–2028 m asl in the Sierra de Baza Natural Park. The fungus produced spores in single species cultures, using *Sorghum vulgare* or *Trifolium pratense* as bait plant. The new species was named *A. viridis* because of its greenish appearance under the dissecting and in the compound microscope. The spores have a smooth surface and are similar in size to several other *Acaulospora* species, such as *A. koskei*, *A. laevis*, *A. capsicula*, *A. colliculosa* and *A. entreriana*. However, the new species can easily be distinguished from all these fungi by the diagnostic staining of the middle wall in Melzer's reagent. Phylogenetic analyses of sequences obtained from the ITS and partial LSU of the ribosomal genes confirm the new species in a clearly separate clade within the Acaulosporaceae.

Key words: arbuscular mycorrhizal fungi, biodiversity, Glomeromycetes, mountainous grasslands, phylogeny.

Introduction

Arbuscular mycorrhizal (AM) fungi belong to the Glomeromycota. They are widespread soil microorganisms able to establish mutualistic associations with most terrestrial plants, and to increase plant fitness and soil quality (Smith & Read 2008). Interest in the study of AM fungal diversity has been stimulated by the evidences about the essential roles played by these plant symbionts in ecosystems (Jeffries & Barea 2012).

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One of the most studied families in the Glomeromycota are the Acaulosporaceae, currently belonging to the order Diversisporales (Oehl et al. 2011b). Members of this family have often been found in acidic soils and in mountainous grasslands environments (Castillo et al. 2006, Oehl et al. 2011a, 2011c, 2012, Veresoglou et al. 2013). In recent years, several species have been described in this family having spores with surface ornamentation (e.g. Błaszowski 2012, Furrázola et al. 2013, Palenzuela et al. 2013), while new species with smooth surfaces (e.g. Schenck et al. 1984, Błaszowski 1988, Błaszowski 1995) have rarely been reported in the last decade (Velázquez et al. 2008).

We found a new *Acaulospora* species with smooth surfaces in seven sites of two adjacent mountains ranges in Andalucía (southern Spain), around roots of endangered plants species like *Artemisia umbelliformis* in the Sierra Nevada National Park, and in a Mediterranean shrubland and a mountainous grassland of Sierra de Baza Natural Park. This AM fungus was cultivated as single species cultures on *Sorghum vulgare* L. and subsequently analyzed morphologically and molecularly as described in Palenzuela et al. (2013). The paper presents its morphological features and its phylogenetic position based on ribosomal gene sequences.

Material and Methods

STUDY SITES AND STUDY PLANTS: Soil samples were taken from several locations at two adjacent mountains ranges in Andalucía, Sierra Nevada and Sierra de Baza, as indicated in Table 1.

PLANT AND SOIL SAMPLING: Five intact plant individuals and the soil attached to the roots were collected at the study sites to establish AM fungal pot cultures. In addition, three samples of the soil surrounding the roots of each plant (0.5–1 kg) were taken with a shovel from a depth of 5–25 cm; they were thoroughly mixed to prepare a composite soil sample per individual plant. These samples were used to isolate AM fungal spores, and to determine soil pH (in a 1:2.5 w/v aqueous solution).

AM FUNGAL POT CULTURES: To cultivate AM fungi, trap cultures were established with the collected native plants in 1500 mL pots (cylindric, 12 cm diam.) filled with the soil originally obtained from around the plant roots in the field. The pots were irrigated three times per week and fertilized every 4 weeks with Long-Aston nutrient solution (Hewitt 1966). The cultures were maintained in the greenhouse of the Estación Experimental del Zaidín (Granada) for more than three years. Single species cultures of the new fungus were first established with *Sorghum vulgare* L. in 350 mL pots as described in Palenzuela et al. (2010). Spores isolated from the trap cultures were stratified during 2 weeks at 4°C before inoculation.

MORPHOLOGICAL ANALYSES: AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The spore morphological characteristics and their subcellular structures were described from specimens mounted in: (i) polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske & Tessier 1983); (ii) a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994); (iii) a mixture of lactic acid and water (1:1); (iv) Melzer's reagent; and (v) water (Spain 1990). The spore structure terminology follows Palenzuela et al. (2011) and Oehl et al. (2011b, 2012) for species with acaulosporoid spore formation. Photographs (Figs 1–12) were taken with a high-definition digital camera (Nikon DS-Fi1) on a compound microscope (Nikon eclipse 50i) or with a Leica DFC 290 digital camera on a Leitz Laborlux S compound microscope using Leica Application Suite Version V 2.5.0 R1 software. Specimens mounted in PVLG and PVLG+Melzer's reagent were deposited at the herbaria Z+ZT (ETH Zurich, Switzerland) and GDA-GDAC (University of Granada, Spain).

MOLECULAR ANALYSES: Five spores were isolated from a pot culture of a single fungal species culture originating from *Artemisia umbelliformis*. They were surface-sterilized with chloramine T (2%) and

Table 1. Geographical data, sampling time and selected soil characteristics at isolation sites of *Acaulospora viridis*.

Plant species At mountain range	Study site	Altitude (m asl)	Latitude	Longitude	Sampling time	Soil type	pH (H ₂ O)
Sierra Nevada							
<i>Artemisia umbelliformis</i> Lam.	Goterón icefalls	2912	37°04'N	3°16'W	October 2006	District Regosol	6.2
<i>Papaver lapeyrouisianum</i> ex Greuter & Burdet	West side of the Mulhacén pick	3200	37°03'N	3°18'W	July 2007	District Regosol	6.6
<i>Sorbus hybrida</i> L.	Upper part of the San Juan ravine	1896	37°07'N	3°22'W	June 2007	Haplic Regosol	6.0
<i>Sorbus torminalis</i> (L.) Crantz	La Estrella path	1270	37°07'N	3°22'W	May 2007	Eutric Regosol	7.1
<i>Laserpitium longiradium</i> Boiss.	S. Jerónimo dehesa	1580	37°06'N	3°26'W	July 2007	Calcaric Cambisol	7.2
<i>Erodium daucooides</i> Boiss.	Prado Redondo ravine	2170	37°07'N	3°25'W	July 2008	Calcaric Cambisol	7.3
Sierra de Baza							
<i>Rosa pouzinzii</i> Tratt.							
<i>Berberis hispanica</i> Boiss. & Reut.							
<i>Prunus ramburii</i> Boiss.	Spiny mediterranean shrubland	1855	37°20'N	2°49'W	March 2005	Calcaric Regosol	8.1
<i>Crataegus monogyna</i> Jacq.							
<i>Festuca iberica</i>							
<i>Trifolium repens</i> subsp. <i>nevadense</i> (Boiss.) Coombe	Mountainous grassland "Prados del rey"	2028	37°22'N	2°51'W	March 2008	Chromic Cambisol	7.7

Soil types were classified according to the World Reference Base for soil resources (FAO 2006).

streptomycin (0.02%) (Mosse 1962) and together crushed with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol et al. 2004). PCRs of the crude extracts were obtained 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 amplified the SSU end, ITS1, 5.8S, ITS2 and partial LSU rDNA fragment using the SSUmAf/LSUmAr and SSUmCf/LSUmBr primers consecutively (Krüger et al. 2009). PCR products were analyzed by electrophoresis in 1.2% agarose gels stained with Gel Red™ (Biotium Inc., Hayward, CA, U.S.A.) and viewed by UV illumination. The expected amplicons were purified using the GFX PCR DNA kit and Gel Band Purification Illustra, cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA, USA), and transformed into One shot® TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells, the 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 (Altschul et al. 1990). The percentage of identity among the *Acaulospora* sequences was calculated using the BLASTn analysis. The new sequences were deposited in the EMBL database under the accession numbers HG421736-HG421738.

PHYLOGENETIC ANALYSES: The phylogeny was reconstructed by independent analyses of the ITS region and partial LSU rDNA. The AM fungal sequences obtained were aligned with other Acaulosporaceae sequences from GenBank in ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999). *Claroideoglossum etunicatum* (W.N.Becker & Gerd.) C.Walker & A.Schüssler 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 2×10^6 generations with a burn in value of 2500) and maximum likelihood (1000 bootstrap) analyses were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), respectively, launched from Topali 2.5, using the GTR + G model. Neighbor-joining and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

Results

***Acaulospora viridis* Palenz., Oehl, Azcón-Aguilar & G.A.Silva sp. nov.** Figs 1–12

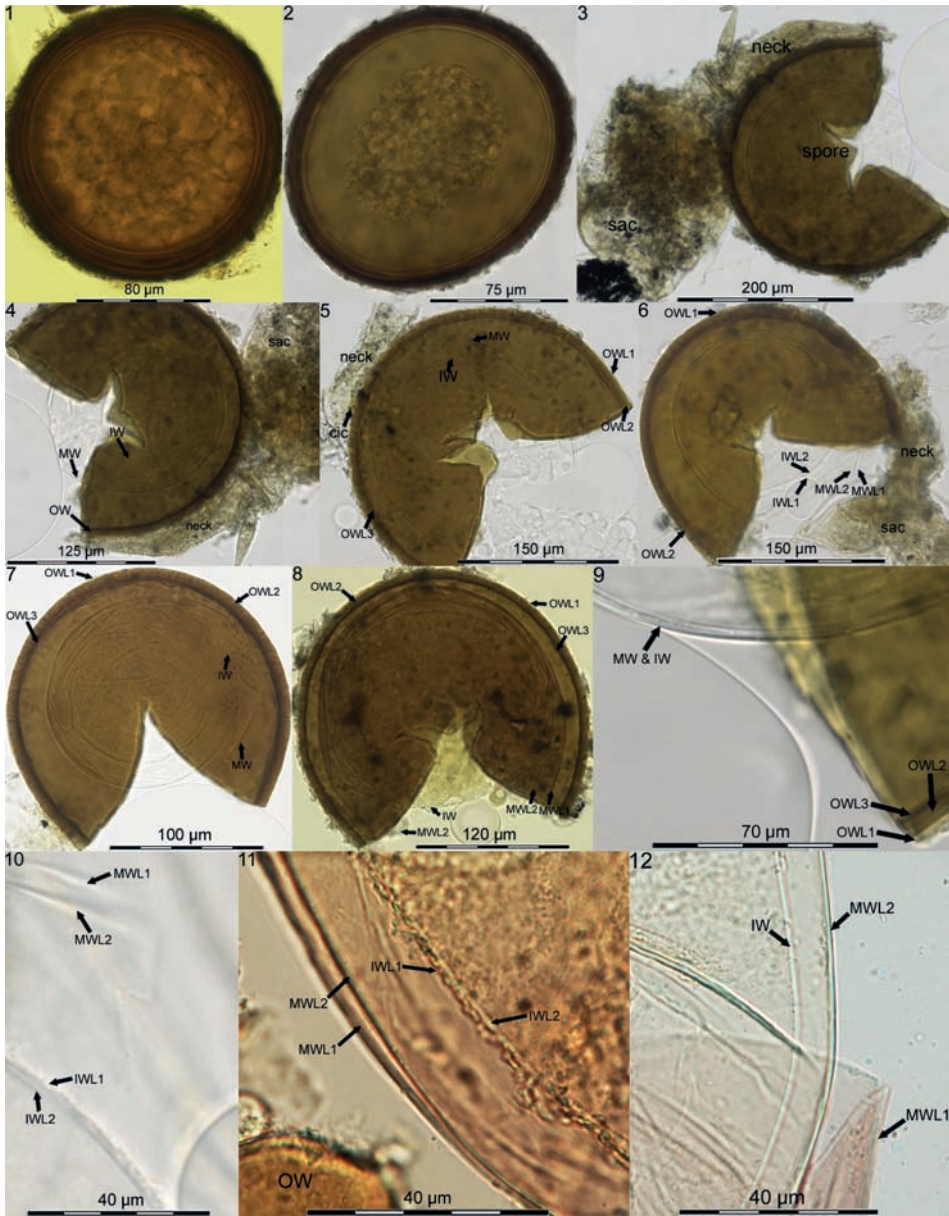
Mycobank MB 804883

ETYMOLOGY: Latin, *viridis*, referring to the greenish color of spores above all when observed in water under the dissecting microscope.

Sporae singulae lateraliter formatae ad sacculum terminalem, viride-fuscae at viride-flavo-fuscae, globosae vel subglobosae vel ovoideae, 140–205 × 140–193 µm in diametro. Tunica media pallide purpurea ad purpurea in Melzeri colorans. Holotypus # 40–4001: Z+ZT (ZT Myc 42872).

HOLOTYPE: Spain, Andalucía, Granada, from single species pot culture generated at the Estación Experimental del Zaidín, on *Sorghum vulgare* (collector J.Palenzuela). Culture was initially inoculated with spores isolated from pot trap cultures of *Artemisia umbelliformis* grown in the original soil substrate deriving from Sierra Nevada (37°04'N; 3°16'W; 2912 m asl). Specimens were deposited at Z + ZT (common mycological herbarium of the University and ETH of Zurich, Switzerland; ZT Myc 42872). Isotypes of the pot culture were deposited at Z+ZT (ZT Myc 42873) and GDA-GDAC (herbarium of the University of Granada, Spain). Paratypes (deposited at Z+ZT and GDA-GDAC) were isolated from rhizospheric soils and from trap cultures of other sites in Sierra Nevada and Sierra de Baza (1855–3200 m asl) (Table 1).

DESCRIPTION: Sporiferous saccules are hyaline and singly formed at the end of mycelial hypha. The saccules are globose to subglobose (145–190 × 140–185 µm), with 1–2 wall layers that are in total 1.5–2.8 µm thick. The saccule neck is 27–51 µm broad at the saccule terminus, about 20–34 µm at the point of spore formation, and tapers to 7–12 µm



Figs 1–12. *Acaulospora viridis*. Figs 1–2. Uncrushed spores, sporiferous saccules detached from the spores. Figs 3–6. Crushed spores with three walls (OWL1-3, MWL1-2, IWL1-2) and degrading saccule necks and saccule terminus (sac) attached. Figs 7–9. Triple-walled, crushed spores; middle wall staining purple in Melzer's reagent (Fig. 8). Figs 10–12. Bi-layered middle and inner walls; MWL1 staining pink to purple in Melzer's (Figs 11–12).

in 120–160 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 (Figs 1–8) form laterally on the neck of sporiferous saccules in distance of 50–120 μm from the saccule terminus. They are globose to subglobose, 140–205 \times 140–193 μm in diameter, and greenish yellow brown to greenish brown to rarely brown, showing a characteristic greenish tint in particular on younger spores under the dissecting microscope in water. The spores have three walls of which the middle react with the Melzer's reagent.

Outer spore wall consists of three layers (OWL1–OWL3) and is in total 6.9–12.1 μm thick (Figs 1–9). Outer layer (OWL1) is subhyaline to pale brown, 1.5–2.6 μm thick, evanescent. Second layer (OWL2) is greenish yellow brown to greenish brown, 4.9–8.5 μm thick. The inner layer of the outer wall (OWL3) is concolorous with OWL2, about 0.5–1.0 μm thick and often difficult to observe, especially when the middle wall does not separate readily from OW.

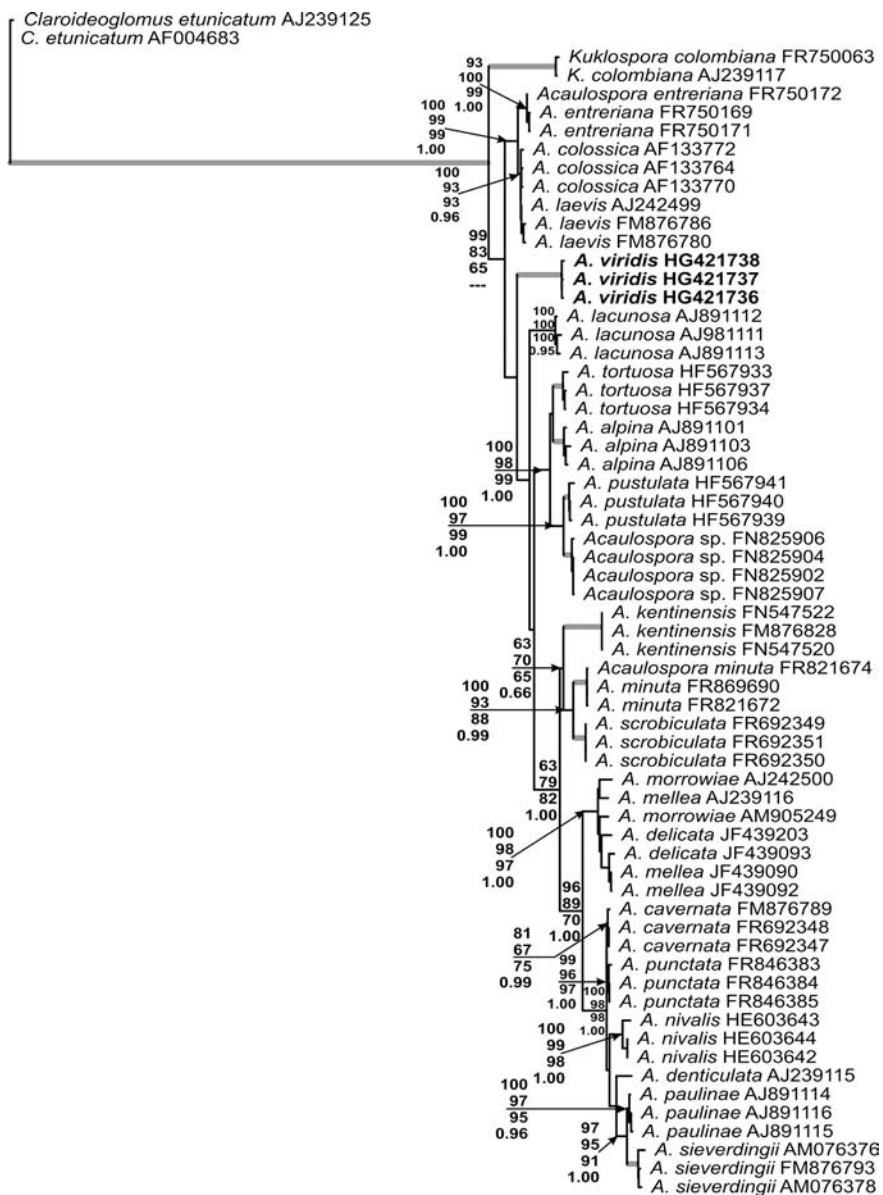
Middle wall is hyaline, bi-layered and thin; 1.4–3.5 μm thick in total (Figs 4–12). Both layers (MWL1 and MWL2) are semi-flexible, tightly adherent to each other and thus, often appear as being only one wall layer. MWL1 stains light purple to purple in Melzer's reagent, while MWL2 regularly does not stain.

Inner wall is hyaline, with two to three layers (IWL1–IWL 3) that are 1.9–3.5 μm thick in total (Figs 4–12). The IWL1 is about 0.8–1.3 μm thick. A 'beaded', granular structure, is evident in water but rarely seen in lactic acid based mountants after a few months of permanent fixing. IWL2 is 0.8–1.6 μm thick and infrequently stains pinkish in Melzer's reagent. IWL3 is very thin (<0.6 μm) and usually very difficult to detect due to the close adherence to IWL2.

Cicatrix (Fig. 5) remains after detachment of the connecting hypha, 10–18(–25) μm wide. The pore is closed by inner lamina of OWL2 and by OWL3.

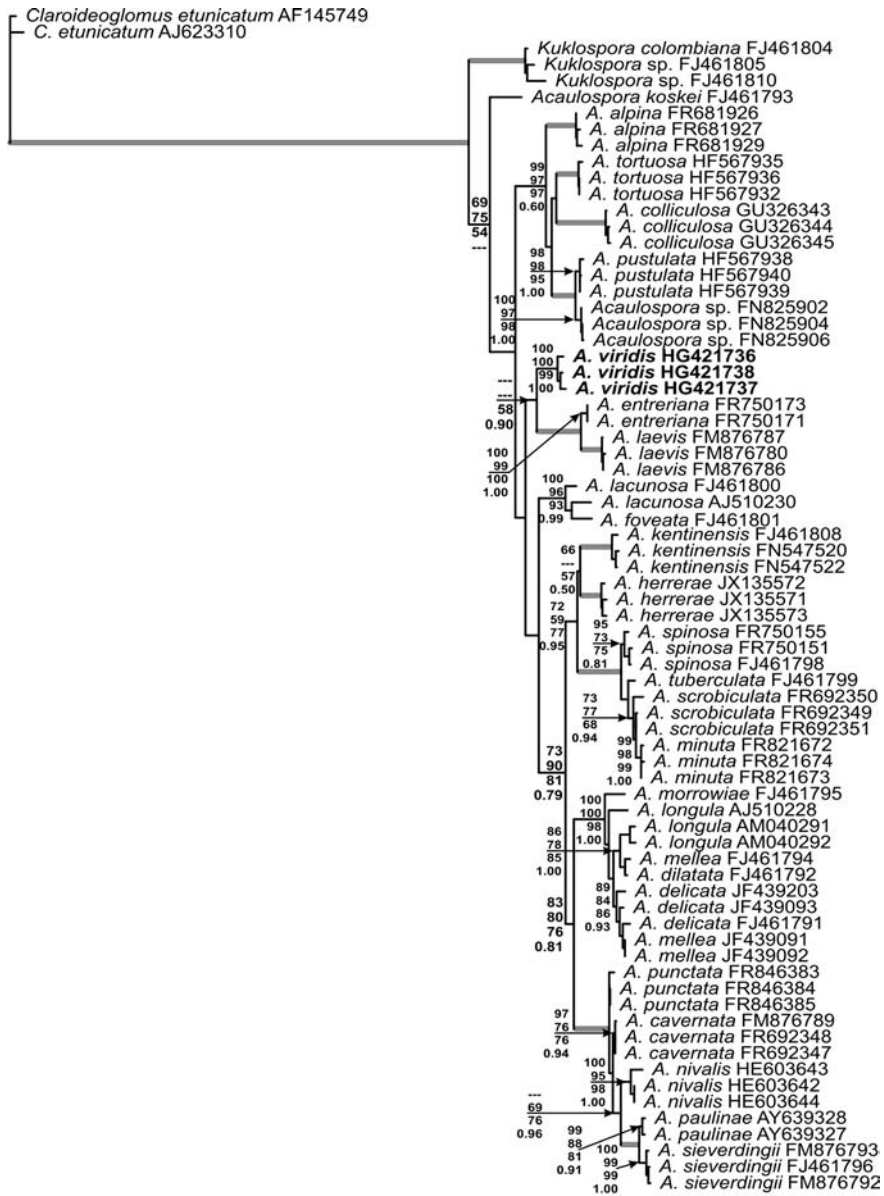
MYCORRHIZA FORMATION: forming vesicular-arbuscular mycorrhizal associations with *Sorghum vulgare* L. and *Trifolium pratense* L. as host plant host in pot cultures. The mycorrhizal structures consisted of arbuscules, vesicles, and intra- and extraradical hyphae staining dark blue in 0.05% trypan blue.

MOLECULAR ANALYSES: The phylogenetic analysis from ITS rDNA sequences places the new fungus in a well separated clade between two major clades of the Acaulosporaceae (Fig. 13). The tree obtained with the partial sequences of the LSU rDNA positions the new fungus also in a well separate clade next to *A. laevis* and *A. entreriana* (Fig. 14), with 92% and 91% of identity, respectively. For the ITS region, the closest species related to *A. viridis* were *Acaulospora entreriana*, *A. colossica* and *A. laevis* with 89%, 88% and 88% of identity, respectively. The intraspecific variations between the different clones of *A. viridis* were about 1–2% for the ITS and LSU rDNA sequences. The environmental LSU rDNA sequence with closest match (98%) to *A. viridis* was found in grass roots (KC515563) sampled in the Tibetan Plateau, Mount Sijilaa (China). No environmental ITS sequences deposited in the GenBank corresponded to the new fungus in the BLASTn analysis.



0.1

Fig. 13. Phylogenetic tree of the Acaulosporaceae obtained by analysis from ITS1, 5.8S rDNA and ITS2 sequences. Sequences are labeled with their database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and bayesian analyses, respectively. Sequences obtained in this study are in boldface. Only support values of at least 50% are shown. Thick branches in grey represent clades with 100% of support in all analyses. The tree was rooted by *Claroideoglossum etunicatum*. (Consistency Index = 0.55; Retention Index = 0.86).



0.1

Fig. 14. Phylogenetic tree of the Acaulosporaceae obtained by analysis from partial LSU rDNA sequences of different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and bayesian analyses, respectively. Sequences obtained in this study are in boldface. Only support values of at least 50% are shown. Thick branches in grey represent clades with 100% of support in all analyses. The tree was rooted by *Claroideoglomus etunicatum*. (Consistency Index = 0.50; Retention Index = 0.84).

DISTRIBUTION of *A. viridis*: The new fungus was found in field soil samples from seven sites in two mountain ranges, i.e. i) in Sierra Nevada National Park between 1270 and 3200 m asl, growing in the rhizosphere of endangered plants species such as *Artemisia umbelliformis*, *Sorbus torminalis* and *Papaver lapeyrousianum*, of critically endangered plant species as *S. hybrida* and *Laserpitium longiradium*, and a vulnerable plant species, *Erodium daucoides*, and ii) in Sierra de Baza Natural Park, at 1855 and 2028 m asl, in the rhizosphere of shrubland plant species like *Berberis hispanica*, *Prunus ramburii*, *Rosa pouzinii* and *Crataegus monogyna* as well as in a mountainous grassland composed of plant species such as *Festuca iberica* and *Trifolium repens* subsp. *nevadense* (among others Table 1).

In Sierra Nevada, the fungus co-occurred with the following AMF species: *Acaulospora laevis*, *Ambispora gerdemannii*, *Claroideoglopus etunicatum*, *Diversispora versiformis*, *Funneliformis caledonius*, *Glomus intraradices*, *Gl. rubiforme* and *Pacispora scintillans*. In Sierra de Baza *Funneliformis mosseae*, *Septoglopus constrictum*, *Gl. intraradices*, *Entrophospora infrequens*, *Scutellospora calospora* and an undescribed *Acaulospora* sp. were the most frequent AMF species accompanying the new fungus at their habitats.

SPECIMEN EXAMINED: Holotype, isotype and paratype specimen, deposited at Z+ZT and GDA-GDAC, and in private collections of J.Palenzuela and F.Oehl.

Discussion

Acaulospora viridis can easily be distinguished from all other *Acaulospora* species, by i) the regularly greenish brown appearance of the spores above all in water, and in PVLG, ii) the pinkish to purple staining feature of the middle wall when exposed to Melzer's reagent, and iii) by its characteristic phylogenetic clade. Of all *Acaulospora* species, only *A. koskei* has a comparable staining reaction in Melzer's reagent (Błaszowski 1995), but this staining is detected on the innermost layer of the outer wall and on the inner wall, while in *A. viridis* the main staining is on the middle wall, but rather weak and infrequently found on the inner wall, and absent on the outer wall. Phylogenetically, *A. viridis* is clearly separate from *A. koskei*.

Without use of Melzer's reagent, spores of *A. viridis* may be confused with spores of *A. laevis*, *A. capsicula*, *A. colliculosa*, *A. entreriana*, *A. koskei* and *A. thomii*, as spores of all these species are about 120–160 µm and, with some divergencies, of a yellow-brownish, or reddish-brown to brown color (Gerdemann & Trappe 1974, Błaszowski et al. 1990, 1995, Schultz et al. 1999, Velasquez et al. 2008). *Acaulospora laevis* forms honey-colored spores (Mosse 1970, Gerdemann & Trappe 1974), *A. capsicula* capsicum-like reddish-brown spores (Błaszowski et al. 1990), while *A. viridis* spores a characteristic greenish tint, and such colour has not been observed for other *Acaulospora* species so far (Oehl et al. 2012). If doubts appear about the identification of those *Acaulospora* spp. it is recommended to measure, besides spore size, the spore walls and wall layers dimensions, and above all to expose some spores to Melzer's reagent to check for the diagnostic feature of *A. viridis*. Finally, *A. thomii* has a persistent, thick hyaline outermost spore wall layer (Błaszowski 1988), while the outermost layer is substantially thinner and evanescent to semi-persistent in *A. viridis*.

The apparently overlapping spore morphological characteristics of the mentioned *Acaulospora* spp. might suggest that some of them have sometimes been misidentified in the past. On the other hand, the almost total absence of environmental sequences for *A. viridis* in public data bases may suggest that the new species is not widely distributed. In Andalucía, however, it was detected in two adjacent mountain ranges on a broad range of soil types, from Dystric Regosols to Calcaric Cambisols and on a rather large gradient of elevation above sea level (1270–3200 m), and in rhizospheric soils of plants belonging to several quite different plant families. The new fungus might play an important role for the survival of endangered host plants in Sierra Nevada and Sierra de Baza, especially in the neutral to slightly alkaline soils where most of the other *Acaulospora* species are regularly absent.

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