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Acaulospora pustulata and *Acaulospora tortuosa*, two new species in the Glomeromycota from Sierra Nevada National Park (southern Spain)

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With 24 figures

Abstract: Two new *Acaulospora* species were found in two wet mountainous grassland ecosystems of Sierra Nevada National Park (Spain), living in the rhizosphere of two endangered plants, *Ophioglossum vulgatum* and *Narcissus nevadensis*, which co-occurred with other plants like *Holcus lanatus*, *Trifolium repens*, *Mentha suaveolens* and *Carum verticillatum*, in soils affected by ground water flow. The two fungi produced spores in pot cultures, using *O. vulgatum*, *N. nevadensis*, *H. lanatus* and *T. repens* as bait plants. *Acaulospora pustulata* has a pustulate spore ornamentation similar to that of *Diversispora pustulata*, while *A. tortuosa* has surface projections that resemble innumerous hyphae-like structures that are more rudimentary than the hyphae-like structures known for spores of *Sacculospora baltica* or *Glomus tortuosum*. Phylogenetic analyses of sequences of the ITS and partial LSU of the ribosomal genes reveal that both fungi are new species within the Acaulosporaceae. They are most closely related to *A. alpina* and undescribed *Acaulospora* species. With 45–72 µm spore size, *Acaulospora pustulata* is the smallest *Acaulospora* species known so far, while *A. tortuosa* has slightly larger spores (61–84(–94) µm), which is in the range known for several other *Acaulospora* species like *A. longula*, *A. alpina*, *A. nivalis* and *A. sieverdingii* that have either smooth or pitted spore surfaces. These two fungi might play an important role in helping their endangered hosts *O. vulgatum and N. nevadensis* to survive under the stressed environments of the high mountains of Sierra Nevada.

Key words: Glomeromycetes, alpine grasslands, biodiversity, conservation, phylogeny, arbuscular mycorrhizal fungi, soil type.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are known to contribute to maintain the productivity and plant species diversity of grasslands (van der Heijden et al. 1998). Particularly, they may play a major role in the survival of critically endangered or endemic plants (Fuchs & Haselwandter 2004, Bothe et al. 2010, Barea et al. 2011). Nevertheless, AMF diversity studies are scarce in mountainous and alpine grasslands (Castillo et al. 2006, Oehl et al. 2011a, Turrini et al. 2012, Azcón-Aguilar et al. 2012), so their actual significance in such ecosystems need to be investigated.

Sierra Nevada is the second highest mountain range in Western Europe being included in the Baetic Cordillera, located in southwestern Europe (Andalucía, provinces of Granada and Almería, Spain) and being one of the 'hot-spots' of European plant diversity (Blanca et al. 2002). In recent years, it has been increasingly shown that the high plant diversity of Andalucía is accompanied by an astonishingly high diversity of AMF, with several species that so far have never (e.g. Palenzuela et al. 2008, 2011, Estrada et al. 2012), or only rarely (Palenzuela et al. 2010, Błaszkowski pers. communication, Cabello, pers. communication) been reported from other regions around the globe.

At two of 27 sites recently investigated in Sierra Nevada, two un-described *Acaulospora* species were detected that form relatively small spores for members of Glomeromycota. The two fungi have distinct pustulate or hyphae-like structures on their spore surfaces and could readily be separated from the AMF communities in the soils and trap cultures where they occurred. The aim of the present study was to investigate the two new species through concomitant analyses on spore morphology and on molecular phylogeny. In the molecular analyses, sequences of the ITS region and partial LSU of the ribosomal genes were generated and used to determine the phylogenetic relationships with other AMF.

The two fungi were found in two soils affected by ground water flow (so-called Gleysols and Histosols, respectively, according to the World Reference Base for Soil Resources, FAO 2006) of wet mountainous grasslands in Sierra Nevada (about 2000 m asl), in the rhizosphere of two critically endangered species, the fern *Ophioglossum vulgatum* and the endemic plant *Narcissus nevadensis*. Because of the ecological singularity of the habitats where both fungi were found and the threatened character of the plants living closely associated to these fungi, it seems reasonable to believe that they might play an important role in helping their endangered hosts to survive under the stressed environments where they develop. Therefore, their isolation, multiplication and characterization are steps forward to get further insights into the functional role of these AMF in alpine ecosystems as those of the high mountains of Sierra Nevada National Park.

Material and methods

Study sites and study plants: At 27 sites of the Sierra Nevada National Park (Spain), the mycorrhizal status of in total 34 higher plant and fern species and the AMF present as spores in the surrounding soils were investigated (Palenzuela et al. 2010, Azcón-Aguilar et al. 2012). These higher plant and fern species are classified either endemic to the Sierra Nevada or threatened with extinction in the region, according to the Red List of Endangered Plant Species of Andalucía (Blanca et al. 1999, 2000)

and the compilation of threatened and endemic flora of Sierra Nevada (Blanca et al. 2002). The new fungi hereafter described were found at two wet mountainous grassland sites where the higher plant *Narcissus nevadensis* L. or the fern *Ophioglossum vulgatum* L. (both endangered) grew, respectively.

Plant and soil sampling: For both *Narcissus nevadensis* and *Ophioglossum vulgatum*, five intact plant individuals and the soil attached to the roots were collected at the sampling sites in July 2007 to immediately 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 and thoroughly mixed to prepare a composite soil sample per individual plant. These samples were used to isolate AM fungal spores, to establish a second set of trap cultures using common plants like *Trifolium pratense* and *Holcus lanatus* as hosts, and to determine the soil pH (in a 1/2.5 w/v aqueous solution) and the content of organic carbon (according to Yeomans & Bremmer 1989).

AM fungal pot cultures: To cultivate AM fungi from Sierra Nevada, trap cultures were established with the collected native plants in cylindrical 1500 mL pots (12 cm diam.) filled with the soil originally around the plants 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 have been maintained in the greenhouse of the Estación Experimental del Zaidín (Granada) for more than 3 years. Single species cultures of the two new fungi were established with *Trifolium pratense* and *Sorghum vulgare* in 350 mL pots as described in Palenzuela et al. (2010) by adding 20 spores isolated from the trap cultures. Spores isolated from the trap cultures were stratified during 2 weeks at 4°C before inoculation. Despite several attempts since 2008, single species culturing of the new fungi has so far not been successful.

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 Oehl et al. (2011b, 2012) for species with acaulosporoid spore formation. Photographs (Figs 1–22) 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 the PVLG+Melzer's reagent were deposited at the herbaria Z+ZT (ETH Zurich, Switzerland), GDA-GDAC (University of Granada, Spain), and URM (Federal University of Pernambuco, Recife).

Molecular analyses: Five spores were isolated per species from the trap cultures inoculated with soils from the two wet grassland sites in Sierra Nevada. They were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) (Mosse 1962) and crushed together with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol et al. 2004). PCRs of the crude extract was 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 a 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 PCR2.1 vector (Invitrogen, Carlsbard, 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 new sequences were deposited in the EMBL database under the accession numbers HF567932-HF567941.

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 glomeromycotan sequences from GenBank in ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999). *Claroideoglomus 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 1×10^6 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 (established with the model cited above) and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

Results

Acaulospora pustulata Palenz., Oehl, Azcón-Aguilar & G.A.Silva **sp. nov.**
Figs 1–10

Mycobank MB 802631

Etymology: Latin, *pustula*, referring to the blister-like structures (i.e. pustules) of the spore surfaces.

Sporae singulae lateraliter formatae ad sacculum terminalem, pallido-fuscae ad flavo-fuscae, globosae vel subglobosae, 45–65(–72) × 44–62(–68) µm in diametro. Tunica exterior pustulibus irregularibus (1.2–)2.4–5.5 µm altis et (1.2–)2.5–5.9(–9.5) µm latis. Holotypus # 47–4701: Z+ZT (ZT Myc 30435).

Holotype: Spain, Andalucía, Granada, Sierra Nevada (37°00'N; 3°22'W, 1980 m asl) on plant species like *Holcus lanatus* and the endangered fern *Ophioglossum vulgatum*, deposited at Z + ZT (common mycological herbarium of the University and ETH of Zurich, Switzerland). Isotypes deposited at Z+ZT (ZT Myc 30436) and GDA-GDAC (herbarium of the University of Granada, Spain). Paratypes (ZT Myc 30437) isolated in another grassland (37°07'N; 3°26'W, 1800 m asl) from rhizospheric soils of the endemic plant species *Narcissus nevadensis* and several other plant species (deposited at Z+ZT, GDA-GDAC and URM).

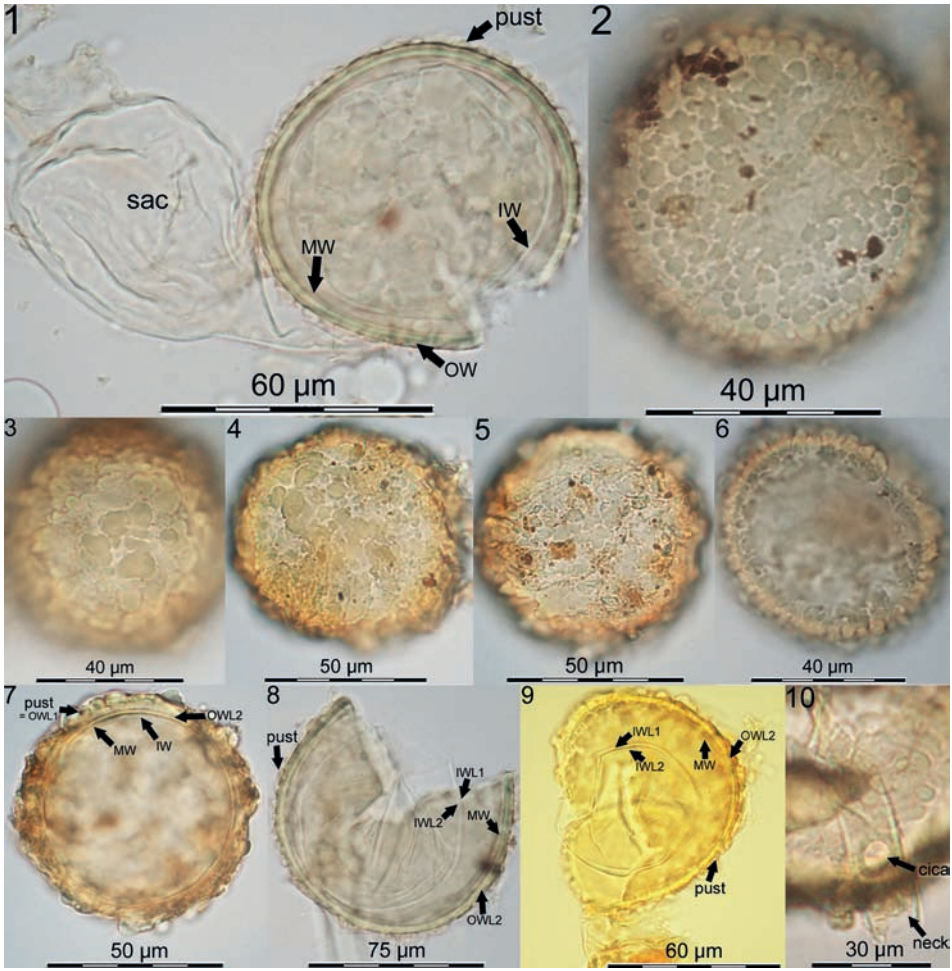
Sporiferous saccules are hyaline and singly formed at the end of mycelial hypha at 20–90 µm distance from the spores that singly arise after saccule formation. The saccule termini are globose to subglobose (48–69 × 48–71 µm), with 1–2 wall layers that are in total 1.1–1.6 µm thick (Fig. 1). The saccule usually collapses after the spore wall has formed and usually is detached from mature spores in soils.

Spores (Figs 1–7) form laterally on the neck of sporiferous saccules. They are globose, to subglobose, 45–65(–72) × 44–62(–68) µm in diameter. They are pale brown when young, becoming yellow brown with age. They have three walls whose layers do not react with the Melzer's reagent.

Outer spore wall consists of three layers (OWL1–OWL3) and is in total 3.5–5.8 µm thick (Figs 1–9). Outer layer (OWL1) is subhyaline to pale brown, 0.5–1.0 µm thick, densely crowded with pustulate projections that are (1.2–)2.4–5.5 µm high and (1.2–)2.5–5.9(–9.5) µm wide at base. Second layer (OWL2) is pale brown becoming yellow brown with age, laminated, 1.5–2.3 µm thick. The inner layer of the outer wall (OWL3) is concolorous with OWL2, about 0.5 µm thick and often hardly to observe, especially when the middle wall does not separate readily from OW.

Middle wall is hyaline, bi-layered and thin; 1.3–2.2 µm thick in total. Both layers (MWL1 and MWL2) are semi-flexible, tightly adherent to each other and thus, regularly appear as being only one wall layer (Figs 7–9).

Inner wall is hyaline (Figs 7–9), with two to three layers (IWL1–IWL 3) that are 1.8–2.8 µm thick in total. The IWL1 is about 0.7–1.2 µm thick. A 'beaded', granular structure,



Figs 1–10. *Acaulospora pustulata*. Fig. 1. Spore formed laterally on the neck of a sporiferous saccule (sac), with three walls (outer, middle, inner; OW, MW, IW) and postulate projections on the spore surface (pust). Figs 2–5. Pustulate ornamentation on increasingly aged spore surfaces in planar view. Fig. 6. Spore with focus on the pustulate ornamentation in almost cross view. Figs 7–8. Uncrushed and crushed spores in cross view with OW (OWL1-OWL2), MW, and bi-layered IW (IWL1-IWL2). Fig. 9. Crushed spore in Melzer's reagent without staining reaction on the walls. Fig. 10. Cicatrix (cica) at the spore base beneath the saccule neck.

is rarely seen in lactic acid based mountants. IWL2 is 0.8–1.5 µm thick. IWL3 is very thin and usually very difficult to detect due to the close adherence to IWL2 (Figs 7–9).

Cicatrix (Fig. 10) remains after detachment of the connecting hypha, 4.5–9.5 µm wide. The pore is closed by some of the inner lamina of OWL2 and by OWL3.

Acaulospora tortuosa Palenz., Oehl, Azcón-Aguilar & G.A.Silva **sp. nov.**
Figs 11–22

Mycobank MB 802632

Etymology: Latin, *tortuosa*, referring to the tortuous projections of the spore surfaces.

Sporae singulae lateraliter formatae ad sacculum terminalem, flavae-aurantiae vel aurantio-fuscae, globosae vel subglobosae, 61–84(–94) × 61–80(–91) µm. Stratum exterior tunicae exterioris fragmentis hypharum vel projectionibus linearibus vel curvatis. Holotypus # 48–4801: Z+ZT (ZT Myc 30438).

Holotype: Spain, Andalucía, Granada, Sierra Nevada (37°00'N; 3°22'W, 1980 m asl) in the rhizosphere of the endangered fern *Ophioglossum vulgatum*, deposited at Z + ZT (common mycological herbarium of the University and ETH of Zurich, Switzerland. Isotypes deposited at Z + ZT (ZT Myc 30439) and at GDA-GDAC (herbarium of the University of Granada, Spain). Paratypes isolated in another grassland (37°07'N; 3°26'W, 1800 m asl) from an endemic plant species *Narcissus nevadensis* deposited at Z + ZT (ZT Myc 30440), GDA-GDAC and URM.

The sporiferous saccules originate terminally from hyphae. They are hyaline and singly formed. After saccule formation, single spores arise laterally on the neck of the saccule, at 35–110 µm distance. The saccule termini are globose to subglobose (60–85 × 55–75 µm), with 1–2 wall layers that are in total 1.4–2.1 µm thick. The saccule usually collapses after the spore wall has formed and regularly is detached from mature spores in soils.

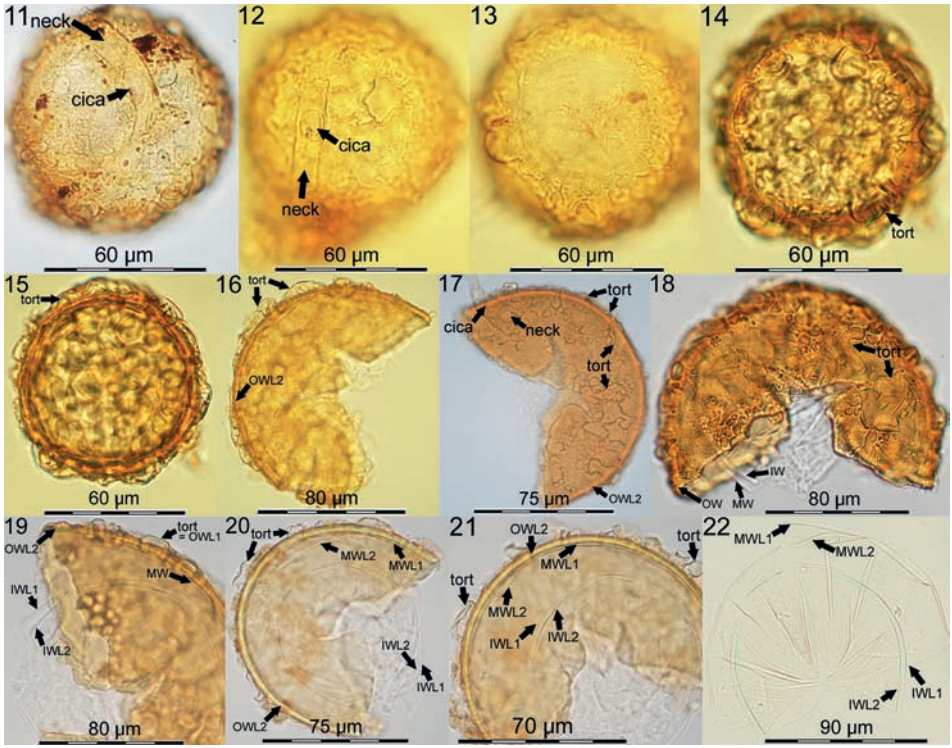
Spores develop laterally on the neck of sporiferous saccules. They are globose, to subglobose, 61–84(–94) × 61–80(–91) µm in diameter. They are yellow brown to yellow orange when young, becoming orange brown with age. They have three walls whose layers do not stain in Melzer's reagent (Figs 11–22).

Outer spore wall consists of three layers (OWL1–OWL3) and is in total 3.7–6.3 µm thick (Figs 16–21). Outer layer (OWL1) is subhyaline to pale yellow, 1.2–2.3 µm thick. It forms irregular hyphae-like structures, that are subhyaline to pale yellow to sometimes dark yellow. They are also highly irregular in length (2.6–10.5(–35) µm), width (2.5–7.5 µm, up to rarely 13 µm) and height (2.4–7.5 µm), and the distances between each other are also quite variable (0.0–6.5 µm). The second layer (OWL2) is yellow brown to yellow orange when young, become orange brown with age, laminate, 2.0–3.2 µm thick. The inner layer of the outer wall (OWL3) is concolorous with OWL2, about 0.5-0.8 µm thick and usually hardly to observe, especially when the middle wall does not separate readily from OW (Figs 19–21).

Middle wall is hyaline, bi-layered and thin; in total 1.2–2.2 µm. Both layers (MWL1 and MWL2) are semi-flexible, tightly adherent to each other and thus, they regularly appear as being only one wall layer (Figs 19–22).

Inner wall is hyaline, with two to three layers (IWL1–IWL 3) that are 2.4–3.5 µm thick in total. The IWL1 is about 0.9–1.3 µm thick. A 'beaded', granular structure, is rarely seen in lactic acid based mountants. IWL2 is 1.2–1.6 µm thick. IWL3 is very thin and usually very difficult to detect due to the close adherence to IWL2 (Figs 19–22).

Cicatrix (Figs 11–12, 17) remains after detachment of the connecting hypha, and is 7–13 µm wide. The pore is closed by some of the inner lamina of OWL2 and by OWL3.



Figs 11–22. *Acaulospora tortuosa*. Fig. 11. Spores formed laterally on the neck of a detached sporiferous saccule terminus. Characteristic cicatrix (cica) at the spore base. Figs 12–15. Spores in planar, almost cross and cross view, and stained with Melzer's reagent but without staining reaction. Spore surfaces with hyphae-like projections (tort) formed by OWL1 that often appear rudimentary. Figs 16–21. Crushed spores with three walls (outer, middle, inner: OW, MW, IW) and multiple wall layers (OWL1-OWL2, MWL1-MWL2, IWL1-IWL2). Fig. 22. MW and IW layers after separation from OW by continued slight pressure on the cover slide.

Molecular analyses: ITS sequences and partial sequences of the LSU rDNA gene for the two new species confirm the fungi in two well separated clades within the Acaulosporaceae next to *A. alpina*, *A. colliculosa*, an un-described *Acaulospora* species, and to each other (Figs 23–24). The intraspecific variation between the different clones of *A. pustulata* and *A. tortuosa* was around 1–2% for the LSU rDNA sequences, and around 2–3% for the ITS sequences. For the ITS region, the closest species related to *A. pustulata* were *A. alpina* (Oehl et al. 2006) and an un-described *Acaulospora* sp. (Oehl et al. 2011d), with 91% and 94% of identity, respectively, in the BLASTn analysis, while the closest species related to *A. tortuosa* was *A. alpina* with 90% of identity. The LSU rDNA sequences from *A. pustulata* and *A. tortuosa* were closest to the undescribed *Acaulospora* sp. mentioned above with 98% and 95% of identity, respectively. The *Acaulospora* sp. clearly is the most similar species related to

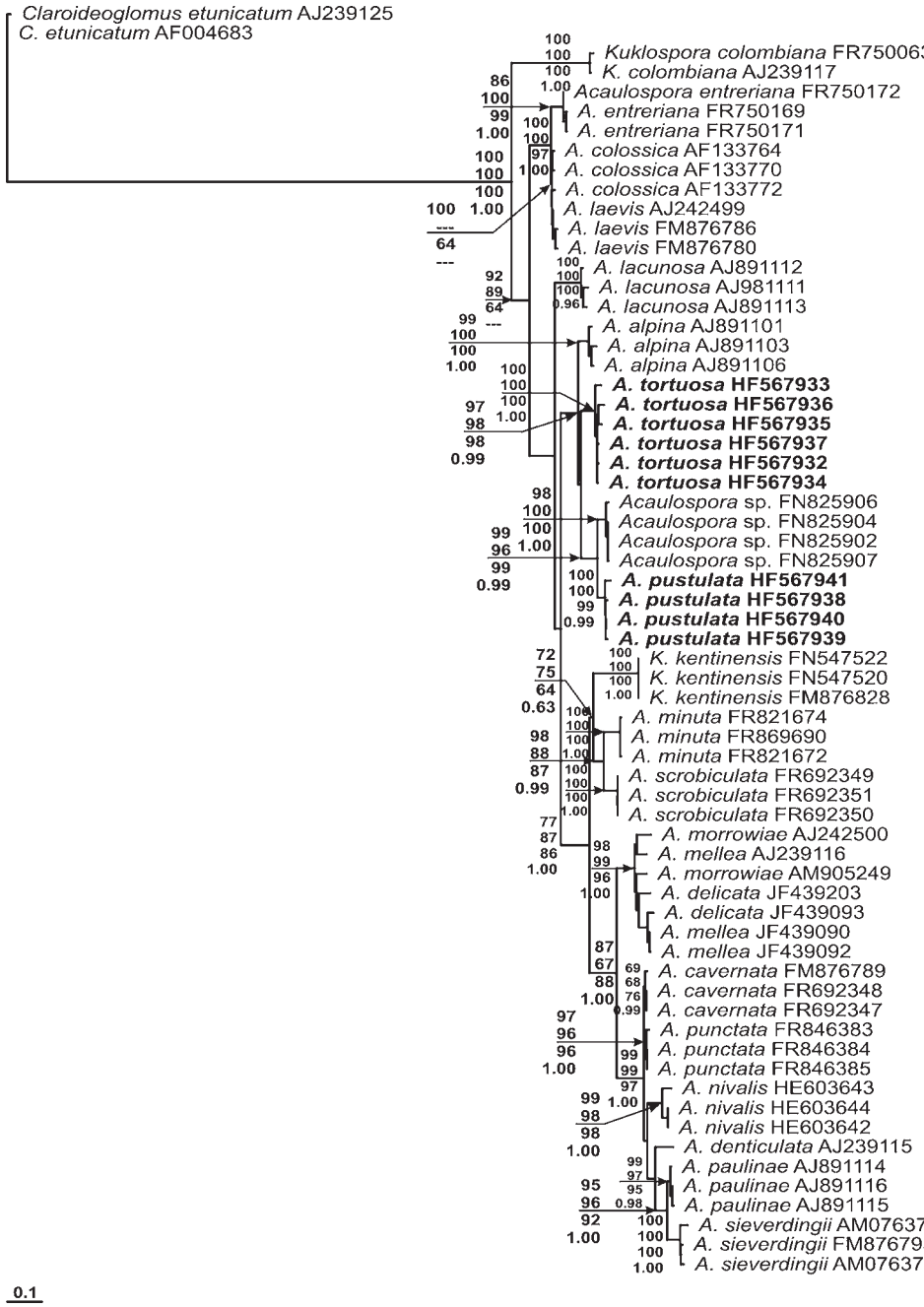


Fig. 23. Phylogenetic tree of the Acaulosporaceae obtained by analysis from ITS1, 5.8S rDNA and ITS2 sequences. Sequences are labeled with their database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and

A. pustulata, but the low similarity in ITS sequences indicates that they are different taxa. Environmental ITS sequences with closest match to *A. pustulata* were found in roots from *Anthoxanthum odoratum* – AJ504636 (94% of identity) sampled from a mountainous grassland in Thuringia (Germany). The LSU region of *A. pustulata* was most similar to environmental sequences amplified in roots from *Prunella vulgaris* – JF717602 (98% of identity) sampled from a grassland in Oregon (USA). *Acaulospora tortuosa* had closest matches to AM fungi inside roots from *Artemisia umbelliformis* (93% – ITS (FN808316) and 95% LSU rDNA (FN808315)) which was sampled from a grassland in the French Alps.

Distribution of *A. pustulata* and *A. tortuosa*: The two new fungi were found in two wet mountainous grassland ecosystems of Sierra Nevada National Park, growing in the rhizosphere of two endangered plant species, *Narcissus nevadensis* and *Ophioglossum vulgatum*, and plants like *Holcus lanatus*, *Trifolium repens*, *Mentha suaveolens*, and *Carum verticillatum* at approximately 1800–2000 m asl. Mean annual air temperatures are around 6.2°C (minimum) and 16.8°C (maximum) for both sites. Soil pH around *N. nevadensis* and *O. vulgatum* roots was 6.4 and 6.5, respectively. Organic carbon was 116 and 301 g kg^{-1}, respectively. Very recently, they were also found in a few Swiss alpine grasslands at Furka Pass (46°33–34'N, 8°24–25'O; at approx. 2500 m asl) with a wider range of soil moisture (well-drained, stagnating water affected and groundwater affected soils), soil pH (3.8–6.5), 1.5 and 300 g kg^{-1} organic carbon. At Furka, *A. pustulata* was found in a Cambisol, a Stagnosol, a Gleysol and a Histosol. *Acaulospora tortuosa* was found in a Podzol, in three well drained Cambisols, and in two alpine Stagnosols. In the Swiss study area, the two fungi co-occurred in each one of the Cambisols and Stagnosols.

In Sierra Nevada, the two fungi co-occurred with the following AMF species: *Acaulospora cavernata*, *A. paulinae*, *Claroideoglomus claroideum*, *Cl. etunicatum*, *Entrophospora infrequens*, *Funneliformis geosporus*, *Septoglomus xanthium*, and an undescribed *Septoglomus* sp. In the Swiss Alps, *Ambispora gerdemannii*, *A. alpina*, *A. punctata* (Oehl et al. 2011e) and *Glomus rubiforme* were the most frequent AMF species accompanying the two new fungi at their habitats.

Specimen examined: Holotype, isotype and paratype specimen from Sierra Nevada, deposited at Z+ZT and GDA-GDAC, and in private collections of J.Palenzuela and F.Oehl. Specimen from Furka Pass in the Swiss Alps, also deposited at Z+ZT and GDA-GDAC.

Discussion

Acaulospora pustulata and *A. tortuosa* represent two distinct species in the Glomeromycota with, so far, unique ornamentation characteristics for species described in the family Acaulosporaceae. *Acaulospora pustulata* has a pustulate spore ornamentation

bayesian analyses, respectively. The tree was rooted by *Claroideoglomus etunicatum*. Sequences obtained in this study are in boldface. Only bootstrap values of at least 50% are shown. (Consistency Index = 0.58; Retention Index = 0.87).

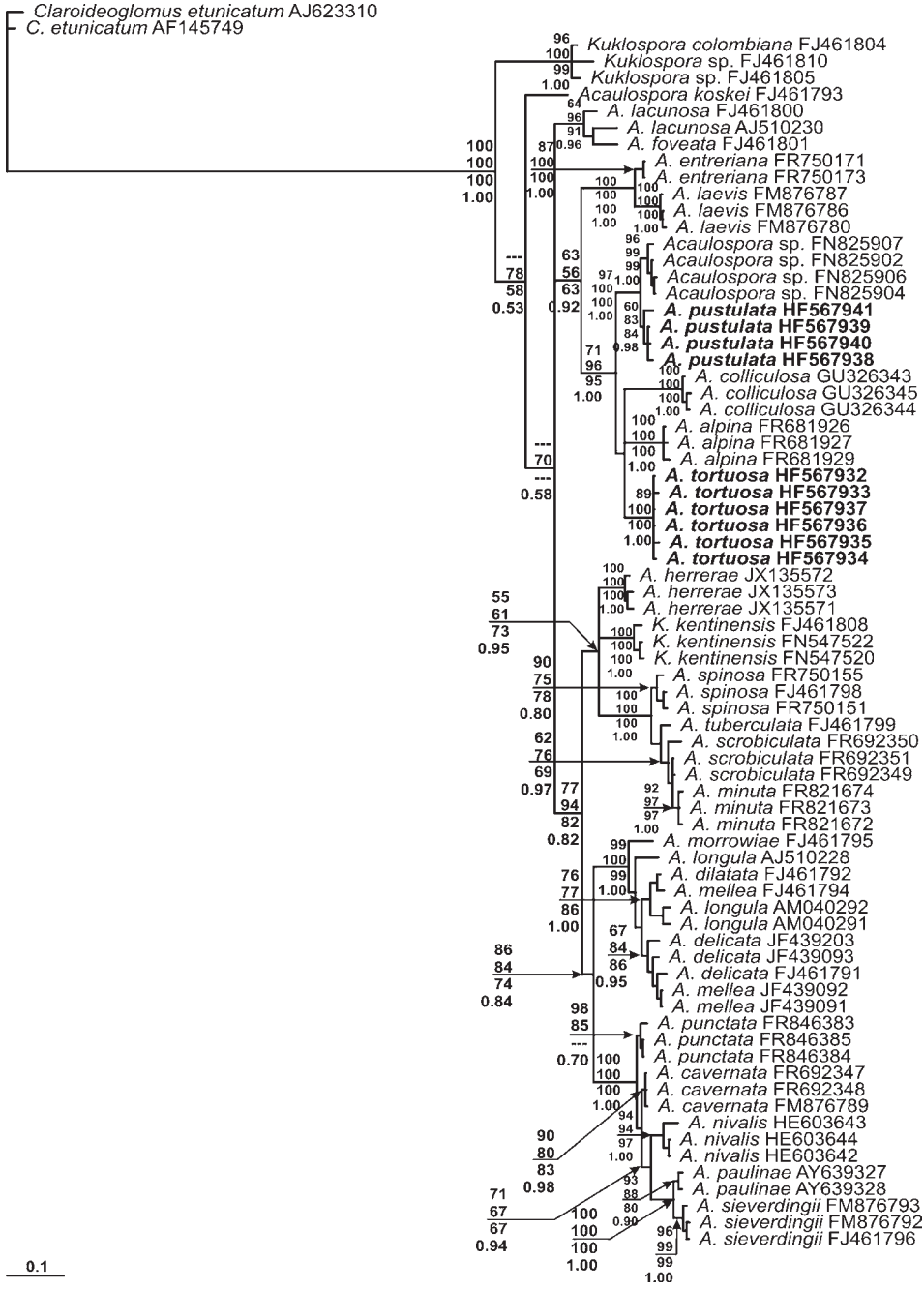


Fig. 24. Phylogenetic tree of the Acaulosporaceae obtained by analysis from partial LSU rDNA sequences of different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum

similar to that of *Diversispora pustulata* (baysionym *Glomus pustulatum*; Koske et al. 1986, Oehl et al. 2011c), *Ambispora brasiliensis* (Goto et al. 2008) and an undescribed *Acaulospora* species (Krüger et al. 2011, Oehl et al. 2011d), while *A. tortuosa* has surface projections that resemble innumerous hyphae-like structures but these appear much more rudimentary than the hyphae-like structures known for spores of *Sacculospora baltica* (basionym *Entrophospora baltica*; Błaszkowski et al. 1998, Sieverding & Oehl 2006, Oehl et al. 2011f), *Glomus tortuosum* (Schenck & Smith 1982), and *Funneliformis mosseae* (basionym *Endogone mosseae*; Nicolson & Gerdemann 1968, Gerdemann & Trappe 1974, Schüßler & Walker 2010) when the spores of the later species are surrounded by a hyphal mantle.

None of the known *Acaulospora* species forms spores smaller than those of *A. pustulata*, and also *A. tortuosa* is among the smallest-spored species of Acaulosporaceae. Other *Acaulospora* species with spores that are regularly smaller than 100 µm are: *A. longula*, *A. morrowiae*, *A. gedanensis*, *A. alpina*, *A. nivalis*, *A. paulinae* and *A. sieverdingii* but all these species have either smooth or pitted spore surfaces (e.g. Błaszkowski 1988, Schenck et al. 1984, Oehl et al. 2006, 2011g, 2012). All other *Acaulospora* species with projection on the spore surface have substantially bigger spores: *A. spinosa*, *A. tuberculata*, *A. bireticulata*, *A. elegans* and *A. denticulata* (Oehl et al. 2012).

The phylogenetic data show that *A. pustulata* and *A. tortuosa* belong to the same major clade as *A. alpina* that form pits on the spore surface instead of projections. There is at least one other major clade in which Acaulosporaceae species with surface depressions (*A. herrerae*, *A. scrobiculata*, *A. minuta* and *Kuklospora kentinensis*) and projections (*A. spinosa* and *A. tuberculata*) group together (Fig. 24; Furrazola et al. 2013). This observation suggests that the type of surface ornamentation, being either depressions or projections, is not a character that could be used to explain the morphological differences between major phylogenetic clades in Acaulosporaceae.

The two new fungi were found in Sierra Nevada in high mountainous grasslands (at approx. 2000 m asl) whose soils have been affected by ground water (Gleysols and Histosols). In the Swiss Alps they were found at slightly higher altitudes (approx. 2500 m asl) in high alpine grasslands where they also occurred in soils affected by ground water (Gleysols and Histosols), but also in soils affected by stagnating water (Stagnosols), and in well-drained Cambisols on slopes. However, in the Central Alps, high alpine Cambisols are exposed, especially at Furka Pass, to high annual precipitations of 2000–2500 mm meaning that also these soils are periodically water-saturated. Hitherto, the two fungi have never been found in the Central or South European lowlands, suggesting that in Europe they might have preferential occurrence in the colder mountainous to alpine areas, and there above all in soils with periodical higher quantities of free water.

likelihood (ML) and bayesian analyses, respectively. The tree was rooted by *Claroideoglomus etunicatum*. Sequences obtained in this study are in boldface. Only bootstrap values of at least 50% are shown. (Consistency Index = 0.53; Retention Index = 0.86).

Acaulospora pustulata and *A. tortuosa* occur in the rhizosphere of two critically endangered plant species in Sierra Nevada, *Ophioglossum vulgatum* and *Narcissus nevadensis*. However, it is not clear if they might have a significant effect on the presence of these two plant species. In the Swiss alpine grasslands, the significance of plant endemism is lower than in Sierra Nevada (Moser et al. 2002), and there was only one endemic or critically endangered plant species co-occurring with the two new fungi at Furka Pass (*Dracocephalum ruyschiana*; Hefel & Stöcklin 2010). However, it will be interesting to find out which plant species in Sierra Nevada and in the Alps especially profit from the presence of these two inconspicuous but very distinctive fungi.

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