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Acaulospora alpina, a new arbuscular mycorrhizal fungal species characteristic for high mountainous and alpine regions of the Swiss Alps

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Abstract: *Acaulospora alpina* sp. nov. forms small (65–85 μm diam), dark yellow to orange-brown spores laterally on the neck of hyaline to subhyaline sporiferous saccules. The spores have a three-layered outer spore wall, a bi-layered middle wall and a three-layered inner wall. The surface of the second layer of the outer spore wall is ornamented, having regular, circular pits (1.5–2 μm diam) that are as deep as wide and truncated conical. A ''beaded'' wall layer as found in most other *Acaulospora* spp. is lacking. The spore morphology of *A. alpina* resembles that of *A. paulinae* but can be differentiated easily by the unique ornamentation with the characteristic pits and by the spore color. A key is presented summarizing the morphological differences among *Acaulospora* species with an ornamented outer spore wall. Partial DNA sequences of the ITS1, 5.8S subunit and ITS2 regions of ribosomal DNA show that *A. alpina* and *A. paulinae* are not closely related. *Acaulospora lacunosa*, which has similar color but has generally bigger spores, also has distinct rDNA sequences. *Acaulospora alpina* is a characteristic member of the arbuscular mycorrhizal fungal communities in soils with pH 3.5–6.5 in grasslands of the Swiss Alps at altitudes between 1800 and 2700 m above sea level. It is less frequent at 1300–1800 m above sea level, and it so far has not been found in the Alps below 1300 m or in the lowlands of Switzerland.

Key words: Alps, Acaulosporaceae, *Acaulospora paulinae*, *Acaulospora lacunosa*, Glomeromycetes, key, molecular identification, mycorrhiza, spore morphology, phylogeny, taxonomy

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INTRODUCTION

At high altitudes, in the mountainous and alpine regions of the Swiss Alps extending from 1000–3000 m above sea level (a.s.l.), we have observed an astonishingly high diversity of arbuscular mycorrhizal (AM) fungal species (Oehl unpubl). Spores of about 60 known species of the Glomeromycota (Schüssler et al 2001) could be identified from different grasslands growing on soils that had developed on siliceous and calcareous bedrocks. Some of the species were new and recently have been described (Oehl and Sieverding 2004, Oehl et al 2005a). Among the AM fungi, species belonging to the genus *Acaulospora* were particularly prominent and relatively much more abundant than in the lowlands of Switzerland. Here we describe a new *Acaulospora* species under the epithet *A. alpina* that was found exclusively in the Alps at altitudes >1300 m a.s.l.

The genus *Acaulospora* was described by Gerdemann and Trappe (1974) who also presented the first key for the two species known at that time. The key differentiated a species known to produce spores with a smooth surface (*A. laevis*) from another one with an ornamented surface (*A. elegans*). Today we know 18 *Acaulospora* spp. with smooth spore surfaces and 15 *Acaulospora* spp. (including *A. alpina*) with ornamentation of the outer spore wall. Schenck et al (1984) presented the latest key to the ornamented species of *Acaulospora*. They used spines, tubercles, ridges, folds, pits or cracks as differentiating features for the spore wall ornamentations. We use similar characteristics and we present an updated key for *Acaulospora* spp. with ornamented spore walls.

In recent years molecular biological tools have been applied to identify AM fungi (Clapp et al 1995, Redecker 2000, Oehl et al 2005a). Environmental rDNA sequences are rapidly increasing in number in the public databases. However only a few DNA sequences of *Acaulospora* originating from morphologically characterized spores are available. This is also true for the highly variable rDNA internal transcribed spacer (ITS) region, which is a useful tool to distinguish many species-level AM fungal taxa (Redecker et al 2003). Some of these database sequences show strong similarity to fungal groups other than the Glomeromycota and are more likely to originate from contaminant organisms (Millner et al 2001). Therefore there is a clear need for rDNA sequences from described *Acaulospora* species.

New species can be adequately characterized only when sequences of morphologically similar species are included in the analysis. To identify the phylogenetic position of *A. alpina* the sequence of the ITS1, 5.8S rDNA and ITS2 region was determined not only from this new species but also from *A. paulinae* Blasz. (Blaszkowski 1988) and *A. lacunosa* J.B. Morton (Morton 1986) which produce morphologically similar spores. Sequences obtained for *A. alpina* also were compared to environmental sequences of *Acaulospora* spp. available from the public databases.

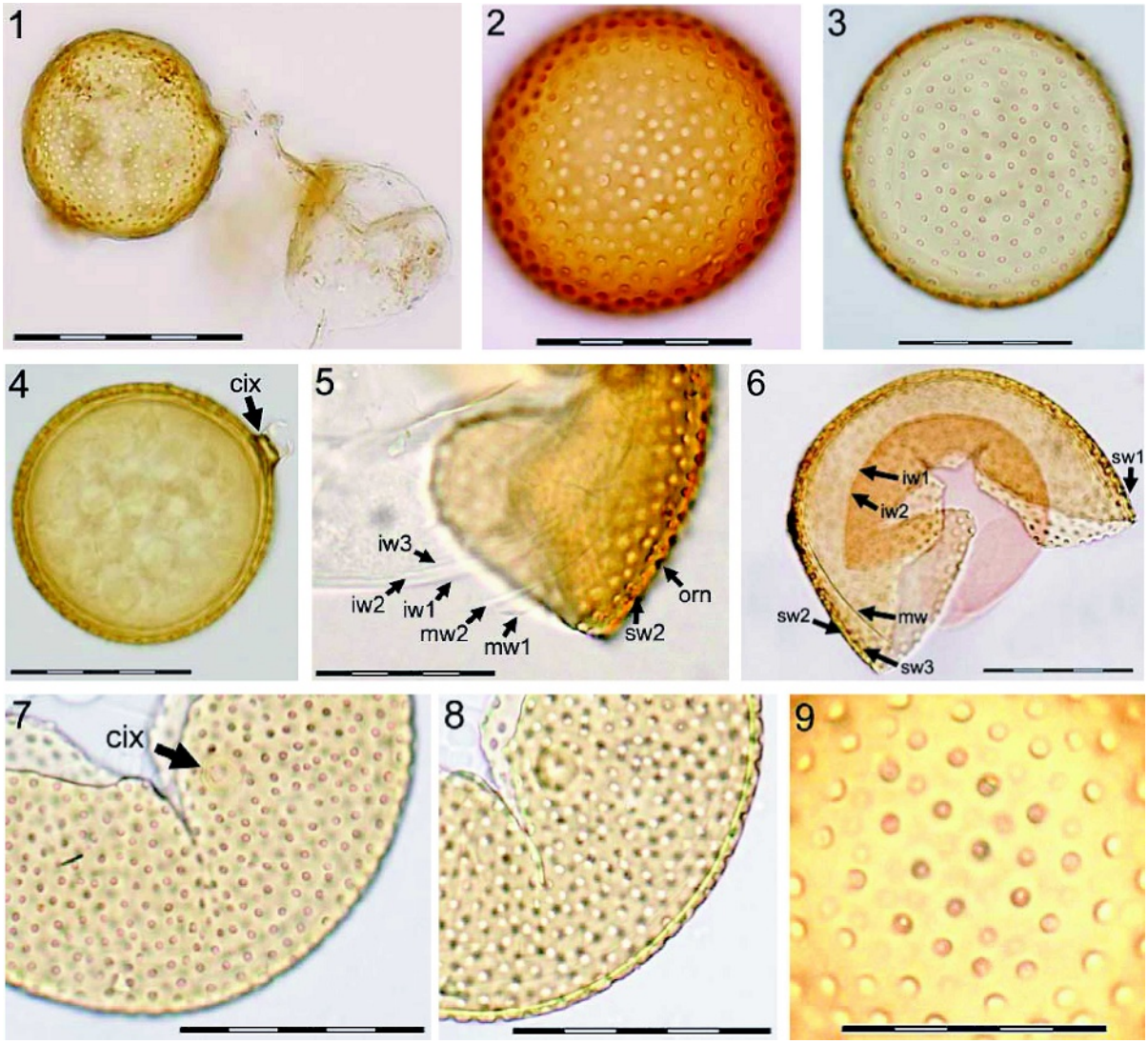
MATERIALS AND METHODS

Soil sampling.—Soil samples were taken from mountainous and alpine grasslands in the Swiss Alps from altitudes of 1000–3000 m a.s.l.; the soils had developed on different geological bedrocks from nutrient poor Jurassic sandstones over granite and gneiss rocks to carbonatic and dolomitic limestones and ultrabasic serpentinites. Undisturbed soil cores 0–10 cm deep were collected at several times Jul–Sep 2003. Spores of AM fungi were separated from the soil samples by a wet sieving process as described by Sieverding (1991).

AM fungal bait cultures.—Bait cultures were established directly after sampling as follows: 1000 mL pots were half filled with 500 g of an autoclaved substrate (Terragreen; American aluminium oxide, Oil Dry US special, type III R; Lobbe Umwelttechnik Iserlohn, Germany) Loess mixture 3 : 1; pH-KCl 6.2; organic carbon 0.3%; available P (Na-acetate) 2.6 mg kg^{-1}; available K (Na-acetate) 350 mg kg^{-1}. Fifty g dry weight field samples were placed at one side on the top of the substrate and covered with another 300 g of autoclaved substrate. Above the soil inocula, about 5–7 seeds of each of the four trap plants, *Plantago lanceolata* L., *Lolium perenne* L., *Trifolium pratense* L. and *Hieracium pilosella* L. were sown. A total of 0.2 mL of a culture broth with *Rhizobium trifolii* (DSM 30138, from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) grown in liquid DSMZ 98 medium at 27 C for 12 h, was added to the 2 wk old *Trifolium pratense* plants in each pot. An automated watering system (Tropf-Blumat, Weninger GmbH, A-6410 Telfs) was installed and the cultures were kept in the greenhouse of the Institute of Botany in Basel under ambient natural light and temperature conditions until the end of 2004. The average annual temperature in Basel is about 9.5 C. The formation of spores in the bait cultures was checked Jun–Dec 2004 at bimonthly intervals as described by Oehl et al (2003, 2004). The new fungus only infrequently produced spores in these bait cultures. All trials of monospecies cultures, either initiated with single or multispores, so far failed to establish a successful symbiosis.

Morphological analyses.—The described morphological characteristics of spores and sporiferous saccules and their subcellular structures are based on observations of specimen mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske and Tessier 1983), in a mixture of PVLG and Melzer's reagent (Brundrett et al 1994), a mixture of lactic acid to water at 1 : 1, Melzer's reagent, and in water. The terminology of the spore structure basically is that of Stürmer and Morton (1999) which was adapted by INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi, www.invam.caf.wvu.edu), but we use different abbreviations for the walls and wall layers. In detail, we call the outer ''spore wall'' layers of the Acaulosporaceae sw1–3, the first flexible inner wall iw1 of Stürmer and Morton (1999), the ''middle wall'' (mw), and the second flexible inner wall iw2 of Stürmer and Morton (1999) the ''inner wall'' (iw). Photographs (FIGS. 1–9, 11–19) were taken with a digital camera (Olympus model DP70-CU) on a compound microscope (Zeiss Axioplan). To improve the quality of the pictures taken of the ornamentation of different *Acaulospora* spp., the software Auto-Montage Essentials 5.00 (Olympus) was used (technique used in FIGS. 1–3, 7, 8, 14, 15). Specimen mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at Z+ZT (Zürich, Switzerland), FB (Freiburg, Germany) and OSC (Corvallis, USA) herbaria.

Molecular analyses.—DNA crude extracts were produced as described by Redecker et al (1997) from spores of *A. alpina* originating from a grassland on a Humic Cambisol at Spadla Alp (at 2700 m a.s.l. near Sent, Engiadina Bassa, Canton Grischun; soil pH 5.0 measured in water) and from isotypes isolated at Grand Muveran (at 2600 m a.s.l. near Ovronnaz/Martigny, Canton Valais; pH 5.6), from Tschima da Flix (at 2400 m a.s.l. near Sur, Surses, Canton Grischun; pH 6.0) and at Stützalp (at 1900 m a.s.l. near Davos, Canton Grischun; pH 6.1). DNA was extracted from approximately 10 spores from each location. Extracts of single spores were used as templates for a two-step polymerase chain reaction (Redecker et al 2003) with the primers NS5/ITS4 and ACAU1661/ITS4i, respectively (Redecker 2000). PCR products were purified with a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany), cloned into pGEM-T (Catalys, Wallisellen, Switzerland), re-amplified from the clones and digested with *Mbo*I and *Hin*fI restriction enzymes (Fermentas, Vilnius, Lithuania). Samples with different RFLP patterns were sequenced with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) for labeling. Samples were run on an ABI 310 capillary sequencer (Applied Biosystems). Sequences of *A. alpina* were submitted to the EMBL database under the accession numbers AJ890446 and AJ891101–AJ891109. To compare the sequences obtained from *A. alpina* to other *Acaulospora* spp. of similar spore morphology, an isolate of *A. paulinae* and the BEG78 isolate of *A. lacunosa* were included in the analysis. The isolate of *A. paulinae* originated from a soil sample taken from a meadow with *Arrhenaterum elatius* L. as the characteristic grass species, at Wintzenheim-La-Forge (Alsace, France) in Apr 2003. The *A. lacunosa*



FIGS. 1–9. *Acaulospora alpina* photographed from type specimen. 1. Spore and sporiferous saccule; bar = 75 µm. 2, 3. Spore showing regular ornamentation; bar = 50 µm. 4. Spore with cylindric pedicel around cicatrix (cix); bar = 50 µm. 5. Cracked spore, with three walls (sw, mw, iw); outer spore wall three-layered (sw1–3) with pitted ornamentation (orn) on sw2; flexible middle wall (mw) with two usually adherent layers (mw1 and mw2; here separated) and inner wall (iw) with three tightly adherent layers (iw1–3); bar = 25 µm. 6. Inner wall (iw2) staining pale purple in Melzer's reagent; iw3 often difficult to observe even in broken spores; bar = 50 µm. 7. Cracked spore showing circular pits on sw2 and the cicatrix (cix); bar = 50 µm. 8. Wall layer sw2 with truncated cone-shaped pits; bar = 50 µm. 9. Outer wall at higher magnification showing regular, round pits on sw2; bar = 25 µm.

isolate originated from a temperate forest in New Hampshire. Sequences were submitted to the EMBL database under the accession numbers AJ89114–AJ891121 (for *A. paulinae*) and AJ891110–AJ891113 (for *A. lacunosa*).

Sequences were aligned in PAUP*4b10 (Swofford 2001) in a dataset comprising rDNA ITS1, 5.8S subunit and ITS2 of other fungi from the family Acaulosporaceae. From a total of 700 positions in the alignment, 315 positions were selected that were in unambiguous alignment. The ITS1 region contains numerous insertions/deletions in long stretches of A or T, which causes serious alignment problems, therefore this region was excluded from the analysis. In all phylogenetic analyses the sequence of the AM fungus *Entrophospora colombiana* was used as outgroup. The appropriate sequence evolution model for maximum likelihood analysis (HKY+G) was determined with Modeltest 3.5. (Posada 2004). Bayesian analysis was performed in MrBayes 3.0 (Ronquist and Huelsenbeck 2003). Four chains were run over 3.6×10^6 generations with a burn-in value of 2000. Neighbor joining analysis was performed with the Kimura 2-parameter model and a gamma shape parameter of 0.5. Maximum likelihood distances obtained by the HKY+G model were used for neighbor joining, which yielded the same tree topology. Bootstrap analysis (Felsenstein 1985) was performed to estimate the robustness of the phylogeny.

TAXONOMIC ANALYSIS

Acaulospora alpina Oehl, Sykorova & Sieverd. sp. nov. (FIGS. 1–9)

Sacculus sporifer hyalinus aut pallido-luteus, globosus vel subglobosus, 65–92 μm diam et formationi sporae praecedens. Sporae singulae lateraliter formatae ad hypham in 40–80 μm distantia ad sacculum terminalem, flavae vel fulvae vel fulvo-aurantiae vel aureae vel aurantio-brunneae, (53–) 65–85 (–97) μm diam, globosae vel subglobosae vel ovoideae vel ellipsoideae vel irregulares (53–) 60–81(–91) × 62–87 (–110) μm. Sporae tunicis tribus: tunica exterior, media et interior. Tunica exterior in totum 2.5–4.0 μm crassa, stratis tribus: stratum exterius hyalinum, tenue et evanescens; stratum medium laminatum vel unitum, flavum vel fulvum vel fulvo-aurantium vel brunneo-aurantium, depressionibus subtilibus, rotundis, 1.5–2.2 (–2.8) μm diam, et conicis, 2.0–2.5 μm profundis, in interiorem strati huius insculptis; stratum interius flavum vel fulvum, subtile. Tunica media tenuis stratis duobus et tunica interior stratis tribus, uterque tunicae hyalinae et flexibiles. Tunica interior 1.2–3.0 μm in totum; solo stratum medium tunicae interioris pallide colorans reagente Melzeri. Typus hic designatus Nu. 41–4101: Z+ZT.

Sporiferous saccule is hyaline, globose (ca. 65–80 μm diam) to subglobose, 65–75 × 75–92 μm, with one wall layer that is generally 1.0–2.1 μm thick (FIG. 1); formed at the end of a hypha in 40–80 μm distance from the spore that arises thereafter. The saccule usually collapses after the spore wall has formed and usually is detached from mature spores in soil samples.

Spores (FIGS. 1–4) form laterally on the subtending hypha of the sporiferous saccule. The spores are dark yellow, orange to brown, globose to subglobose, (53–) 65–85(–97) μm diam, rarely ovoid to irregular, (53–) 60–81(–91) × 62–87(–110) μm diam.

Outer spore wall consists of three layers (sw1, sw2 and sw3), in total 2.5–4.0 μm thick (FIGS. 5, 6). Outer layer (sw1) is hyaline, unit, 0.5–1.0 μm thick, sloughing, evanescent and thus, usually absent in mature spores. Second layer (sw2) is light to dark yellow to yellow-orange to orange-brown, laminated, 2.0–3.0 μm thick including the ornamentation with regular, round and truncated conical pits that are 1.5–2.2(–2.8) μm diam and at least as deep as wide (FIGS. 7–9). Due to their truncated cone shape the pits often appear to have a dark central point, but there is no second depression or a projection within the pit. The distance between the pits is (3.0–)4–6(–7) μm. The inner spore wall layer (sw3) is concolorous with sw2, 0.5–1.3 μm thick, usually tightly adherent to sw2 and often difficult to observe when <1.0 μm. None of these wall layers stains in Melzer's reagent.

Middle wall is hyaline, bilayered and thin; in total 0.5–1.2 μm; both layers (mw1 and mw2) are semiflexible (FIG. 5), tightly adherent to each other and thus, often appearing as being one wall layer (FIG. 6). None of the layers reacts to Melzer's.

Inner wall is hyaline, with three layers (iw1–3) that are 1.2–3.0 μm thick in total (FIG. 5). The iw1 is about 0.5 μm thick, and not ''beaded''; iw2 is 1.2–2.0 μm thick; iw3 is about 0.5 μm thick and usually difficult to detect due to the close adherence to iw2. Only iw2 shows a light, pale pink reaction to Melzer's reagent (FIG. 6) usually visible only in cracked spores and not observed in all specimen.

Cicatrix (FIGS. 4, 7) remains after detachment of the connecting hypha (FIG. 4), (5–)7–12 μm wide. The layer sw2 often continues for a small distance (0.8–2.2[–3] μm) into the detaching hypha forming a short cylindrical pedicel around the pore. While the pore itself is not ornamented, the tapering pedicel wall often has the pitted ornamentation of sw2. The pore is closed by some of the inner laminae of sw2 and by sw3.

Etymology. Latin, *alpina*, referring to the Swiss Alps where the species was first found.

Specimen examined. SWITZERLAND. GRISCHUN: Sent, Alp Spadla, at 2000–2700 m a.s.l. (HOLOTYPE: Z+ZT); GRISHUN: Pontresina, Diavolezza at 2000–2700 m; GRISCHUN, San Murezzano (St. Moritz), Corviglia at 2700 m; GRISHUN: La Punt, Passo D'Alvra-Piz Üertsch, at 2300–2600 m, GRISHUN: Sur, Tschima da Flix, at 2000–2500 m; GRAUBÜNDEN: Davos, Parsennhütte and Stützalp, at 1800–2300 m (ISOTYPE: OSC); GRAUBÜNDEN: Chur-Haldenstein, at 1620–2300 m; GRISHUN: Sumvitg-Surrein, Alp Nadels, at 1950–2500 m; TICINO, Olivone, Piz Corvo-Paso di Lucomagno, at 1800–2500 m; TICINO: Airolo, Passo di Gotthardo, at 1800–2000 m; URI and VALAIS: Realp-Oberwald, Furkapass, at 1850–2650 m; BERN: Axalp, Axalphorn, at 1700–2300 m; BERN: Grindelwald, Grosse Scheidegg/Gemschberg, at (1350–)1800–2500 m (ISOTYPE: FB); VALAIS: Ovronnaz, Grand Muveran, at 1720–2600 m; VALAIS: Champez, Le Cartogne, at (1350–)1800–2600 m; VALAIS: Col de Grand St Bernhard, Pointe de Drône, at 2300–2500 m a.s.l.

Commentary. Spores of *A. alpina* were abundantly isolated from the rhizosphere of alpine grasslands (soil pH 3.5–5.5) with vegetation dominated by *Carex curvula* All. and/or *Nardus stricta* L. Spores were less frequent in alpine grassland soils with pH > 6.0 and plant species communities dominated by *Carex ferruginea* Scop., *Carex sempervirens* Vill. or *Sesleria caerulea* (L.) Scop., or in lower altitude grasslands (1500–1800 m a.s.l.) with plant species communities dominated by *Nardus stricta* or by *Trisetum flavescens* (L.) P. Beauv. The new species was found in a broad range of soils that developed on acidic sandstones, siliceous gneiss and granite rocks, up to ultrabasic serpentinite and calcareous ''Bündner Schiefer'' schists and carbonatic and dolomitic limestones.

Molecular biological analysis.—Sequences of approximately 550 bp long were obtained, comprising ITS1, the 5.8S rDNA subunit and ITS2. Phylogenetic analysis firmly placed all sequences of *A. alpina* into the genus *Acaulospora* and in a single clade, which is clearly distinct from the other *Acaulospora* spp. that have been analyzed. In particular *A. lacunosa* and *A. paulinae* are not closely related to *A. alpina* or each other. The sister group of *A. alpina* is made up of environmental sequences obtained from roots from the Schiefergebirge mountains of Thuringia, Germany (Renker et al 2003) and an alpine meadow near Ramosch (Canton Grischun, Switzerland) (FIG. 10). One of the environmental sequences from *Anthoxanthum* roots (ASP504636) appears to be an outlier, grouping somewhat intermediate between *A. alpina* and the other sequences obtained from roots. These environmental sequences are different from those of *A. alpina* as indicated by the bootstrap values. Together *A. alpina* and the environmental sequences form a monophyletic clade that is supported by the bootstrap distance analyses and Bayesian probabilities test. Five major clades were recovered consistently within *Acaulospora* (FIG. 10) by distance, maximum likelihood and Bayesian analyses and received good support: (i) *A. paulinae/denticulata*, (ii) *A. morrowiae/mellea*, (iii) *A. alpina* and environmental sequences from mountainous areas, (iv) *A. lacunosa*, (v) *A. laevis/colossica*. With the exception of the first two, which were identified as sister groups, the deeper relationships among clades were not resolved well.

Acaulospora spp. with ornamented outer spore walls.—Including *A. alpina*, 15 *Acaulospora* spp. have been described that have an ornamented outer spore wall. These are *A. elegans* Trappe & Gerd. (Gerdemann and Trappe 1974), *A. scrobiculata* Trappe (Trappe 1977), *A. bireticulata* F.M. Rothwell & Trappe (Rothwell and Trappe 1979), *A. spinosa* C. Walker & Trappe (Walker & Trappe 1981), *A. foveata* Trappe & Janos and *A. tuberculata* Janos & Trappe (Janos and Trappe 1982), *A. rehmii* Sieverd. & S. Toro and *A. denticulata* Sieverd. & S. Toro (Sieverding and Toro 1987), *A. taiwania* H.T. Hu (Hu 1988), *A. undulata* Sieverd. (Sieverding 1988), *A. cavernata* Blasz. (Blaszkowski 1989), *A. excavata* Ingleby & C. Walker (Ingleby et al 1994), and *A. lacunosa* and *A. paulinae*. Below we present a key, to help to distinguish the ornamented *Acaulospora* spp. For this report species that have depressions or pits on the outer spore wall are of particular interest, and we include color photographs for those species (FIGS. 11–19). We did not have access to specimen of *A. taiwania*, thus a photo of this species is not presented. Photographs of the pitted *Acaulospora* spp. spores generally were taken from type or isotype material. We included *A. denticulata* in the picture series because it has a pit (cavity) in each of the broad projections of which the ornamentation of this species consists. It is however a member of the group of species with spines or projections on the spore surface.

KEY TO *ACAULOSPORA* SPP. WITH ORNAMENTED OUTER SPORE WALLS

1. Spores with spines or polygonal projections with or without a reticulum 2
1. Spores with depressions (pits) or cerebriform folds 6
2. Spore spines or projections with a reticulum . . 3
2. Spore spines or projections without a reticulum 4
3. Reticulum three-layered enclosing polygonal projections ± 1 × 1 µm; spores generally 150–200 µm. . . . . *A. bireticulata*
3. Reticulum one-layered, overlaid over crowded, densely-organized spines ± 2 µm high; spores 140–280 µm . . . . . *A. elegans*
4. Spores with fine spines or tubercles 5
4. Spores with circular to oblong projections, 4–5(–9) µm wide and up to 3.2 µm high; each projection with a center cavity . . . . . *A. denticulata* (FIG. 11)
5. Spores with fine crowded, densely organized spines, 1–4 µm tall, 1 µm at base and tapering to 0.5 µm at the tip. . . . . *A. spinosa*
5. Spores with fine tubercles 0.7–3.5 µm long and 1.5 µm broad at the base, tapering to 0.7–1.1 at the rounded tip, irregular distances (0.5–3 µm) between single tubercles . . . . . *A. tuberculata*
6. Spores with pits 7
6. Spores with cerebriform folds . . . . . *A. rehmii* (FIG. 12)
7. Spores in sporocarps, spores 75–80 µm diam, ornamentation of 0.5–1µm wide, 4–5 side pits, 1.2 × 0.5–1 µm across, ridges form mesh . . . . . *A. taiwania*
7. Spores formed singly in soil, not in sporocarps. . . . 8
8. Pits of irregular shape 9
8. Pits of regular round shape 10
9. Spores 100–240 µm diam, subhyaline to light olive, circular to ellipsoid to y-shaped pits, 1.0–1.5 × 1.0–3 µm diam . . . . . *A. scrobiculata* (FIG. 13)
9. Spores 100–180 µm diam reddish-yellow to yellow-brown, with irregular, saucer-shaped pits, 0.2–3 × 0.2–6 µm diam . . . . . *A. lacunosa* (FIG. 14)
10. Spores with regular round pits, spores regularly <100 µm diam 11
10. Spores with regular round pits, spores regularly >100 µm diam 12

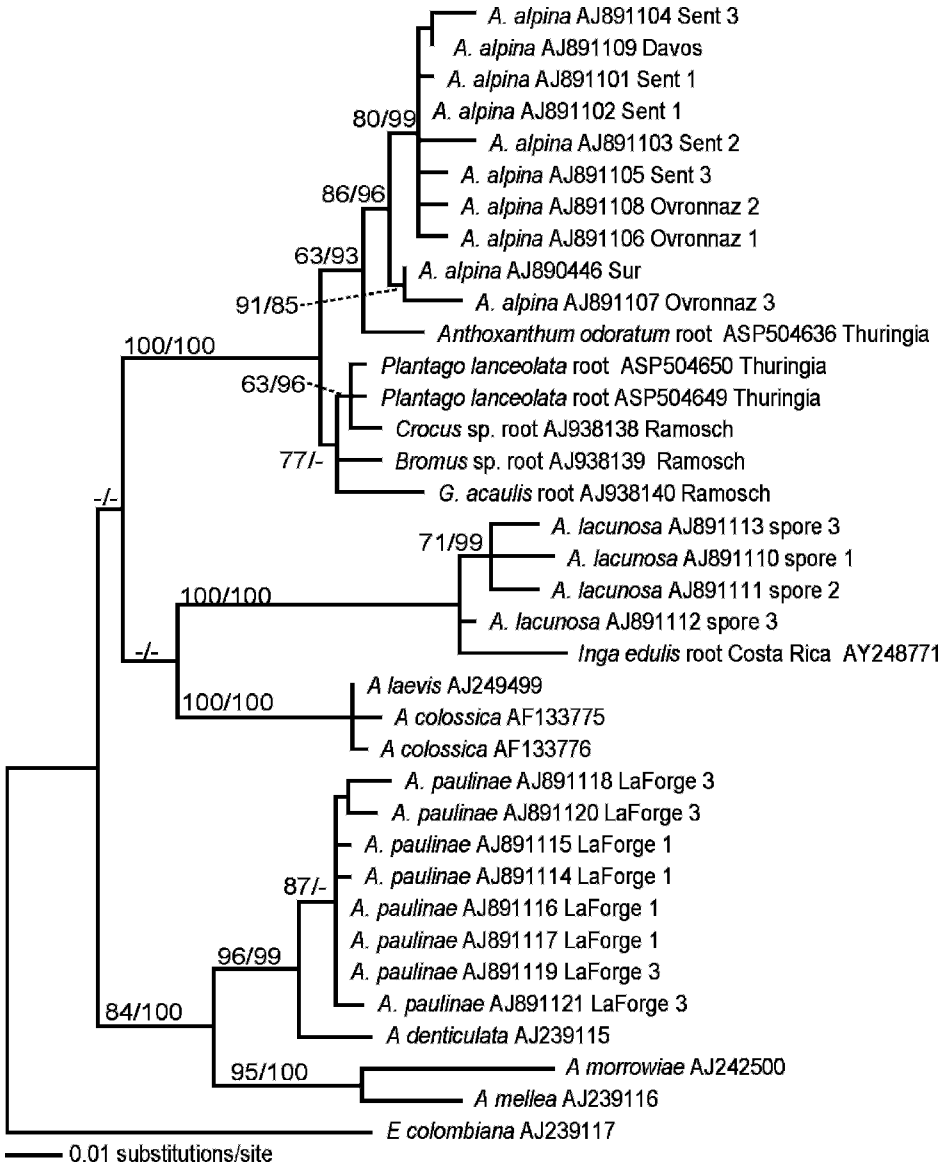
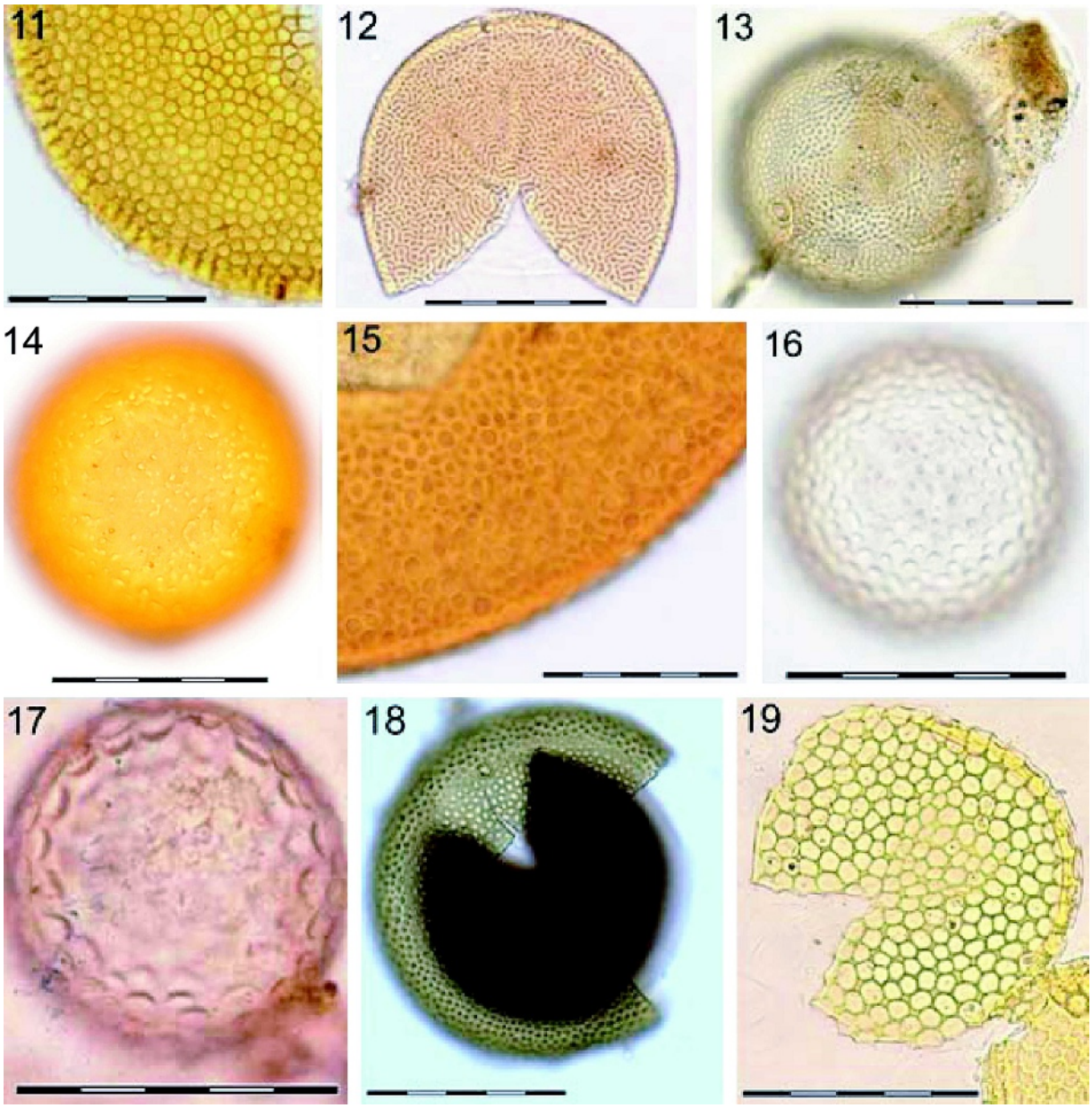


FIG. 10. Phylogenetic tree of *Acaulospora* spp. obtained by maximum likelihood analysis of 5.8SrDNA and ITS2 sequences. The left number above the line of each branch denotes the bootstrap value obtained from 1000 replicates of neighbor joining analysis (Felsenstein, 1985); the right number after the slash indicates the credibility value from Bayesian analysis. Sequence labels show the source organism, the database accession number and the locality. Multiple spores analyzed from the same site are numbered 1, 2, 3.

11. Spores hyaline to subhyaline 13
11. Spores yellow to orange brown, truncated cone shape pits of widest diameter of 1.5–2.2 µm . . . . . *A. alpina* (FIGS. 2, 3)
12. Spores regularly 100–180 µm diam. 14
12. Spores regularly >185 µm diam with concave round pits of widest diameter 4–10 µm . . . . . .*A. foveata* (FIG. 15)
13. Spores hyaline to subhyaline, concave round pits of widest diameter < 3.5 µm . . . . *A. paulinae* (FIG. 16)
13. Spores hyaline to subhyaline, concave round pits of widest diameter > 3.5 µm . . . . *A. undulata* (FIG. 17)
14. Spores yellow brown, 115–170 µm diam with concave round pits of widest diameter 2–5 µm. . . . . *A. cavernata* (FIG. 18)
14. Spores ochre to brown, 100–180(–200) µm diameter with concave round pits of widest diameter 4–20 µm. . . . . *A. excavata* (FIG. 19)

DISCUSSION

Spores of the genus *Acaulospora* share several features. With the exception of *A. undulata* and *A. myriocarpa* Spain, Sieverd. & N.C. Schenck (Schenck et al 1986) (see below), they have three walls



FIGS. 11–19. *Acaulospora* spp. with ornamented outer spore wall (sw). 11. *A. denticulata* (ISOTYPE, OSC No. 46,713) with polygonal knobby projections with a central depression on the rounded tops; bar = 50 µm. 12. *A. rehmii* (ex type, C–116–6, Sieverding collection) with ridges and depressions appearing as cerebriform folds; bar = 100 µm. 13. *A. scrobiculata* (ISOTYPE, BOLIVIA, Santa Cruz de la Sierra, Oehl collection) with regular or y-shape pits, bar = 100 µm. 14. *A. lacunosa* (isotype obtained from the International Bank for the Glomeromyceta, BEG78) with irregular, saucer-shape pits; bar = 100 µm. 15. Big-spored *A. foveata* (culture C–48–1, Sieverding collection, described in Schenck et al (1984); bar = 100 µm. 16. *A. paulinae* (ISOTYPE, SWITZERLAND, Therwil (Basel), deposited by Oehl at Z+ZT) with round concave pits; bar = 75 µm. 17. *A. undulata* (ex type, Sieverding collection) with large depressions (undulations), bar = 75 µm. 18. *A. cavernata* (ISOTYPE, GERMANY, Black Forest, Glottertal; deposited by Oehl at Z+ZT) with round pits, 3–5 µm wide, inner wall layer stained purple in Melzer's reagent; bar = 100 µm. 19. *A. excavata* (ISOTYPE, OSC No. 83,345) with large and deep globose depressions; bar = 100 µm.

(Stürmer and Morton 1999): an outer spore wall (sw), a middle wall (mw) and an inner wall (iw) using our spore wall terminology. The innermost wall (iw) is the wall from where spores germinate, and a so-called germination orb may be involved in the germination process (Spain 1992). The outer wall generally is trilayered, the middle wall is bilayered and the germinal inner wall is bi- or trilayered. Some of these

layers are often difficult to discern (e.g. the innermost layer of the outer wall and some of the layers of the inner wall). Also the mounting medium can have a strong influence on the visibility of fine wall layers and, therefore, difficult to see layers should be observed in water (Spain 1990). Despite these common features in all Acaulosporaceae, the spores of the new species *A. alpina* can be distinguished easily from all others by the unique surface ornamentation and by a combination of several other morphological characteristics. These are the small spore size, the dark yellow to orange-brown spores, the apparent absence of a ''beaded'' layer in the inner wall and the weak, sometimes absent, staining reaction of iw2 in Melzer's reagent.

Three AM fungal species have similarities in spore morphology with *A. alpina*. *Acaulospora taiwania* shares spore size and spore color but forms the spores in sporocarps and not singly in the soil as *A. alpina*. Furthermore the ornamentation on the spore wall of *A. taiwania* consists of 4–5-sided pits that give the appearance of a mesh. The morphological definition of *A. paulinae* is broad and there is overlap with *A. alpina*. However the ornamentation structures of *A. paulinae* are coarser, less regular and consist of concave pits or depressions (FIG. 16), and not of truncated conic depressions as in *A. alpina* (FIGS. 5, 8). Also spores of *A. paulinae* have a significant ''beaded'' inner wall layer, and the innermost layer stains strongly in Melzer's reagent. Spores of *A. lacunosa* are similar in color to those of *A. alpina* but they are bigger. Moreover *A. lacunosa* has irregular depressions on the spore surface, a beaded inner wall layer, and one of the inner wall layers stains dark purple in Melzer's reagent.

Spores size of *A. undulata* is similar to that of *A. alpina*. However they are white to creamy and the round concave pits are generally wider in diameter. Furthermore in *A. undulata* the middle wall is lacking and the inner wall bears some similarity to the inner wall of some species of the genus *Archaeospora* J.B. Morton & D. Redecker (Morton and Redecker 2001). The root infection structures of *A. undulata* stain only weakly with trypan-blue, and vesicles were scarce (E. Sieverding unpublished observations). These features are typical for members of the genus *Archaeospora*. Spores of *A. myriocarpa* also lack the middle spore wall (see above) and the root infection structures of *A. mycriocarpa* (Schenck et al 1986) resemble those of Archaeosporaceae (Morton and Redecker 2001) too. Based on these observations it is possible that *A. undulata* and *A. myriocarpa* both are members of Archaeosporaceae.

Phylogenetically both *A. paulinae* and *A. lacunosa* clearly are separated from *A. alpina* (FIG. 10). These three species are not related. Comparison of sequences obtained from spores to those from field-collected roots allows additional insights into the occurrence and ecological range of AM fungal taxa. The closest relatives to *A. alpina* were detected in roots from a site close to one of our spore sampling sites (Ramosch, Engadin) and from a mountainous grassland (710 m a.s.l.) in central Germany (Renker et al 2003) (FIG. 10). These data suggest that species related to *A. alpina* may occur at alpine as well as lower altitude mountainous areas. Our analyses also show that previously unnamed environmental sequences from Costa Rica (AY248771) apparently belong to *A. lacunosa*. Some other previously published sequences from this species (Millner et al 2001) are not related to *A. lacunosa*, and even not to the Glomeromycota. It is likely that the sequences belong to non-Glomeromycota fungi inhabiting AM fungal spores.

Acaulospora lacunosa was described from lower pH soil and soils with high aluminium concentration in West Virginia (Morton 1986). *Acaulospora paulinae* was reported to be widespread in grasslands and arable lands of Poland (Blaszkowski 1993). We found *A. paulinae* frequently in grasslands and arable lands of the upper Rhine lowland in France, Germany and Switzerland (Oehl et al 2003, 2004, 2005b) and lower mountainous regions, but restricted to decarbonated soils with pH of 4–6.5. With increasing altitude in the Alps spores of *A. paulinae* were found in decreasing spore numbers, but spores were found even up to 3000 m a.s.l. (www.nfp48.ch/projekte/projectdocs/17/Wiemken.pdf). In contrast spores of *A. alpina* were most abundant in grasslands of the high mountainous and alpine regions at 1900–2600 m a.s.l. Above 2700 m a.s.l. the species rarely was found. So far it was never detected in the lowlands or in the Swiss Alps in mountainous grasslands at altitudes below 1300 m a.s.l.

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