Nova Hedwigia, Vol. 108 (2019), Issue 3–4, 475–488
Published online December 5, 2018; published in print May 2019

Acaulospora tsugae, a new species in the *Glomeromycetes* from Taiwan, and a key to species in Acaulosporaceae

Tzu-Chao Lin[1], Gladstone Alves da Silva[2] and Fritz Oehl[3]*

[1] Endemic Species Research Institute, Jiji, Nantou, Taiwan

[2] Universidade Federal de Pernambuco, Av. da Engenharia s/n, Cidade Universitária, 50740-600, Recife, PE, Brazil

[3] Agroscope, Competence Division for Plants and Plant Products, Ecotoxicology, Müller-Thurgau-Strasse 29, CH-8820 Wädenswil, Switzerland

* Correspondence to: fritz.oehl@agroscope.admin.ch

With 8 figures

Abstract: *Acaulospora tsugae* sp. nov. was found in the rhizosphere soil of the alpine plant *Tsuga chinensis* var. *formosana* in Taiwan. It is here presented based on concomitant morphological and molecular spore analyses. The spores of the new fungus are 64–74×84–99 µm in diameter, subglobose to elliptical, pale yellow to yellow, and have three spore walls, which develop inside the neck of a sporiferous saccule. The spores have two persistent opposite cicatrices and differentiate a beaded inner wall that is characteristic for species of the Acaulosporaceae. Based on ITS sequences obtained from the ribosomal gene, a monophyletic clade within a major clade of the Acaulosporaceae is formed, next to several other species that were for the first time detected from montane to high alpine areas in Europe, such as *A. alpina*, *A. pustulata* and *A. tortuosa*. *Acaulospora tsugae* was also detected at one of multiple sites analyzed in the Swiss alpine regions, suggesting for this conspicuous fungus an infrequent, but intercontinental distribution. An updated identification key for all species in Acaulosporaceae is also included in this study.

Key words: Alps, arbuscular mycorrhiza, Asia, *Entrophospora,* Switzerland, Taiwan

Introduction

During the survey of hypogeous fungi colonizing alpine ecosystems in central mountains of Taiwan in 2008, soil samples were taken from the rhizosphere of the alpine plant *Tsuga chinensis* var. *formosana* (*Pinaceae*). A so far unreported species of the family *Acaulosporaceae* (Glomeromycetes) was found that forms entrophosporoid spores, i.e. within

www.borntraeger-cramer.com
DOI: 10.1127/nova_hedwigia/2018/0513

the hyphal neck (sometimes also called stalk) of terminal to intercalary sporiferous saccules.

Species with such characteristics were originally classified in the genus *Entrophospora* R.N. Ames & R.W. Schneid. (Ames & Schneider 1979). Sieverding & Oehl (2006) reorganized all five entrophosporoid species, described at that time, in three major groups, based on the spore formation relative to the saccule, spore wall characteristics, type and position of the proximal and distant cicatrices on the spore bases within the saccules, and on root colonization structures: i) *E. infrequens* and *E. baltica* remained in the genus *Entrophospora* of the new family Entrophosporaceae, ii) *Entrophospora colombiana* and *E. kentinensis* were transferred to the genus *Kuklospora* Oehl & Sieverd. within the family Acaulosporaceae, and *E. schenckii* was transferred to the genus *Intraspora* of the Archaeosporaceae. Kaonongbua et al. (2010) rejected *Kuklospora* as a valid monophyletic group based on molecular phylogenetic findings, while Oehl et al. (2011) continued with *Kuklospora* as the most ancestral genus within the family based on the type species *K. colombiana*. Moreover, Oehl et al. (2011) transferred another species of *Entrophospora*, *E. nevadensis*, to a newly erected genus, *Tricispora* with *T. nevadensis* (Diversisporaceae) and *E. baltica* to a new genus, *Sacculospora* with *S. baltica*, in the new family Sacculosporaceae (Diversisporales). Consequently, *Entrophospora* became a monospecific genus. The family Entrophosporaceae was included to Glomerales after detailed morphological and phylogenetic analyses. Willis et al. (2016) confirmed the genus *Sacculospora* as a legitimate, monophyletic genus within the order Diversisporales for *S. baltica* and the new species *S. felinovii*. In the meanwhile, Oehl et al. (2012) published a first extensive identification key for all species of the whole genus *Acaulospora* and acknowledged *A. kentinensis* (former *K. kentinensis*) as a member of the genus *Acaulospora* (Oehl et al. 2014), while *K. colombiana* remained in *Kuklospora.*

The objective of the present study was to elucidate the phylogenetic position of the new species of the genus *Acaulospora* isolated from *T. chinensis* based on concomitant morphological and phylogenetic spore analyses, and to present an identification key for all species of the family Acaulosporaceae.

Material and methods

Collection sites, isolation, and pot culturing: In August 2008 soil samples were collected from the rhizosphere (0–20 cm depth) of alpine plants *Tsuga chinensis* var. *formosana* in the central mountains in Taiwan. The collection site was on the southwest of the Taroko National Park, situated at 24°09’57” N and 121°14’12” E (2475 m above sea level). The soil is characterized by 13.4% organic matter, pH (H_2O) of 4.1 and 8.2 mg kg-1 available P. The climate is cool temperate climate, the highest and lowest monthly average temperatures are 11.5 °C and 1.3 °C, respectively, and the mean annual precipitation is 4300 mm.

Spores were isolated from the field soils by wet sieving (Gerdemann & Nicolson 1963) and sucrose centrifugation (Jenkins 1964), suspended in water, and divided into morphological groups using a stereomicroscope. Then, the categorized spores were used to inoculate *Sorghum bicolor* (*Gramineae*) growing in 500 mL pots (30 spores per pot) filled with autoclaved sand-vermiculite substrate (2:1; w/w), and placed in a greenhouse in the Taiwan Endemic Species Research Institute.

Morphological analyses: About 100 field-collected spores were mounted in PVLG, PVLG + Melzer's reagent, H_2O, and their microscopic characters examined (Brundrett et al. 1994). The spore structure terminology follows Goto & Maia (2006) and Oehl et al. (2012, 2014). Permanent slides are conserved at Taiwan Endemic Species Research Institute, Chichi, Nantou County, Taiwan (TAIE).

Molecular analyses: After isolation from field soil samples, spores were washed in ultrapure water and sonicated three times. The DNA was extracted from individual spores placed on a slide in a drop (5–10 µl) of ultrapure water, and crushed with a sterile needle. Crude DNA extract used as template to amplify the ITS rDNA region by nested PCR, using, in the first PCR, the universal eukaryotic primers SSU-Glom1 (Renker et al. 2003) and LSU-Glom1b (Walker et al. 2007), and the ITS1 and ITS4 (White et al. 1990) primers in the second run. Cycling parameters for the amplification of the DNA from ITS region were as described by Walker et al. (2007). PCR product from the second round of amplification was resolved by agarose gel electrophoresis, stained with ethidium bromide, and purified by using PCR Fragment Extraction Kit (Geneaid, Taiwan). The purified PCR fragment was cloned into yT&A Cloning Vector (Yeastern Biotech Co., Taiwan). The ligation product was used to transform DH5α*E. coli* competent cells and selection was performed on LB agar supplemented with 50 µg/ml ampicillin. The colonies were screened for the presence of insert by colony PCR. DNA sequencing was done by using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, v3.1 (Applied Biosystems) on the ABI PRISM 3730XL DNA Analyzer by Mission Biotech Co. (Taiwan). The new sequences were deposited in the GenBank database under the accession numbers MH045497-MH045498 and MH333277-MH333280.

Phylogeny: The AM fungal sequences obtained were aligned with other related glomeromycotean sequences from GenBank in ClustalX (Larkin et al. 2007) and edited with Bioedit (Hall 1999). *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüssler was included as the outgroup taxon. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian inference (two runs over 3×10^6 generations with a sample frequency of 300 and a burn-in value of 25%) 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 HKY + G model.

Results

Acaulospora tsugae T.C.Lin & Oehl sp. nov. (Figs 1–7)

Mycobank MB 827528

Diagnosis: *Acaulospora tsugae* differs from *A. kentinensis* by the lack of surface ornamentation of their spores, which are 64–74 × 84–99 µm in diameter and pale yellow to yellow, formed within the neck of sporiferous saccules.

Holotype extracted from the rhizospheric soil of the alpine plant *Tsuga chinensis* var. *formosana*, TAIWAN, Nantou County, Ren-ai Township, 24°09'56.9"N; 121°14'19.9"E; 31 Aug. 2008, coll. T.C. Lin. (TAIE, slide no. Ltc267; ZT Myc 59203), deposited in the Mycological Herbarium at ETH Zurich (Z+ZT, in Switzerland).

Isotypes: Ltc 265-266 (TESRI), 31 Aug. 2008; deposited at Taiwan Endemic Species Research Institute (TESRI), Chichi, Nantou County, Taiwan, and at Z+ZT: (ZT Myc 59254; slides Ltc 267-268; Ltc/OF 269-271, mounted 16 August 2018).

Etymology: Latin *tsugae,* referring to the host, on which the new species was first found in rhizospheric soil of *T. chinensis* in Taiwan.

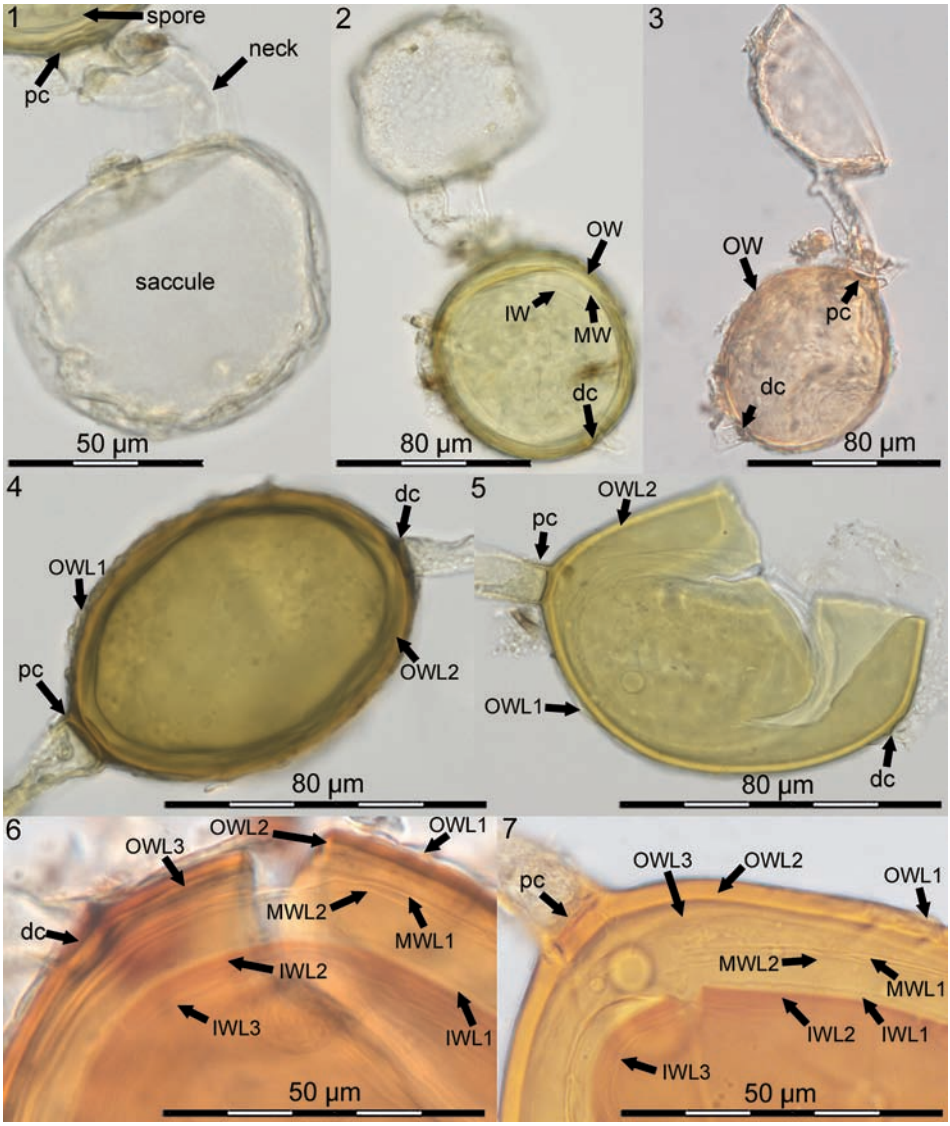
Description: The sporiferous saccules are hyaline to subhyaline, globose to subglobose, 65–100 µm, in diameter, with 1–2 wall layers that are in total 1.3–2.6 µm thick. The saccule neck is 16–34 µm broad at the saccule terminus, about 13–21 µm at the point of spore formation, and tapers to 8–13 µm in about 100 µm distance from the spore towards the mycelium.

Spores singly formed in soil by swelling within the hyphal neck of sporiferous saccules in 17–33 µm distance from the saccule termini. They are subglobose to elliptical, 64–74×84–99 µm in diameter and pale yellow to yellow brown. Spores have three walls (OW, MW & IW).

Outer spore wall consists of three layers (OWL1-OWL3). OWL1 is hyaline and continuous with the wall of the neck and the saccule, 0.8–1.5 µm thick, evanescent. OWL2 is pale yellow to yellow, laminate, smooth, 3.3–5.4 µm thick. OWL3 is concolorous with OWL2, 0.4–0.6 µm thick and often difficult to observe, but sometimes readily separates from OWL2 under pressure of the cover slide.

Middle wall is hyaline, bi-layered. Both layers (MWL1 and MWL2) are flexible, tightly adherent to each other and 2.1–3.5 µm thick in total. MWL1 is about 1.0–1.3 µm, and MWL2 is about 1.0–1.5 µm.

Inner wall is hyaline, with three layers (IWL1-IWL3). IWL1 is about 0.8–1.4 µm thick with a granular ‘beaded’ structure and tightly adherent to IWL2, which is 0.9–2.5 µm thick. IWL3 is about 0.5–1.0 µm thick and often also tightly adherent to IWL2, but sometimes readily separates through pressure applied to the cover slide. IWL2 stains faint purple to purple in Melzer’s reagent.



Figs 1–7. *Acaulospora tsugae*:1. Spore base with proximal cicatrix (pc) and neck of a sporiferous saccule towards the saccule terminus. 2–3. Spores formed intrahyphally in the neck of a sporiferous saccule, and thus with an additional distal cicatrix (dc) on the spore towards the hyphal mycelia, on which the sporiferous saccule had initially formed. Spores with three walls (OW, MW, IW). 4–5. Proximal (pc) and distal (dc) cicatrices on intact or crushed spores. Note the short, but clear continuation of the pigmentation of wall layer OWL2 into the neck of the sporiferous saccule, while the evanescent, hyaline outer layer OWL1 already is partly missing on the spore surface. 6–7. Spore segments with triple-layered OW (OWL1-3), bi-layered MW (MWL1-2), and triple-layered IW (IWL1-3). IWL2 staining faintly purple to purple in Melzer's reagent.

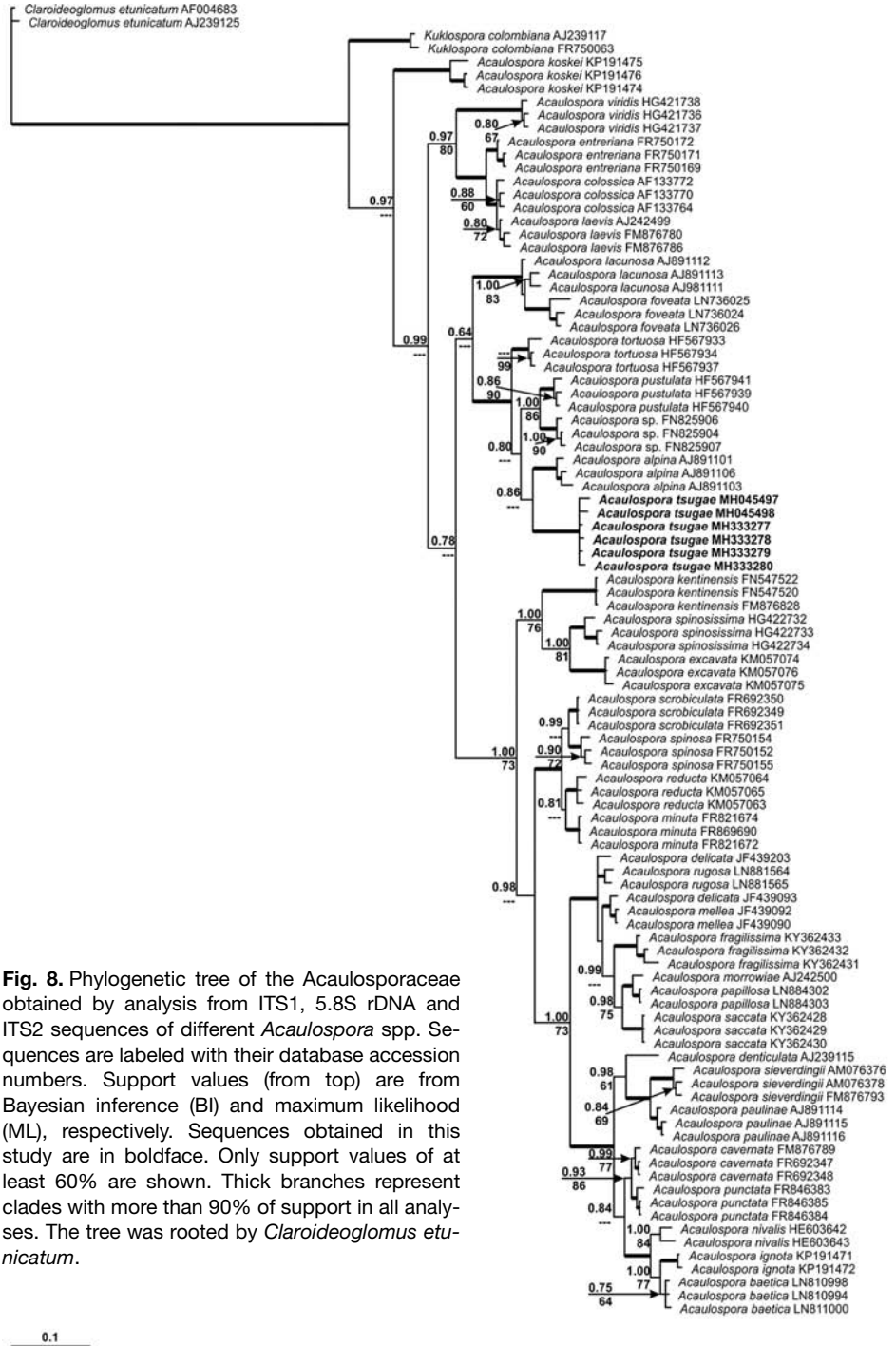


Fig. 8. Phylogenetic tree of the Acaulosporaceae obtained by analysis from ITS1, 5.8S rDNA and ITS2 sequences of different *Acaulospora* spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), respectively. Sequences obtained in this study are in boldface. Only support values of at least 60% are shown. Thick branches represent clades with more than 90% of support in all analyses. The tree was rooted by *Claroideoglomus etunicatum*.

The spore pore proximal to the sporiferous saccule is closed by continuation of the laminae of OWL2, and by OWL3, and forms a cicatrix (pc), 13–21 µm diameter. The second cicatrix (dc), also formed by OWL2 on the spore, but distant to the sporiferous saccule towards the mycelia hypha, is 7.9–11.2 µm in diameter. As formed on OWL2, both cicatrices are persistent and on the spore and concolorous with OWL2 of the spore. Remarkably, OWL2 generally reaches for 5–25 µm from the proximal cicatrix into the neck of the sporiferous saccule, while from the distal cicatrix only a hyaline neck hypha tapers towards the mycelia hyphae.

Mycorrhiza formation: unknown. Attempts to establish pure cultures of the fungus have failed since 2008.

Distribution: Hitherto known only from Taiwan, and from a few locations also from Switzerland. Spores of this species were collected only from the central mountains of Taiwan at high altitudes (about 2,500–2,900 m a.s.l.) in sites dominated by *chinensis* var. *formosana* (*Pinaceae*). In Switzerland, *A. tsugae* was found in a plant species-rich alpine pasture dominated by *Festuca violacea* at Furka pass at 2450 m a.s.l., where the young soil has formed basically by alternated freezing and thawing, called solifluction (46°34'16" N; 8°25'09" E; Oehl & Seitz 2018).

Specimen examined: Holotype (slide Ltc 267, deposited at Z+ZT), isotypes (slides Ltc 264-266, deposited at TESRI), isotypes (Ltc 268 and Ltc/OF 269-271, deposited at Z+ZT).

Phylogenetic analyses: The phylogenetic analyses from the ITS sequences placed the new fungus in a separate clade within the Acaulosporaceae, close to *A. alpina* (Fig. 8). The support values for the clade of the new species were 100% in all analyses. In the BLASTn analysis, the environmental ITS rDNA sequence with a closest match (94%) to *A. tsugae* was recovered from a grassland of Tibetan plateau in China (KF206474).

Key for *Acaulosporaceae* species sensu Oehl et al. (2014). A few species were not included in the key, since it has been assumed that they do not belong to the genus *Acaulospora* (e.g. *A. soloidea*; see Oehl et al. 2012) or might be synonymous with earlier described species (*A. walkeri* with *A. laevis*), or confused with species from other glomeromycotean orders ('*A. brasiliensis*' with *Ambispora brasiliensis*).

1 Spores apparently not formed on stalk of sporiferous saccules 2

1' Spores generally formed on/in stalk of sporiferous saccules 3

2 Spores white to pale ochraceous, 69–85 µm, IWL1 hyaline, beaded; IWL2 1.5–3.5 µm in PVLG, deep purple in Melzer's reagent . . . . . *A. saccata* D. Redecker et al.

2' Spores creamy white to pale ochraceous, 60–83 µm. IW not staining in Melzer's reagent except for IWL1, which may become light greenish yellow-ochre . . . . . *A. fragilissima* D. Redecker et al.

3 Spores with a single cicatrix at spore base 4

3' Spores with two persistent cicatrices at the spore, proximal and distal to the saccule terminus . . 47

4 Spores without ornamentation on the outer spore wall 5

4’ Spores with ornamentation on the outer spore wall 21

5 Spores generally < 100 µm 6

5’ Spores generally > 100 µm 13

6 Spores hyaline, white to subhyaline, to pale cream 7

6’ Spores significantly pigmented, darker than pale cream 8

7 Spores hyaline to white, (80–)94 (–115) µm . . . . . *A. polonica* Błaszk.

7’ Spores pale yellowish cream, 80–125 × 80–110 µm; OW turning slightly darker yellow but IW orange-red in Melzer’s . . . . . *A. delicata* C. Walker et al.

8 Spores yellow or ochreaous to light yellow brown 9

8’ Spores dark yellow brown to orange brown, (72–)95–105(–126) µm . . . . . *A. mellea* Spain & N.C. Schenck

9 Spores with a smooth surface 10

9’ Spores with a roughened, either papillae or rugose surface 11

10 Spores without reaction in Melzer’s reagent, spores pale yellow to yellow brown, (55–)65(–75) µm, a beaded wall hitherto not observed . . . . . *A. gedanensis* Błaszk.

10’ Spores with reaction in Melzer’s reagent 12

11 Spores with roughened surface due to the presence of small papillae (0.5–1.1 µm wide, 0.5–1.2 µm high, and in 0.5–1.1 µm distance from each other), yellow white to light yellow to creamy, 65–100 µm . . . . . *A. papillosa* C.M.R. Pereira & Oehl

11’ Spores with a rugose surface, generally with ballooning OWL1 in lactic acid based mountants, subhyaline to straw colored, (49–)92(–118) µm. . . . . *A. rugosa* J.B. Morton

12 Spores with a mucilaginous wall, dull to pale yellow, 75–90(–100) µm . . . . . *A. longula* Spain & N.C. Schenck

12’ Spores, without mucilaginous wall, bright yellow, sparkling in reflected light, 79–92(–120) µm . . . . . *A. morrowiae* Spain & N.C. Schenck

13 Spores hyaline to brilliant white, 145–317 µm . . . . . *A. splendida* Sieverd. et al.

13’ Spores with pigmented spore wall 14

14 Spores yellow, honey colored to yellow brown 15

14’ Spores reddish orange, orange brown to red brown, or greenish brown to dark brown. 16

15 Spores deep yellow to brownish yellow, (78–)106(–130) µm, with a roughened surface often resembling a minute, papillate surface . . . . . *A. dilatata* J.B. Morton

15’ Spores honey colored, dull yellow to yellow brown to olive brown, 120–300 × 120–520 µm . . . . . *A. laevis* Gerd. & Trappe

16 Spores without staining reaction on the outer wall in Melzer’s reagent 17

16’ Spores staining reddish brown on the inner layer of the outer wall in Melzer’s; spores reddish orange, (80–)183(–340) µm, IW purple to dark purple in Melzer’s. . . . . *A. koskei* Błaszk.

17 Spores with a rather thin, evanescent to rarely semi-persistent outer hyaline spore wall layer . . 18

17' Spores with a thick, semi-persistent to persistent outer hyaline spore wall layer, (150–)185 (–240) µm, dark brown to black . . . . . *A. thomii* Błaszk.

18 Spores without staining reaction on the inner wall in Melzer's. 19

18' Spores with a purple to dark purple staining reaction on IW in Melzer's 20

19 Spores brown, 260–330 µm . . . . . *A. entreriana* M.S. Velázquez & Cabello

19' Spores orange brown to dark red brown, 180–380 µm. . . . . *A. colossica* P.A. Schultz et al.

20 Spores orange-red to capsicum-red, (170–)298–330 µm. . . . . *A. capsicula* Błaszk.

20' Spores greenish yellow brown to greenish brown, 140–205 × 140–193 µm . . . . . *A. viridis* Palenz. et al.

21 Spores with spines, warts, pustules, or other regular to irregular projections 22

21' Spores with depressions (pits) or cerebriform folds 31

22' Spores with projections without a reticulum 23

22 Spores with projections and a reticulum 30

23 Spores with spines, tubercles, warts, pustules or other regular projections 24

23' Spores with tortuous hyphae-like structures on the surface that are subhyaline to pale yellow to sometimes dark yellow. These structures 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); spores yellow orange to orange brown, 61–84(–94) × 61–80(–91) µm . . . . . *A. tortuosa* Palenz. et al.

24 Spores with spines, tubercles, warts, or pustules 25

24' Spores with circular to oblong projections, 4–5(–9) µm wide and up to 3.2 µm high; each projection with a central cavity; (112–)130–175 µm. . . . . *A. denticulata* Sieverd. & S. Toro

25 Spores with spines, tubercles, or warts 26

25' Spores crowded with pustules; pale brown when young, becoming yellow brown with age, 45–65(–72) × 44–62(–68) µm. . . . . *A. pustulata* Palenz. et al.

26 Spores with spines or tubercles 27

26' Spores with evenly distributed warts or flattened elevations on OWL2, up to 1 µm high on the upper surface, frequently deteriorating with age, and then gradually becoming invisible; spores yellowish white to orange-yellow, 65–80 µm . . . . . *A. ignota* Błaszk. et al.

27 Spores with spines or tubercles formed on hyaline to subhyaline, evanescent to (semi-) persistent outer layer(s) of OW 28

27' Spores crowded with fine spines formed below the evanescent OWL1 on the upper surface of the structural, laminated, pigmented layer; spines about 1.0–2.9 µm high, 0.9–1.4 µm at the base, pointed to 0.5 µm broad at the top, and <1 µm apart; spores yellow brown to brown 74–98(–107) × 73–98 µm . . . . . *A. spinulifera* Oehl et al.

28 Structural layer on OW generally < 2.5 µm thick; spores light yellow when young, becoming bright yellow to brownish-yellow, 120–187 × 116–180 µm; second evanescent layer (OWL2) subhyaline, densely crowded with short spiny projections that are 0.5–1.1 µm high and 0.4–0.8 µm wide at base. . . . . *A. spinosissima* Oehl et al.

28' OW generally > 2.5 µm thick 29

29 Spores with fine crowded, densely organized spines, 1–4 µm tall, 1 µm at base and tapering to 0.5 µm at the tip; spores (110–)140–330 µm, yellow brown to brown to rarely dark brown . . . . . *A. spinosa* C. Walker & Trappe

29' 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; spores 250–340 µm, dark honey brown to reddish black. . . . . *A. tuberculata* Janos & Trappe

30 Reticulum three-layered enclosing polygonal projections ±1 × 1 µm; spores light brown to brown, generally 150–200 µm . . . . . *A. bireticulata* F.M.Rothwell & Trappe

30' Reticulum one-layered, overlaid over crowded, densely-organized spines ±2 µm high; spores yellow brown to dark brown, 140–330 µm. . . . . *A. elegans* Trappe & Gerd.

31 Spores with pits 32

31' Spores with cerebriform folds, (82–)112–175 µm . . . . . *A. rehmii* Sieverd. & S. Toro

32 Spores in sporocarps, 75–80 µm, ornamentation of 0.5–1 µm wide, 4–5 side pits, 1.2 × 0.5–1 µm across, ridges form mesh. . . . . *A. taiwania* H.T. Hu

32' Spores formed singly in soil, not in sporocarps 33

33 Spores regularly < 100 µm. 34

33' Spores regularly > 100 µm 40

34 Pits of irregular shape 35

34' Pits of regular round shape 37

35 Spores with a reticulum forming ridges between the pits; spores yellow, becoming mostly yellowish brown when mature, (50–)70–95(–112) µm, or occasionally ellipsoidal or ovoid 79–126 × 50–92 µm; OWL2 yellowish brown, 2.1–3.5 µm thick, uniformly ornamented with rounded (0.5–1.4 µm) to elliptical pits, 1.3–1.9 µm long, 0.9–1.4 µm wide, and 0.6–2.3 µm deep; some pits vermiform or 'rugulate', 2.2–4.8 long and 0.5–1.0 µm wide . . . . . *A. herrerae* Furrazola et al.

35' Spores generally without a reticulum 36

36 Spores 65–85 µm, hyaline to subhyaline to rarely light yellow, irregular pits resembling small dots (0.8–1.8 µm) or lines (0.5–1.2 × 1.8–2.5 µm . . . . . *A. sieverdingii* Oehl et al.

36' Spores 81–100 × 75–82 µm, bright yellow to dark yellow, with irregular pits, 0.9–1.5 × (0.9–) 1.5–3.5(–5.5) µm wide and 1.2–2.5 µm deep. . . . . *A. nivalis* Oehl et al.

37 Spores hyaline, subhyaline, pale yellow to creamy 38

37' Spores yellow to orange brown, or creamy brown to light brown 39

38 Spores hyaline to subhyaline, concave round pits of widest diameter <3.5 µm; (60–)72(–95) µm; pits 2.0–2.5 × 3.0–3.5 µm, when seen in a plan view, 0.8–1.0 µm deep . . . . . *A. paulinae* Błaszk.

38’ Spores pale yellow to creamy, concave round pits of widest diameter > 3.5–7.8 µm; 70–95 × 60–70 µm . . . . . *A. verna* Błaszk.

39 Spores yellow to orange brown, truncated cone shape pits of widest diameter of 1.5–2.2 µm; spore 65–85 µm . . . . . *A. alpina* Oehl et al.

39’ Spores creamy brown to light brown, often appearing with a grayish tint in water; spores 65–92 µm; pits about 0.8–1.6 × 0.7–1.4 µm wide, 0.6–1.3 µm deep and about (1.5–)2.2–5.1 µm apart . . . . . *A. baetica* Palenz. et al.

40 Pits of irregular shape 41

40’ Pits of regular round shape 42

41 Spores 100–240 µm, subhyaline to light olive, circular to ellipsoid to y-shaped pits, 1.0–1.5 × 1.0–3.0 µm . . . . . *A. scrobiculata* Trappe

41’ Spores 100–180 µm, reddish-yellow to yellow-brown, with irregular, saucer-shaped pits, 0.2–3 × 0.2–6 µm. . . . . *A. lacunosa* J.B. Morton

42 Spores regularly 100–180 µm 43

42’ Spores regularly >185 µm with concave round pits of widest diameter 4–10 µm. . . . . .*A. foveata* Trappe & Janos

43 Pits regularly < 2.0 µm 44

43’ Pits regularly > 2.0 µm 45

44 Spores subhyaline to yellow-white, 105–129 µm, pits 1.1–2.0(–2.7) µm wide and at least as deep (1.4–3.5 µm) as wide; distances of (1.1–)2.0–3.2 µm between the pits . . . . *A. punctata* Oehl et al.

44’ Spores bright yellow orange to orange brown, 150–220 µm, crowded with minute pits that are 0.5–1.2(–1.8) µm wide and 0.5–1.1 µm deep. . . . . *A. minuta* Oehl et al.

45 Spores without small pits and ridges within the large pits. 46

45’ Spore with secondary small pits (ca. 0.5 µm broad and deep) and fine ridges within irregularly shaped, often edged to sometimes dumbbell-shaped pits (5.5–19 × 3.5–8.6 µm) large pits; spore whitish yellow, dark yellow to light brown, 135–205 µm . . . . . *A. reducta* Oehl et al.

46 Spores yolk yellow to light brown, 115–170 µm, with concave round pits of widest diameter 2.5–5.0 µm . . . . . *A. cavernata* Błaszk.

46’ Spores pale ochraceous to yellow orange, 100–180(–200) µm, with concave round pits of widest diameter 4–20 µm . . . . . *A. excavata* Ingleby & C. Walker

47 Spores described to be formed either laterally on or within the neck of the sporiferous saccule, while saccule terminus was never observed; spores hyaline or subhyaline to pale yellow, 75–140 µm; OWL1 covered with irregularly spaced, hemispherical, hyaline to subhyaline protrusions, 0.5–3 mm wide and up to 1 mm high . . . . . *A. colliculosa* Kaonongbua et al.

47’ Spores exclusively formed intrahyphally within the neck of the sporiferous saccule 48

48 Spores with a strong proximal cicatrix, which continues from a few to several µm into the saccule neck 49

48’ Spores with a fine proximal cicatrix, which resembles a ring and does not continue into the saccule neck 50

49 Spores with smooth surfaces, pale yellow to yellow brown, 64–74×84–99 µm . . . . . *A. tsugae* T.C. Lin & Oehl

49’ Spores with pitted spore ornamentation, pale yellow to yellow brown, 85–140 × 95–210 µm; pits 1–3 µm, separated by ridges 2–6 µm. . . . . *A. kentinensis* (C.G. Wu & Y.S. Liu) Kaonongbua et al.

50 Spores with smooth surfaces, pale yellow to light golden brown, (75–)100–115(–135) µm . . . . . *Kuklospora colombiana* (Spain & N.C. Spain) Oehl & Sieverd.

50’ Spores with spiny spore ornamentation, subhyaline to yellowish-white, 100–250 µm; OWL2 crowded with fine spines, 2–3 µm high and 0.5 µm. . . . . *K. spinosa* B.P. Cai et al.

Discussion

The spore diameter of *Acaulospora tsugae* overlaps with several other *Acaulospora* species, but by its spore formation within the neck of the sporiferous saccule and its smooth spore surfaces, it can be easily distinguished from all of those species. Only *Kuklospora colombiana* has, like the new species, smooth spores, formed within the neck of the saccule, but its spores are generally larger, and the cicatrix proximal to the saccule is less pronounced than in *A. tsugae*.

The phylogeny confirms the distinct clade of *A. tsugae* within the *Acaulospora* species. Phylogenetically, the most closely related species to *A. tsugae* remarkably were all described from montane to alpine altitudes, but those have all ornamented surfaces, either pitted (*Acaulospora alpina*; Oehl et al. 2006) or with pustulate or tortuous projections (*A. pustulata* and *A. tortuosa*; Palenzuela et al. 2013).

The new identification key for Acaulosporaceae species comprises 51 species. Many of these species were described within the last twelve years based on concomitant morphological and phylogenetic analyses. The combination of both methods allowed an enormous progress on both morphological and molecular based species identification in a group of fungi that had only 2–4 members about 40 years ago, when the first two species of the family were described. Currently, the family Acaulosporaceae comprises one of the best studied arbuscular mycorrhizal fungal families, and several major clades can be recognized on the phylogenetic clades. Nevertheless, it was concluded that this fungal group is much more diverse than currently known, an assumption, which might be valid not only for its tropical (Oehl et al. 2014), but also for its arctic and alpine members (Oehl et al. 2006, 2012).

Acknowledgements

T.C.L is grateful to the Council of Agriculture (Taiwan) for financial support. F.O. acknowledges SNF for continuous support (current projects IZ73Z0_152740 and IZ76Z0_173895).

References

Ames, R.N. & Schneider, R.W. (1979): *Entrophospora*, a new genus in the Endogonaceae. – Mycotaxon 2: 347–352.

Brundrett, M., Melville L. & Peterson, L. (1994): Practical Methods in Mycorrhizal Research. – University of Guelph, Guelph, Ontario, Canada, Mycologue Publications.

Gerdemann, J.W. & Nicolson, T.H. (1963): Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. – Trans. Br. Mycol. Soc. 46: 235–244.

Goto, B.T. & Maia, L.C. (2006): Glomerospores, a new denomination for the spores of *Glomeromycota*, a group molecularly distinct from *Zygomycota*. – Mycotaxon 96: 129–132.

Guindon, S. & Gascuel, O. (2003): A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. – Syst. Biol. 52: 696–704.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucl. Acids Symp. Ser. 41: 95–98.

Jenkins, W.R. (1964): A rapid centrifugal-flotation technique for separating nematodes from soil. – Plant Dis. Rep. 48: 692.

Kaonongbua, W., Morton, J.B. & Bever, J.D. (2010): Taxonomic revision transferring species in *Kuklospora* to *Acaulospora* (Glomeromycota) and a description of *Acaulospora colliculosa* sp. nov. from field collected spores. – Mycologia 102: 1497–1509.

Larkin, M.A., Blackshields G., Brown, N.P., Chenna, R., McGettigan, P.A. et al. (2007): Clustal W and Clustal X version 2.0. – Bioinformatics 23: 2947–2948.

Milne, I., Wright, F., Rowe, G., Marshal, D.F. & Husmeier, D. et al. (2004): TOPALi: Software for automatic identification of recombinant sequences within DNA multiple alignments. – Bioinformatics 20: 1806–1807.

Oehl, F., Palenzuela, J., Sánchez-Castro, I., Kuss, P., Sieverding, E. & Silva, G.A. (2012): *Acaulospora nivalis*, a new fungus in the Glomeromycetes, characteristic for high alpine and nival altitudes of the Swiss Alps. – Nova Hedwigia 95: 105–122.

Oehl, F. & Seitz, B. (2018): Endomykorrhiza: mikroskopische Bodenpilze. – In: Hiltbrunner, E. & Körner, C. (eds.): Hotspot Furka. Biologische Vielfalt im Gebirge. Alpine Forschungs- und Ausbildungsstation Furka und Universität Basel. 60 p., ISBN 978-3-033-06701-1, available online on www.alpfor.ch.

Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K. & Silva, G.A. (2011): Advances in Glomeromycota taxonomy and classification. – IMA Fungus 2: 191–199. doi: 10.5598/imafungus.2011.02.02.10

Oehl, F., Sykorová, Z., Redecker, D., Wiemken, A. & Sieverding, E. (2006): *Acaulospora alpina*, a new arbuscular mycorrhizal fungal species characteristic for high mountainous and alpine regions of the Swiss Alps. – Mycologia 98: 286–294.

Oehl, F., Tchabi, A., Silva, G.A., Sánchez-Castro, I., Palenzuela, J., Monte Júnior, I.P., Lawouin, L.E., Coyne, D. & Hountondj, F.C.C. (2014): *Acaulospora spinosissima*, a new arbuscular mycorrhizal fungus from the Southern Guinea Savanna in Benin. – Sydowia 66: 29–42.

Palenzuela, J., Azcón-Aguilar, C., Barea, J.M., Silva, G.A. & Oehl, F. (2013): *Acaulospora pustulata* and *Acaulospora tortuosa*, two new species in the Glomeromycota from Sierra Nevada National Park (southern Spain). – Nova Hedwigia 97: 305–319.

Renker, C., Heinrichs, J., Kaldorf, M. & Buscot, F. (2003): Combining nested PCR and restriction digest of the internal transcribed spacer region to characterize arbuscular mycorrhizal fungi on roots from the field. – Mycorrhiza 13: 191–198.

Ronquist, F. & Huelsenbeck, J.P. (2003): MrBayes 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics 19: 1572–1574.

Sieverding, E. & Oehl, F. (2006): Revision of *Entrophospora*, and description of *Kuklospora* and *Intraspora*, two new genera in the arbuscular mycorrhizal Glomeromycetes. – J. Appl. Bot. Food Qual. 80: 69–81.

Walker, C., Vestberg, M., Demircik, F., Stockinger, H., Saito, M., Sawaki, I. Nishmura, H. & Schüssler, A. (2007): Molecular phylogeny and new taxa in the Archaeosporales (Glomeromycota): *Ambispora fennica* gen. sp. nov., Ambisporaceae fam. nov., and emendation of Archaeospora and Archaeosporaceae. – Mycol. Res. 111: 137–153.

White, T.J., Bruns, T.D., Lee, S.B. & Taylor, J.W. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. – In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (eds.): PCR protocols a guide to methods and applications, Academic Press, New York, pp. 315–322.

Willis, A., Błaszkowski, J., Prabhu, T., Chwat, G., Góralska, A., Sashidhar, B., Harris, P., D'Souza, J., Vaingankar, J. & Adholeya, A. (2016): *Sacculospora felinovii*, a novel arbuscular mycorrhizal fungal species (Glomeromycota) from dunes on the west coast of India. – Mycol. Progress 15: 791–798.

Manuscript received: August 24, 2018
Accepted: September 24, 2018