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Taxonomic revision transferring species in *Kuklospora* to *Acaulospora* (Glomeromycota) and a description of *Acaulospora colliculosa* sp. nov. from field collected spores

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Abstract: In a phylogenetic study of arbuscular mycorrhizal fungal species in *Acaulospora* (Acaulosporaceae, Glomeromycota) we discovered that species classified in genus *Kuklospora*, a supposed sister clade of *Acaulospora*, did not partition as a monophyletic clade. Species in these two genera can be distinguished only by the position of the spore relative to a precursor structure, the sporiferous saccule, as either within (entrophosporoid) or laterally (acaulosporoid) on the saccule subtending hypha. Subsequent spore differentiation follows identical patterns and organization. Molecular phylogeny reconstructed from nrLSU gene sequences, together with developmental data, support the hypothesis that the entrophosporoid mode of spore formation evolved many times and thus represents a convergent trait of little phylogenetic significance. Therefore genus *Kuklospora* is rejected as a valid monophyletic group and it is integrated taxonomically into genus *Acaulospora*. Thus *Acaulospora colombiana* and *Acaulospora kentinensis* are erected as new combinations (formerly *Kuklospora colombiana* and *Kuklospora kentinensis*). Mode of spore formation is demoted from a genus-specific character to one that is included with other traits to define *Acaulospora* species. In addition we describe a new AM fungal species, *Acaulospora colliculosa* (Acaulosporaceae), that originated from a tallgrass prairie in North America. Field-collected spores of *A. colliculosa* are small (<100 μm diam), hyaline or subhyaline to pale yellow and form via entrophosporoid development based on structure and organization of cicatrices and attached hyphae. Each spore consists of a bilayered spore wall and two bilayered inner walls. A germination orb likely forms after the completion of spore development to initiate germination, but this structure was not observed. A character distinguishing *A. colliculosa* from other *Acaulospora* species is hyaline to subhyaline hemispherical protuberances on the surface of the outer spore wall layer. A phylogeny reconstructed from partial nrLSU gene sequences unambiguously placed *A. colliculosa* in the *Acaulospora* clade.

Key words: Acaulosporaceae, *Entrophospora*, molecular identification, phylogeny, spore ontogeny, tallgrass prairie

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are important soil microbes because of their often beneficial roles in agricultural crop productivity, sustainability of plant communities and ecosystem functions (van der Heijden and Sanders 2002, Varma 2008). They are distributed worldwide in almost all habitats, and they form symbiotic relationships with a majority of land plants (Smith and Read 2008). They are thought to be ancient asexual organisms with unique biology and genetics (Sanders 2002, Bever et al. 2008). Our current understanding of the contribution of AM fungi to ecosystem functions is incomplete (van der Heijden et al. 2004) in part because of our limited knowledge on the biodiversity of AM fungi and the difficult nature of AM fungal taxonomy and species identification (Morton et al. 1995, Bever et al. 2001).

AM fungal taxonomy traditionally has been based on morphology of resting spores. Higher taxonomic rankings would be based primarily on mode of spore formation while species identification relied on spore size, color, number and organization of spore wall and other internal wall components, and the reaction of any wall layers to various mycological stains (Walker 1983, Morton 1988, Morton and Benny 1990). Ontogeny also was used to define morphological characters according to their origins and developmental states (Morton 1986, 1995; Franke and Morton 1994; Bentivenga and Morton 1995; Sturmer and Morton 1997, 1999). In the past few years many AM fungal researchers have been exploiting molecular approaches to identify species of AM fungi both from cultures and from field samples (Redecker et al. 2000, Morton and Redecker 2001, Kowalchuk et al.

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2002, Rosendahl and Stukenbrock 2004, Gamper and Leuchtmann 2007, Mummey and Rillig 2007). Combined with morphological data, this trend has led to several major taxonomic changes in Glomeromycota and new species descriptions (Blaszkowski et al. 2006, Oehl et al. 2006, Redecker et al. 2007, Walker et al. 2007, Palerizuela et al. 2008).

Family Acaulosporaceae J.B. Morton & Benny was described first in 1990 and included two genera, *Acaulospora* Gerd. & Trappe and *Entrophospora* R.N. Ames & R.W. Schneid. The family was defined by spores forming from the sporiferous saccule (Morton and Benny 1990). While members of *Acaulospora* form spores laterally on or to the side of the saccule neck (hereafter referred to as acaulosporoid development), *Entrophospora* species produce spores intercalary or inside the neck of the saccule (hereafter referred to as entrophosporoid development). These two modes of spore formation then result in either only one cicatrix (a scar marking the location where the spore has been attached) on acaulosporoid spores or two cicatrices on entrophosporoid spores. Berch (1985) emended genus *Acaulospora* to accommodate also species that form spores in loose aggregates. Since then several members of *Acaulospora* sensu Gerd. & Trappe emend. S.M. Berch have been transferred to other taxa based on combined morphological and molecular evidence (Morton and Redecker 2001, Walker et al. 2007).

Entrophospora sensu R.N. Ames & R.W. Schneid. was partitioned into three distinct groups based on morphological features (Sieverding and Oehl 2006). Two species were retained in *Entrophospora* (*E. infrequens* [I.R. Hall] R.N. Ames & R.W. Schneid. and *E. baltica* Blasz., Madej & Tadych), but the genus was placed in a new family, Entrophosporaceae Oehl & Sieverd., with uncertain phylogenetic relationship. *Entrophospora schenckii* Sieverd. & S. Toro was transferred to a new genus, *Intraspora* Oehl & Sieverd., in an ancestral family, Archaeosporaceae J.B. Morton & D. Redecker. Two species, *E. colombiana* Spain & N.C. Schenck and *E. kentinensis* C.G. Wu & Y.S. Liu, remained in family Acaulosporaceae but were transferred to a new genus, *Kuklospora* Oehl & Sieverd.

The only ontogenetic distinction of species in *Acaulospora* and *Kuklospora* is mode of spore formation (acaulosporoid versus entrophosporoid respectively). These modes however have no effect on subsequent spore development and organization (Sturmer and Morton 1999). In fact without saccules or cicatrices to distinguish spore position spores of *Kuklospora* species are indistinguishable from those of species in *Acaulospora*. Rodriguez and co-workers (2001) hypothesized convergent evolution (specifically a development of sporiferous saccule before spore formation) among *E. infrequens* and other members of *Acaulospora*. Although their data with regards to *E. infrequens* were ambiguous, the molecular phylogenetic tree suggested that *Kuklospora* (represented by *A. colombiana*) was a distinct monophyletic clade in Acaulosporaceae. Oehl and Sieverding (2006) cited this work in their morphological arguments justifying erection of *Kuklospora*.

We discovered an undescribed species from field samples with evidence of an entrophosporoid mode of spore formation and other morphological characters that indicated membership in *Kuklospora*. Phylogenetic analysis of the large subunit of the nuclear ribosomal (nrLSU) gene sequences however placed this species firmly in the *Acaulospora* clade. We use both the morphological and molecular evidence of convergent evolution of the entrophosporoid mode of spore formation to show that *Kuklospora* is not a monophyletic genus and therefore must be rejected. *Acaulospora* is emended and members of *Kuklospora* as well as the new species, *Acaulospora colliculosa*, are incorporated in this genus. Because only field-collected spores of *A. colliculosa* were analyzed some characters were missing or ambiguous. A robust description of this species therefore was possible only through interpretation of observed physical traits, extrapolation of other traits based on phylogenetically conserved characters present in known related species and verification of relationships within *Acaulospora* based on nrLSU gene evolution.

MATERIALS AND METHODS

Sample collections and culture attempts.—Several soil samples were taken from a tallgrass prairie remnant in the Iroquois State Wildlife Area, Iroquois, Illinois, USA, Jul 2006–Apr 2007. Soils were sampled to a depth of 40 cm, placed in plastic bags and stored at 4 C. AM fungal spores were extracted from these samples by wet sieving and decanting followed by a 60% sucrose gradient centrifugation (Sylvia, Kaonongbua and Bever 2007). Subsamples also were used to establish pot cultures containing a mixture of tallgrass prairie native species (*Andropogon gerardii* Vitman, *Coreopsis palmata* Nutt., *Amorpha canescens* Pursh and *Lespedeza capitata* Michx) and growing in a local greenhouse for up to 2 y. Cultures produced spores of a range of AM fungal species but not of the entrophosporoid spore morphotype described in this paper. Healthy-appearing spores from the field also were inoculated onto roots of 10–14 d old *Sorghum bicolor* (L.) Moench seedlings, but these cultures also failed to produce fungal growth. Thus all specimens of the new fungal species characterized in this study originated from the field.

Morphological analyses.—Spore size of the new species was measured with an ocular micrometer, and color of spores

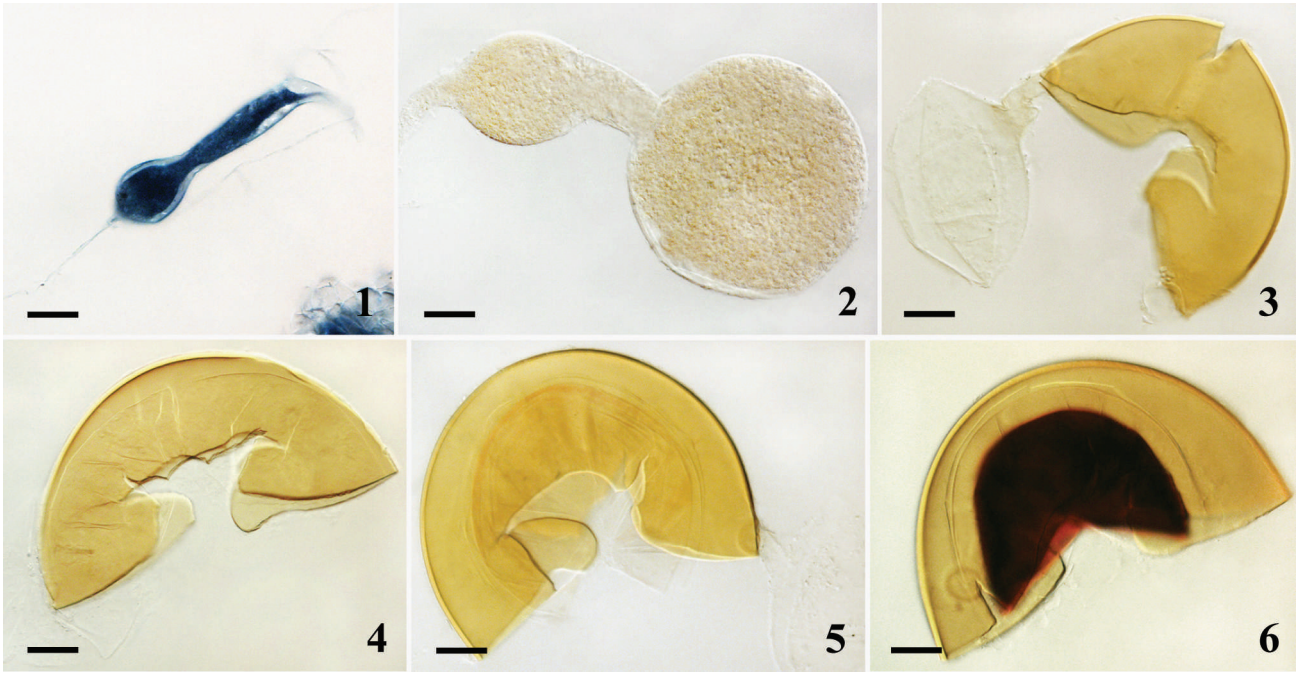
was determined under reflected light with a stereomicroscope. Spores were mounted in polyvinyl alcohol lactoglycerol (PVLG) and PVLG mixed with Melzer's reagent (1 : 1 v/v) to describe and measure dimensions of spore subcellular structures. Voucher specimens have been deposited at OSC (Corvallis, Oregon) and FH (Cambridge, Massachusetts) herbaria. Additional specimens also were deposited in the slide collection of the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, http://invam.caf.wvu.edu) at West Virginia University and in the personal collections of W. Kaonongbua and J.D. Bever. Selected images of spores were captured by a Nikon DXM1200C digital camera on a Nikon Eclipse E600 Microscope (Nikon Instruments, Melville, New York). Scanning electron microscope images were obtained at the microscopic facility of the Indiana Molecular Biology Institute, Indiana University, with standard protocols. Healthy-looking spores were treated 10 s in autoclaved 0.05% Tween 20 solution with a generic tabletop ultrasonic cleaner. Treated spores then were rinsed with autoclaved deionized water and deposited on a glass cover slip glued to a metal stub and air dried before being sputter coated with palladium-gold mixture. Coated spores were observed with a JSM-5800LV scanning electron microscope (JEOL USA, Peabody, Massachusetts) at 15 kv and the images captured digitally. The terminology for the spore structures is based on distinctions elucidated from ontogenetic patterns of origin and transformations (Sturmer and Morton 1999).

Molecular analyses.—Crude DNA was extracted by crushing single sonic-treated spores with either a plastic pipette tip or disposable plastic pestle in a 0.5 mL plastic tube containing 10 μL TE buffer (pH 8.3). The tubes were immersed in boiling water 2 min, snapped cool on ice and contents used either immediately as templates for polymerase chain reaction (PCR) or stored at −20 C until needed. DNA extracts were centrifuged at 12 000 rpm 2 min, and 5 μL supernatant from each extract was used as a template in a 25 μL reaction of nested PCR approach with the Premix ExTaq™ kit following manufacturer's instruction (Takara Bio, Madison, Wisconsin). The first round of PCR amplification used the primer pair ITS1 and NDL22 (van Tuinen et al. 1998), and the second round used primers LR1 and FLR2 (Trouvelot et al. 1999). The latter primers specifically targeted a region spanning the D1 and D2 regions of the nrLSU gene. One microliter primary PCR products, diluted to 1 : 20 with water, was used as template for the nested PCR procedure. Amplification sets consisted of an initial denaturation at 95 C for 4 min, 35 cycles at 94 C for 30 s, annealing at 58 C for 1 min, extension at 72 C for 1 min, and a final extension step at 72 C for 10 min. PCR products (5 μL) then were separated by electrophoresis on 1% agarose gel in TBE buffer, stained with ethidium bromide and viewed by UV illumination. Amplicons were purified with QIAquick PCR Purification kit (QIAGEN, Germantown, Maryland), and products were cloned with the TOPO TA cloning kit (Invitrogen, USA) following manufacturers' protocols. Positive clones were confirmed with direct colony PCR technique using M13F and M13R primers included in the cloning kit. Amplicons were purified as described above and later used as templates for sequencing with the BigDye® Terminator 3.1 on the 3730*xl* DNA analyzer (Applied Biosystems, USA). Each clone was sequenced from both directions with either M13F or M13R as sequencing primers.

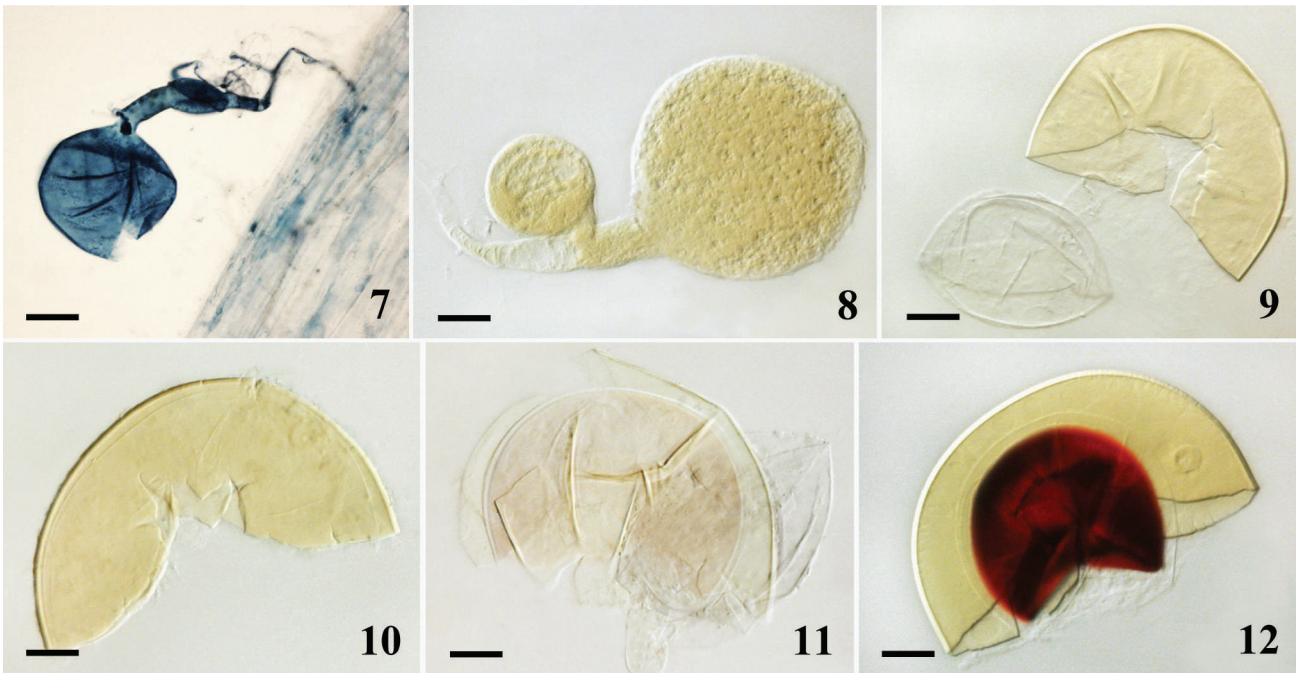
Phylogenetic analyses.—Sequences of both strands for each clone were aligned with BioEdit software (7.0.9.0, http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) and subjected to a BLAST query (Altschul et al. 1990) on the NCBI databases to verify homology with other glomeromycotan sequences. The nrLSU gene sequences of the new AM fungal species have been deposited at the NCBI's GenBank (GU326339–52). Within-species sequence similarity also was calculated with BioEdit software. Sequences of the new AM fungus together with representatives of other Glomeromycete nrLSU gene sequences, particularly those of members of Acaulosporaceae sensu Oehl & Sieverd., available in the public sequence databases, were analyzed together. Here we present only the details of our analyses on the Acaulosporaceae dataset. All sequences were aligned with Clustal X (2.0) (Larkin et al. 2007). The alignment was inspected and edited manually with BioEdit software before analysis for best fitted model with jModeltest software (Posada 2009). Aligned sequences then were used to reconstruct phylogenetic trees by the neighbor joining (NJ) method with Kimura two parameter and the maximum likelihood (ML) method with GTR + GAMMA + I model. MEGA4 (Tamura et al. 2007) was used to generate the NJ tree and an Online version of the RAxML (Stamatakis, Hoover and Rougemont 2008) as implemented by CIPRES (http://www.phylo.org/) to generate the ML tree. Sequences of *Ambispora appendicula* (AJ271712) (mistakenly labeled *Ambispora gerdemannii* in GenBank) and *Ambispora gerdemannii* (AJ510233) were used as outgroups. Statistical confidence was calculated by bootstrap analysis of 1000 replications for both analyses. All trees were viewed and manipulated with the FigTree software (1.2.2, http://tree.bio.ed.ac.uk/).

EMENDATION OF *ACAULOSPORA* AND NEW COMBINATIONS

Sieverding and Oehl (2006) distinguished species placed in genus *Kuklospora* from those in *Acaulospora* solely by position of spores in association with the subtending hypha of a sporiferous saccule. However these workers failed to consider the significance of parallel ontogenetic patterns of species in both genera. When discrete stages in ontogeny of *K. colombiana* CL356 (labeled *A. colombiana*) from saccule attached to a mycorrhizal root to mature spore (FIGS. 1–6) were compared with those of *A. mellea* CL649 (FIGS. 7–12), the only distinguishing morphological feature between these species is the mode of spore formation. The same comparison holds true when comparing *K. kentinensis* with any ornamented *Acaulospora* such as *A. scrobiculata* (not shown). These patterns indicate that divergence in spore position is a localized change that does not affect any later stages of spore development.



FIGS. 1–6. Discrete stages in ontogenesis of spores of *Acaulospora colombiana* CL356. 1. Saccule developing from hypha attached to a mycorrhizal root. 2. Spore initial forming within the hypha subtending a sporiferous saccule. 3. Spore with the spore wall differentiated; no germinal inner walls formed. 4. Spore with the first germinal inner wall synthesized. 5. Spore with the second germinal inner wall formed but not yet fully differentiated as indicated by pale reaction of the innermost layer in Melzer's reagent. 6. Fully differentiated spore in Melzer's reagent. Bars = 25 µm.



FIGS. 7–12. Discrete stages in ontogenesis of spores of *Acaulospora mellea* CL649. 7. Saccule developing from hypha attached to a mycorrhizal root. 8. Spore initial forming laterally from the hypha subtending a sporiferous saccule. 9. Spore with the spore wall differentiated; no germinal inner walls formed. 10. Spore with the first germinal inner wall synthesized. 11. Spore with the second germinal inner wall formed, but not yet fully differentiated as indicated by pale reaction of the innermost layer in Melzer's reagent. 12. Fully differentiated spore in Melzer's reagent. Bars = 25 µm.

Molecular evidence supported conclusions from morphological evidence. Phylogenetic trees of nrLSU gene sequences with either NJ or ML algorithms failed to support *Kuklospora* as a monophyletic clade distinct from the *Acaulospora* clade (FIGS. 13, 14). NJ and ML trees differed in topology of clades containing *K. colombiana* (labeled *A. colombiana*) and *A. koskei*, but both trees showed strong support for multiple independent origins of the entrophosporoid spore development in the Acaulosporaceae clade (FIGS. 13–14). For instance *K. kentinensis* (labeled *A. kentinensis*) is not a sister taxon of *K. colombiana* but instead clusters with strong support with *A. spinosa* and *A. tuberculata* and is nested within the Acaulosporaceae clade (FIGS. 13, 14).

The nrLSU gene evolution and ontogenetic evidence together provide strong evidence that the entrophosporoid mode of spore formation is not a unique innovation that results in a divergent monophyletic lineage. Instead this change appears to reflect a mutation within species that otherwise share both genetic and morphological traits with other *Acaulospora* species. Consequentially *Acaulospora* is emended to include new combinations that integrate congruent morphological and molecular datasets.

Acaulospora Gerd. & Trappe emend. Kaonongbua, J.B. Morton & Bever

Basionym: *Acaulospora* Gerd. & Trappe, Mycol. Mem. 5:31. 1974.

Synonym: *Kuklospora* Oehl & Sieverd., J. Applied Bot. Food Quality 80:74. 2006.

Type species: *Acaulospora laevis* Gerd. & Trappe, Mycol. Mem. 5:33. 1974.

Sporiferous saccule forms before spore formation, typically having a thin wall relative to that of the spore. Saccule may form terminally or intercalary near a hyphal tip. Spores are formed singly in soil mostly and more rarely in plant roots or in sporocarps. Spores form either on the side of or within the subtending hypha of the sporiferous saccule. A single cicatrix is found on spores that are borne laterally on the saccule neck and two cicatrices indicate intercalary development within the subtending hypha of the sporiferous saccule. Spores are globose, subglobose, ellipsoid or broadly fusiform and typically consist of a spore wall with 2–3 layers and at least one bilayered hyaline germinal wall synthesized within the spore independently (and consecutively in case of species with two germinal walls). The outer layer of the spore wall is continuous with the wall of the saccule's subtending hypha, whereas other layers of the spore wall develop de novo and therefore are formed independently of saccule or hyphal components. Spore wall ranges from hyaline to dark brown or even black, with a surface that can be smooth to highly ornamented. Layers of the germinal wall proximal to the spore wall when present are thin (<2 μm) and often are tightly adherent and thus sometimes difficult to separate. Neither layer reacts in Melzer's reagent in species discovered to date. The outer layer of the innermost germinal wall typically is covered with granular excrescences (or beads), while the inner layer either reacts slightly pink, light to dark red-brown, dark red-purple in Melzer's or no reaction at all, depending on species. A germination orb is formed between the two germinal walls or between the germinal wall and the spore wall when only a single germinal wall is present. Mycorrhizal structures consist of intraradical hyphae that often coil near entry points and typically grow parallel to the long axis of roots between cortical cells, arbuscules that stain with varying intensity and vesicles that vary considerably in shape.

Acaulospora colombiana (Spain & N.C. Schenck) Kaonongbua, J.B. Morton & Bever comb. nov.
MycoBank MB515730

Basionym: *Entrophospora colombiana* Spain & N.C. Schenck, Mycologia 76:693. 1984.

Synonym: *Kuklospora colombiana* (Spain & N.C. Schenck) Oehl & Sieverd., J. Appl. Bot. Food Quality 80:74. 2006.

Acaulospora kentinensis (C.G. WU & Y.S. Liu) Kaonongbua, J.B. Morton & Bever comb. nov.
MycoBank MB515731

Basionym: *Entrophospora kentinensis* C.G. WU & Y.S. Liu, Mycotaxon 53:287. 1995.

Synonym: *Kuklospora kentinensis* (C.G. WU & Y.S. Liu) Oehl & Sieverd., J. App. Bot. Food Quality 80:74. 2006.

NEW SPECIES DESCRIPTION

Acaulospora colliculosa Kaonongbua, J.B. Morton & Bever sp. nov. FIGS. 15–22
MycoBank MB515732

Sporocarpium ignotum. Sacculus sporifer non observatus. Sporae singulatim in solo factae, intra collum sacculi sporiferi, teste praesentiae cicatricum duarum in sporis singulis. Sporae globosae usque subglobosae (75–)84.25–84.72(–99.6) μm diam, et hyalinae vel subhyalinae usque leviter luteae luce reflexa. Spora unaquaeque e parietibus tribus formata: paries sporae et parietes duo interiores. Paries sporae e stratis duobus formatus, crassitie composita 3–4 μm. Stratum exterius subhyalinum usque leviter luteum, rigidum, et 2–3 μm crassum. Hujus strati superficies exterior protuberationibus hemisphaericis, irregulariter dispositis, hyalinis usque subhyalinis, 0.5–3 μm latis et plerumque <1 μm altis induta. Stratum interius subhyalinum usque leviter luteum, <1 μm crassum, et arcte ad stratum exterius adhaerens. Paries interior primus hyalinus, bistratus, et

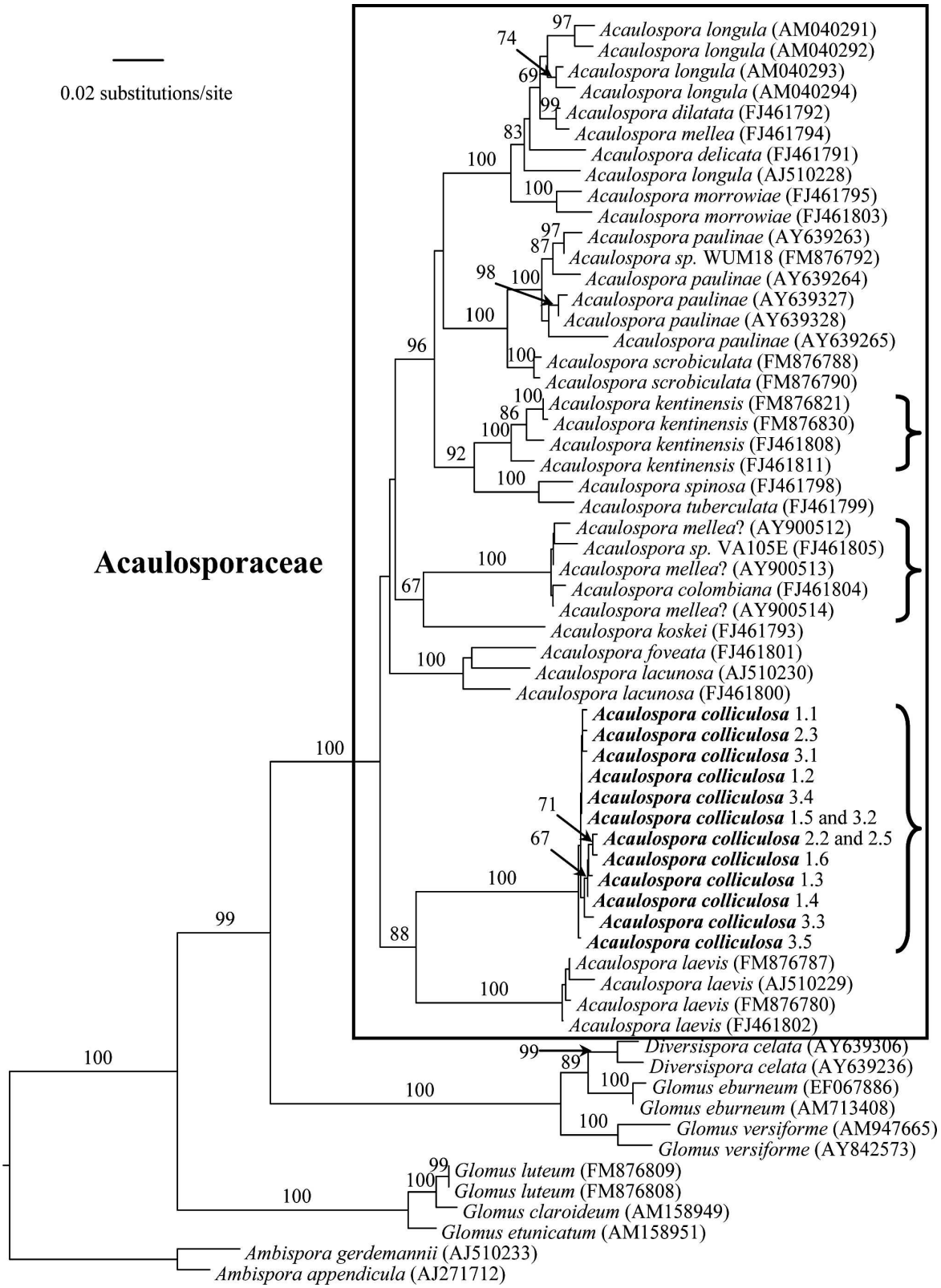


FIG. 13. Neighbor joining tree inferred from partial nrLSU sequences showing multiple origins of an entrophosporoid mode of spore formation within the Acaulosporaceae and the phylogenetic position of *A. colliculosa* in genus *Acaulospora*. Sequences from species with the entrophosporoid development are indicated with curly brackets while sequences of *A. colliculosa* also are shown in boldface followed by the first numbers represent spore numbers and the second numbers after the

crassitie composita <1 μm. Hic paries flexibilis, stratis ambobus arcte adhaerentibus et ergo saepe stratum unicum simulantibus. Paries interior secundus hyalinus, bistratus, et crassitie composita 1 μm. Hic paries flexibilis, stratis ambobus saepe arcte adhaerentibus, sed interdum separabilibus. Nulla excrescentia granularia in strato exteriore hujus parietis observata. Nulla strata parietum solutioni Melzeri respondentia.

Sporocarp has not been found.

Sporiferous saccule is not observed but assumed to have been formed and then sloughed based on presence of cicatrices on spores (FIGS. 15–18). Spores are formed singly in soil, within the subtending hypha of a saccule based on the presence of two cicatrices and attached hyphae on individual spores (FIGS. 17, 18). Walls of the attached hyphae are up to 1 μm thick and are covered with ornamentations similar to those of the spore wall, but smaller in width and height (FIGS. 15, 16). Presence of these ornamentations along the length of the hyphal remnants suggests that the sporiferous saccule might be covered with similar ornamentations. Remnant hyphae widen from 3–4 μm at the point of attachment to 8 μm in observed specimens.

Spores are usually globose to subglobose, (75–) 84.25–84.72(–99.6) μm diam, and hyaline or subhyaline to pale yellow in reflected light. Because of surface ornamentations (FIGS. 19, 20), they appear to have a dull surface under a stereomicroscope. Spores are composed of three discrete walls (FIGS. 21, 22) differentiated by ontogenetic patterns in related species (FIGS. 1–12).

Spore wall consists of two layers, with a composite thickness of 3–4 μm. The outer layer is subhyaline to pale yellow, rigid and 2–3 μm thick (FIGS. 21, 22). This layer is covered on the outer surface with irregularly spaced, hemispherical protrusions that are hyaline to subhyaline, 0.5–3 μm wide and usually up to 1 μm high (FIGS. 19, 20). The inner layer tightly adheres to the outer layer and gives the inner surface of the spore wall a slightly wrinkled appearance. When separated and visible, this layer is subhyaline to pale yellow and up to 1 μm thick. None of the spore wall layers reacts with Melzer's reagent.

Germinal wall 1 is hyaline, bilayered (FIG. 21) and with a composite thickness of up to 1 μm. This wall is flexible with both layers often tightly adherent and therefore appearing as one layer (FIG. 22). Neither layer reacts with the Melzer's (FIGS. 21, 22).

Germinal wall 2 is hyaline, bilayered, with a combined thickness of 1 μm. This wall is flexible with both layers often tightly adherent (FIG. 21) but sometimes separable (FIG. 22). No granular excrescences (beads) were observed on the outer layer of this wall. No reaction to Melzer's has been observed (FIGS. 21, 22).

Germination orb was not observed in collected specimens, and so details of its structure are not known at this time.

Etymology. Latin, *colliculosa*, meaning ''covered with little rounded or hillock-like elevations'' and referring to the unique ornamentation of the outer layer of the spore wall of this species.

Specimens examined. USA. ILLINOIS: Iroquois State Wildlife Area, 40°59′N, 87°33′W. Spores extracted from soil samples collected 14 Jul 2006. Type materials consisted of spores mounted on slides, *W. Kaonongbua*, ABS00040 (HOLOTYPE: OSC), *W. Kaonongbua*, ABS00022 (PARATYPE: OSC), *W. Kaonongbua*, ABS00001–2 (ISOTYPE: FH).

Distribution and habitat. So far *A. colliculosa* is known only from a single location in Iroquois State Wildlife Area (Illinois) in a patch of tallgrass prairie remnant of the Kankakee Sand region.

Molecular analyses. A total of 14 nrLSU gene sequences of approximately 660 bp long were obtained from three spores of *A. colliculosa*. These sequences were at least 98.9% similar. BLAST queries of all 14 sequences confirm their affinity to those of other members of Acaulosporaceae. NJ and ML phylogenetic analyses produce trees of similar topology and placed *A. colliculosa* within genus *Acaulospora* with strong phylogenetic supports (100% bootstrap support for the NJ analysis [FIG. 13] and 93% support for the ML analysis [FIG. 14]). Regardless of phylogenetic algorithm, *A. colliculosa* always clustered with *A. laevis*, the type species of *Acaulospora*.

DISCUSSION

Revision of Acaulospora.—Contrary to the taxonomic analysis by Sieverding and Oehl (2006), our phylogenetic analyses of the nrLSU gene clearly indicate that *Kuklospora* is a paraphyletic group and that the entrophosporoid mode of spore formation has multiple origins within *Acaulospora*. Regardless of the method of phylogeny reconstruction, *A. kentinensis* comb. nov., *A. colombiana* comb. nov. and *A. colliculosa* sp. nov. were distributed in separate clades

←

period represent clone numbers. The numbers on the tree braches are bootstrap support values based on 1000 replications of the neighbor joining analysis (values are not shown if below 67%). Sequences of *Ambispora appendicula* (AJ271712) and *Ambispora gerdemannii* (AJ510233) were used as outgroups. The scale indicates the number of base substitutions per site.

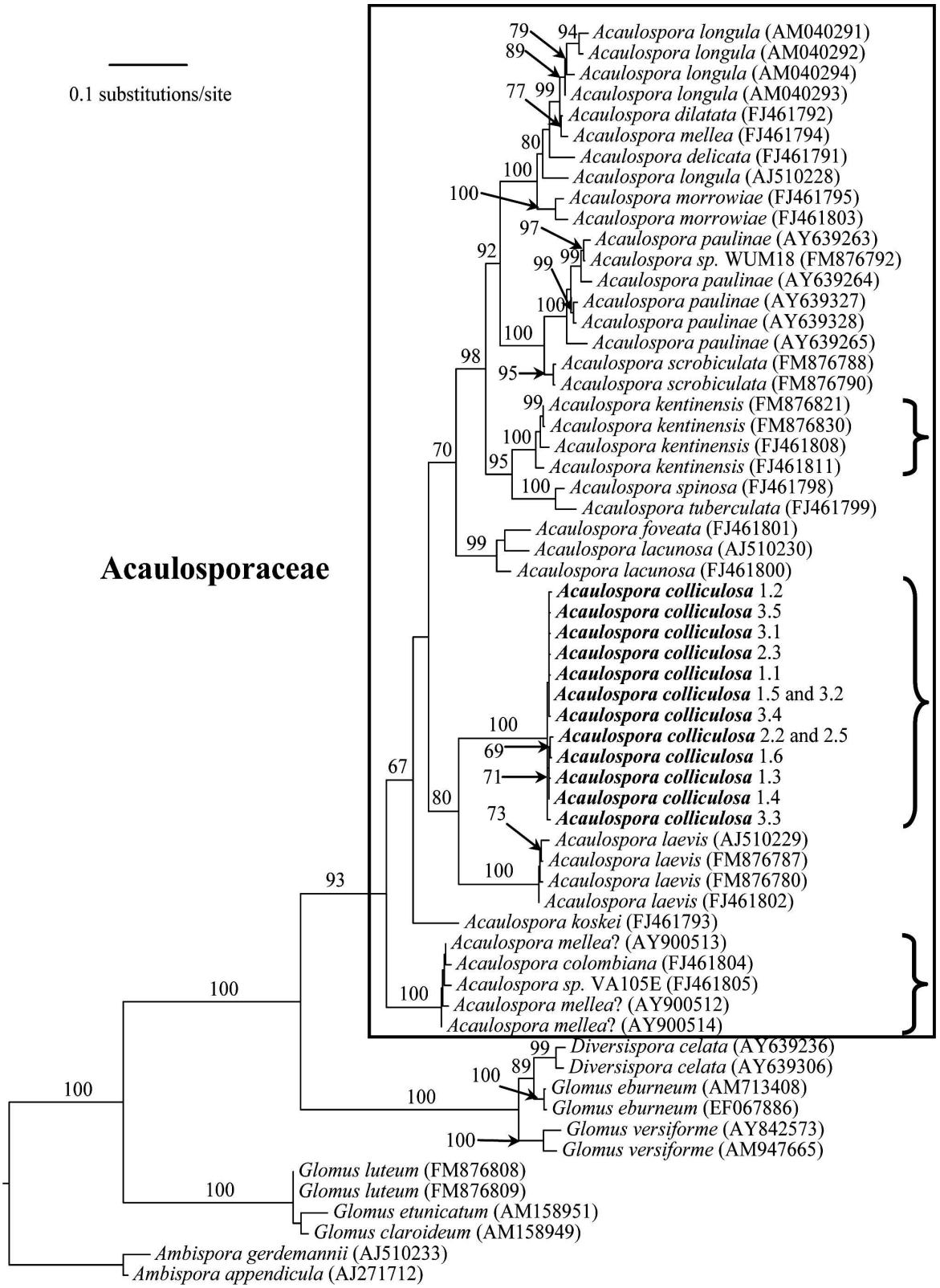
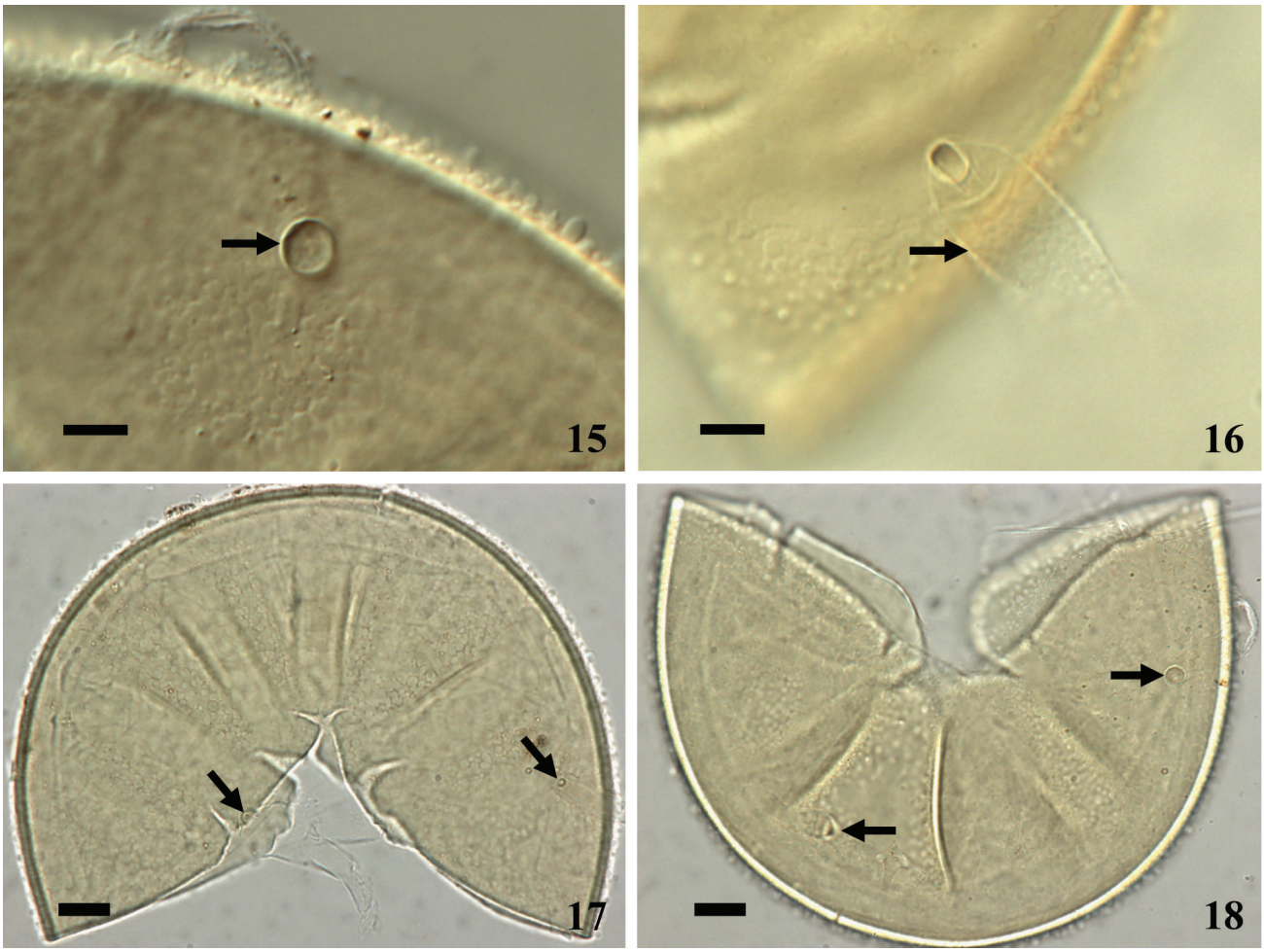


FIG. 14. Maximum likelihood tree inferred from partial nrLSU sequences showing the convergent evolution of the entrophosporoid developments in the Acaulosporaceae. The tree also indicates the phylogenetic position of *A. colliculosa* in genus *Acaulospora*. Sequences from species with entrophosporoid development are indicated with curly brackets while sequences of *A. colliculosa* also are shown in boldface followed by the first numbers represent spore numbers and the second



FIGS. 15–18. *Acaulospora colliculosa*. 15. Cicatrix (arrow) on spore surface with remnant of attached hypha. 16. Attached hypha (arrow) connected to a cicatrix and thus the spore. Note similar ornamentations on the attached hypha to that on the spore surface. 17, 18. Crushed spores in PVLG (17) or PVLG + Melzer's reagent (18) showing ornamentation of the spore wall and two attached hyphae/cicatrices (arrows). Bars: 15–16 = 5 µm, 17–18 = 10 µm.

(FIGS. 13, 14). Analysis of the SSU nrRNA gene also indicated that *A. colombiana* and an undescribed entrophosporoid species (WV201) nest within *Acaulospora* instead of as a unique monophyletic group (Walker et al. 2004). Similarly *A. colombiana* and *A. kentinensis* evolved from different immediate ancestors based on a phylogeny of partial sequences of the β-tubulin gene (Msiska and Morton 2009). Thus gene phylogenies within Acaulosporaceae indicate the entrophosporoid mode of spore formation is not a synapomorphy that defines a unique monophyletic group.

Three other congruent lines of morphological evidence support this conclusion. First, and most important, this trait arose in at least three lineages, each with their own unique suite of morphological characters (Sieverding and Oehl 2006). Second, a shift from acaulosporoid to entrophosporoid positioning (or vice versa) of the spore in the subtending hypha of the saccule does not alter later events in spore ontogenesis indicative of a localized mutation instead of an emergent trait in a common ancestor of these species (FIGS. 1–12). Third, the number of species with the entrophosporoid mode of formation is disproportionately rare compared to acaulosporoid initiation of spores. Of the 37 species in Acaulosporaceae currently described that share a number of phylogenetically informative traits only three are

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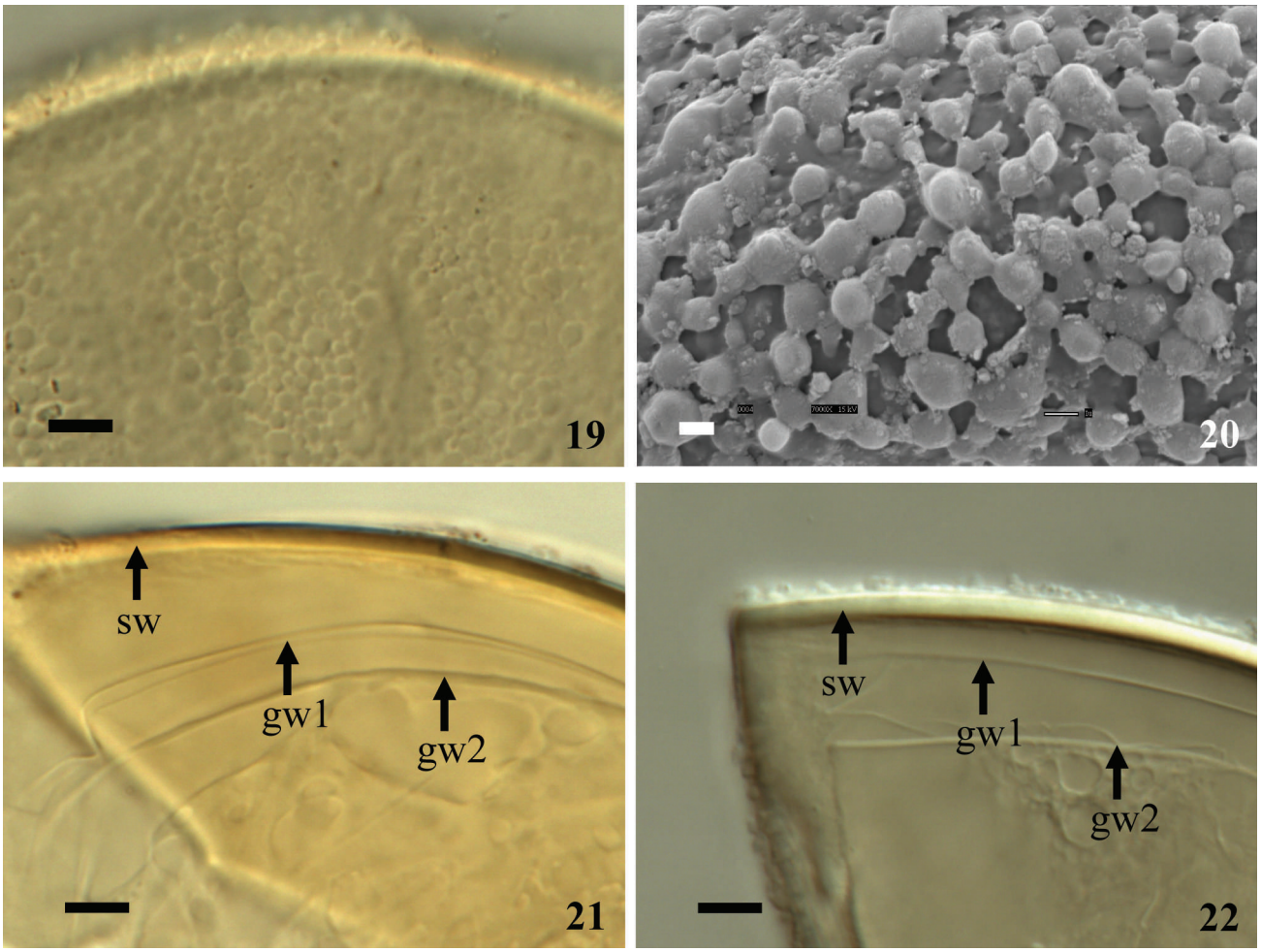
numbers after the period represent clone numbers. The numbers on tree braches are bootstrap support values based on 1000 replications of the ML analysis (values are not shown if below 67%). Sequences of *Ambispora appendicula* (AJ271712) and *Ambispora gerdemannii* (AJ510233) were used as outgroups. The scale indicates the number of base substitutions per site.



FIGS. 19–22. *Acaulospora colliculosa*. 19, 20. Detailed images showing the surface ornamentation of the spore wall under a light microscope (19) or an SEM (20). Note the irregular spacing between each of the hemispherical protuberances. 21, 22. Details of crushed spores in PVLG (21) or PVLG + Melzer's reagent (22) showing subcellular structures. Arrows indicate spore wall (sw) with typical three sublayers, the first (gw1) and second (gw2) bilayered germinal inner walls. Bars: 19, 21, 22 = 5 μm, 20 = 1 μm.

entrophosporoid. Collectively this evidence strongly supports the hypothesis that entrophosporoid mode of spore formation arose independently in different species with no perturbations in any other events during fungal ontogenesis, either in mycorrhizal development (not shown) or spore differentiation. Consequentially we reject *Kuklospora* as a valid monophyletic taxon and revise and expand the definition of genus *Acaulospora* to accommodate both modes of spore formation.

New species description.—A combination of morphological and molecular characters clearly places *A. colliculosa* in genus *Acaulospora*, family Acaulosporaceae. nrLSU gene sequences clearly identify *A. colliculosa* as a distinct and strongly supported lineage, thus providing justification for ranking the fungus as a new species (FIGS. 13, 14). The sequence data however was not helpful in distinguishing between acaulosporoid and entrophosporoid mode of spore formation because the latter evolved independently among species of Acaulosporaceae. Morphologically no sporiferous saccules were observed, but the presence of two cicatrices with remnant hyphae on numerous spores indicates an entrophosporoid mode of spore formation. Spores of *A. colliculosa* were similar in organization and subcellular structures to that in spores of other species in *Acaulospora*, which consists of a spore wall with 2–3 layers and one or two bilayered, hyaline, germinal wall(s) (Sturmer and Morton 1999).

Under a stereomicroscope spores of *A. colliculosa* are similar in size, shape and color to those of *A. morrowiae* and *A. delicata*. All these species produce

spores that are subhyaline to pale yellow-brown, although those of *A. colliculosa* generally are lighter and grade toward colorless (hyaline). Mean spore diameter of all three species is less than 100 μm and spores are almost uniformly globose to subglobose. However spores of *A. colliculosa* are ornamented (FIGS. 19, 20) so they have a duller appearance in reflected light than either *A. morrowiae* or *A. delicata*. Under a compound microscope surface ornamentations are easily discerned and are unique among *Acaulospora* species with ornamented spores for two reasons. First, most ornamented species produce spores covered with variations in pits instead of with projections (e.g. *A. kentinensis*, *A. lacunosa*, *A. paulinae* and *A. scrobiculata*). Second, the protuberances on the spore of *A. colliculosa* are hemispherical as compared with spines or tubercules on spores of *A. spinosa* and *A. tuberculata* respectively.

Although the spore wall of *A. colliculosa* has been observed to consist of two layers, it is likely that this wall also might have a missing outermost layer that is continuous with the wall of the saccule's subtending hypha. This layer is present on spores of *Acaulospora* species where the saccule is still attached, which is common in young pot cultures (Sturmer and Morton 1999). This layer typically sloughs when the spore detaches from the saccule.

Variation in germinal inner wall phenotypes in spores of *Acaulospora* species is limited mostly to the inner layer of the innermost wall (Sturmer and Morton 1999). This variation organizes species into three diagnostic (nonphylogenetic) groups where (i) this layer is thin and nonreactive to slightly pink in Melzer's reagent, (ii) slightly thicker and red-brown (dextrinoid) in Melzer's and (iii) ''amorphous'' or highly plastic and dark red-purple (amyloid) in Melzer's. These histochemical reactions are clearly discernible in newly formed spores but can be weak or absent in older or senescing field-collected spores (Morton 1986). A question thus arises whether the non-staining reaction in field-collected spores of *A. colliculosa* is a true phenotype or an artifact of environment and age. Close examination of this germinal innermost wall shows that both layers are thin (FIGS. 21, 22). The inner layer would be at least double the thickness observed if field conditions had modified this germinal inner wall from a dark-staining phenotype (Morton 1986). Therefore we conclude that healthy spores of *A. colliculosa* have a germinal innermost wall with a non- to pale-staining reaction similar to that of *A. laevis*, *A. delicata* and *Acaulospora* sp. VA105E. Of note, *A. laevis* is depicted as a sister species of *A. colliculosa* based on nrLSU gene sequence data (FIGS. 13, 14), although spores of *A. laevis* are larger, orange to red-brown and with a smooth surface.

Acaulospora colliculosa was detected with high frequency in an unplowed native prairie, having been collected from 11 of 16 samples. However no spores were recovered from intensive samplings of nearby post-agricultural fields suggesting the fungus might be sensitive to disturbance.

Additional phylogenetic inferences.—The clade containing *A. colombiana* (FJ461804) includes other sequences (accession No. AY900512–4) labeled *A. mellea* in public databases and an undescribed entrophosporoid *Acaulospora* species (VA105E, FJ461805) from INVAM (FIGS. 13, 14). However a sequence of acaulosporoid *A. mellea* (CR316B, FJ461794) from INVAM grouped within the *A. morrowiae* clade. Spores of *A. colombiana* easily could be mistaken as *A. mellea* in the absence of evidence for mode of spore formation (FIGS. 6, 12). Because *A. colombiana* and *A. mellea* sequences used here were obtained from monospecific cultures maintained for more than a decade in INVAM, the sequences AY900512–4 likely were misidentified from morphological criteria.

Nucleotide variation between the five sequences from *A. colombiana* lineage was low (FIGS. 13, 14), and this clade also included *Acaulospora* sp. VA105E. Spores of the latter species are morphologically identical to those of *A. colombiana* except that the innermost layer of the second germinal inner wall produces at most a pale pink in Melzer's compared to a dark red-purple reaction in *A. colombiana* spores. These observations suggest that the morphological groupings defined by Melzer's reaction of the second germinal inner wall are not phylogenetically significant, even though they are highly conserved character states and are shared by different species. The reaction to Melzer's appears to have evolved convergently, as evidenced also by species from divergent clades sharing a nonreactive germinal inner wall (*A. delicate*, *A. laevis* and *Acaulospora* sp. VA105E) (FIGS. 13, 14).

Although high variation of inter- and intraspecific nrLSU gene sequences have been reported for some species (Clapp et al. 2001, Rodriguez et al. 2001, Rodriguez et al. 2005), results of this study indicate that this gene correlates with morphology in the separation and identification of AMF species. Species such as *A. colliculosa*, *A. laevis*, *A. kentinensis*, and *A. paulinae* are clearly discernible lineages with adequate sampling of sequences from different isolates and even different spores of each species. Some sequences, such as those from *A. longula*, are interspersed among morphologically similar species

(FIGS. 13, 14). This pattern could be another instance of mislabeling or even an example of erroneously defining species based on morphological differences that represent population-level variation. On the other hand, the two clades of *A. longula* indeed might represent two different species, albeit ones with trait differences difficult to discern. Resolution of this issue will require collecting and analyzing additional data.

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