Acaulospora koreana, a New Species of Arbuscular Mycorrhizal Fungi (Glomeromycota) Associated with Roots of Woody Plants in Korea

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ABSTRACT
A new species of arbuscular mycorrhizal fungi (Glomeromycota), Acaulospora koreana, was isolated from forest soils in South Korea. This novel fungus was collected from the rhizosphere of Lindera obtusiloba and Styrax obassia in forest and propagated with Sorghum bicolor in pot. Morphological characteristics of spores of A. koreana are rarely distinguished from Acaulospora mellea, which is reported as one of the most abundant mycorrhizal species in Korea. However, molecular evidence of rDNA sequence using improved primers for glomeromycotan fungal identification strongly supported that A. koreana is different from A. mellea but also any other species belonging to the genus Acaulospora. This is the first novel glomeromycotan fungus introduced in South Korea, but it suggests that there is a high possibility for discovering new arbuscular mycorrhizal fungi considering the abundance of plant species and advanced phylogenetic analysis technique.

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1. Introduction
Arbuscular mycorrhizal fungi (AMF) belonging to Glomeromycota are the most common fungus of the terrestrial ecosystem and form a symbiotic relationship with the plant roots [1]. Instead of obtaining photosynthetic carbon from plants, AMF promote absorption of inorganic nutrients from the soil and increase the fitness of the host plant in harsh environments. The ecological importance of AMF has been widely recognized as it is known to affect the diversity and productivity of plant communities [2–4], but there are still difficulties in studying fungal characteristics of AMF because the systematics of AMF has not yet been fully understood. Identification of AMF is based on morphological characteristics such as spore size, color, spore structure, and rDNA sequence analysis [5–10]. However, they have a few morphological characteristics, which may vary depending on the development [11].

In addition, as some characteristics result from convergent evolution and do not reflect the phylogenetic status and relationships of AMF, the morphology-based identification of species has been the cause of misidentification in the past.

AMF have been identified using molecular phylogenetic analysis. Over, the past decade, a variety of molecular markers that can amplify various regions of DNA has been developed and the most frequently used markers for identification of species are on rDNA region [12]. Although the internal transcribed spacer (ITS) regions in rDNA are available as a valid molecular marker for taxa within Ascomycota and Basidiomycota, the ITS sequences of Glomeromycota is not suitable for identification of closely related species due to the high level of variation in intra-species, but 18S rDNA region has been widely used for identification of the fungi [13–16]. The primers proposed by Krüger et al. [9] are capable of amplifying a nucleotide sequence of about 1500 bp including the complete ITS and partial 18S and 28S regions of rDNA, and exhibit the best resolution for discrimination of AMF at the species level. Thus, the Krüger fragment has been proposed as the DNA barcoding region of AMF [10].

In this study, field soils were collected from the rhizosphere of various woody plants in the central part of Korea. AMF spores produced through trap cultures and single species culture were examined, and a previously undescribed Acaulospora species were found. The morphological characteristics of the spores and molecular phylogenetic analysis confirmed that the new strain belongs to the genus...
Figure 1. Morphology of *Acaulospora koreana*. (A) Spore size. (B) Murograph. 1 – evanescent, 2 – laminated, 3,4,5 – unit, 6 – membranous, 7 – amorphous. (C) Spores in dish water. (D–F) Spores of *Acaulospora koreana* in PVLG. Spore wall structures were indicated in E (L2, L3) and two bilayered germination wall (gw1, gw2) were showed in F. (G and H) Spore of *Acaulospora koreana* in Melzer’s reagent. Innermost wall (L2 of gw2) was stained as light pink in Melzer’s reagent. I: Various cicatrix of *Acaulospora koreana*. 
Acaulospora. We herein describe it as
Acaulospora koreana.

2. Materials and method

2.1. Soil sampling, single species cultures
Rhizosphere soils of Lindera obtusiloba Blume and Styx obasia Siebold et Zucc. were collected in Munan mountain (37° 37'19.02"N, 127° 19'47.16"E, altitude 535 m) of Namyangju in the central part of Korean peninsula. Soils and roots were collected within a radius of 20 cm around the root of the host plant and transferred to the laboratory. The field soil transferred to the laboratory was trap-cultured in the greenhouse. Sands sterilized at 121°C for 60 min and field soils were mixed at a ratio of 1:5:1 and then placed in a pot (15 cm x 17 cm) washed with 3% NaOCl and Sorghum bicolor seed sterilized with 70% ethanol was sown. Then plants were watered once a day and cultured for 12 weeks with low P (1/20) Hoagland solution once a week. AMF spores were extracted using a wet sieving and sucrose density gradient centrifugation method [17, 18]. Single spore obtained from the trap cultivation was inoculated into the roots of S. bicolor to mass-produce spores of single species. Roots of S. bicolor were stained with trypan blue (Acros Organics, Morris, NJ), and morphological features of mycorrhiza were observed [19].

2.2. Morphological characterization
After mounting spores in polyvinyl alcohol lactic acid glycerol (PVLG) and Melzer’s reagent, morphological characteristics and wall structures of spores were observed under microscopes and microphotographs were recorded by DIC microscope (AXIO Imager A1 & AXIO Cam ICc 1; Carl Zeiss, Oberkochen, Germany). Description of morphological characteristics of spore was performed as outlined by Stürmer and Morton [20] and Walker [21], and voucher specimens were deposited in Department of Biology Education, Korea National University of Education (Cheongiu, Korea).

2.3. DNA extraction, PCR, and phylogenetic analysis
Phylogenetic analysis was performed using the nucleotide sequences of the rDNA region. A healthy and clean spore was surface-sterilized with 70% ethanol and disrupted to extract the genomic DNA. Analysis of the Krüger fragments was performed in the DNA barcoding region of AMF for phylogenetic analysis. PCR was carried out using genomic DNA of crushed spores (Mycycler™ Thermal cycler; Bio-Rad, Hercules, CA) [9]. After the second PCR, 2 µl of the reaction mixture was loaded onto a 1.5% agarose gel containing Ecodye Nucleic Acid Staining Solution (BIOFACT, Seoul, Korea), and the results

Figure 2. (A–C) Arbuscule (AR), vesicles (V), hyphal coils (HC) of mycorrhizas of Acaulospora koreana stained with trypan blue. Scale bar = 50 µm.
were confirmed by electrophoresis (100 V, 25 mM). A band of about 1500 bp was identified and sequenced (Solgent, Daejeon, Korea). The resulting sequences were searched by NCBI (http://www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool). Each sequence was aligned using a MEGA 5 program and phylogenetically analyzed using Maximum-likelihood analysis [22]. Bayesian phylogenetic analysis was also performed using Mrbayes 3.2 [23], and the species was revealed by combining the results of the two systems. A GTR model was set for the nucleotide substitution model of maximum-likelihood and Bayesian phylogenetic analyses. Phylogenetic tree robustness were assessed by bootstrapping test (1000 replicates) for the maximum-likelihood tree and four incrementally heated chains (100,000 generations) for the Bayesian inference.

3. Results

3.1. Taxonomy

Acaulospora koreana E. H. Lee, S. H. Park & A. H. Eom, sp. nov. Figures 1 and 2.

Holotype: KNUE S1021

The spores were extracted from rhizosphere soils (Munan mountain in Korean peninsula, 37°37’19.02”N, 127° 19’47.16”E, altitude 535 m, 7 September 2010) of Lindera obtusiloba Blume and Styrax obassia Siebold et Zucc. The DNA sequence information for this specimen was deposited in GenBank (Accession number KY 565427-KY 565429)

Etymology: The specific epithet koreana was taken from the name of the nation from which the specimens were collected.

Spores are formed solely in soil. Small oil droplets shine in the spores, and the color is mostly light brown to dark brown. Spore shape is mostly round, with some oval and irregular. The size of the spore was (96–) 127 (–153) × (99–) 132 (–158) μm (n = 200) (Figure 1(A)). The ratio of the width to the long axis length of the spores, the width/length ratio, was 0.96, which was close to the circle. The spore walls consist of three layers of spore walls (L1–L3) and two layers of germinal walls (gw1, gw2) (Figure 1(B)). The outermost spore wall L1 was vitreous, thin and flexible with a thickness of less than 1 μm, and it is observed that other substances adhere to the surface of the spore, and it was associated with L2 and L3. The second spore wall L2 was a laminae layer consisting of small tacky layers, light yellow and 2–6 μm thick. The third spore wall L3 was a thin layer with a thickness of 1–4 μm and yellowish brown and not well separated, but it was a somewhat flexible wall when separated from the spore wall L2 and had a wrinkle similar to the inner germination wall (Figure 1(D–F)). The germination wall consisted of two walls (gw1, gw2) and was easily separated from the spore wall by a flexible glass wall (Figure 1(F, H)). The germination wall 1 (gw1) was composed of two transparent layers (L1, L2) like glass and was easily separated from the spore walls. Both layers were less than 1 μm in thickness, with two layers attached, most of which were seen as one layer and only two layers of spores.

The germination wall 2 (gw2) had two glassy layers (L1 and L2) bonded, but the reaction to Melzer’s reagent was different and was easily distinguishable. L1 of gw2 was vitreous with a thickness of less than 1 μm, and the outer surface had a droplet shape and looked rough when viewed under a microscope (Figure 1(H)). When the spore is broken, the inner layer containing L1 of gw2 may be separated from the spore and separated. There was no color change in Melzer’s reagent and easily distinguishable from L2 of gw2. L2 of gw2 was a thin, vinyl-like layer of glass, less than 1μm thick and stained with a deep purple color in Melzer’s reagent. It was confirmed that the color changed to light purple color with time. The spores were formed on the side of the hypha of the mycelium, which was adhered to the hyphal terminus, which swelled to 90–100 μm in diameter. The hyphal terminus was white and gradually become empty during spore formation, resulting in the formation of a hollow glassy pouch attached to the spore. Transparent hyphae attached on spores were observed. The hyphal terminus remained attached to the young spore and remained in water. The cicatrix on the surface of the spores shows the area of contact between the spore and the neck of sporiferous saccule during spore formation (Figure 1(I)). The scar was circular or elliptical with a diameter of 6–10 μm.

3.2. Mycorrhizal associations

Hyphal coil, arbuscules, and vesicles were observed when the infecting form was observed in the root (Figure 2(A–C)). Mycorrhizal structure and hyphae in roots were stained with trypan blue. Rectangular vesicles were occasionally formed and distributed locally, and hyphal coils were also distributed.

3.3. Phylogenetic analysis

Phylogenetic analysis showed that A. koreana is a distinctive lineage from other Acaulospora species. Morphological characteristics of A. koreana such as
spore size, color, wall structure and Melzer’s reagent staining of *A. koreana* were close to those of *A. mellea*, but BLAST results showed that the sequences of *A. koreana* have a relatively low similarity (<86%) with other species of the genus *Acaulospora*. Both Bayesian and maximum-likelihood analyses showed that *A. koreana* is closely related to *Acaulospora lacunosa* rather than *A. mellea* (Figures 3 and 4). However, *A. lacunosa* with irregularly shaped depressions in the outer

Figure 3. Phylogenetic relationship of *Acaulospora koreana* sp. nov. and allied species of *Acaulospora*, inferred by maximum-likelihood analysis using Krüger fragment (partial 18S rDNA, ITS1, 5.8S rDNA, ITS2, partial 28S rDNA). Numbers at nodes indicate bootstrap support values (1000 replicates).
spore wall exhibited morphologically distinctive characteristics from *A. koreana*, and morphologically similar *A. mellea* was one of the most distant phylogenetic groups from *A. koreana*.

### 4. Discussion

A total of 37 species have been reported in the genus of *Acaulospora* so far [24], and these species show distinct differences in the developmental process of spores and germination walls with glomoid spores accounting for most of AMF [11]. The spores of *Acaulospora* have no attached mycelium and have an additional wall structure called germination wall. The most key characteristics identifying *Acaulospora* species are spore wall surface structures and germination wall structure, especially the staining reaction to Melzer’s reagent of the innermost germination wall. However, the surface structures of spore walls is likely to be damaged in the field soil environment, and the staining reaction of Melzer’s reagent on the germination wall may be different according to the stage of spore development, or may not reflect the phylogenetic relationships [24]. Identification of *Acaulospora* species is incomplete by morphological characteristics. Therefore, it should be based on molecular evidence. In the present study, phylogenetic analysis showed that *Acaulospora koreana* was quite distant from *A. mellea* despite morphological similarity. Phylogenetic relationship with *A. mellea* is more apparent in the phylogenetic analysis using the Krüger fragment.
including 18S, 28S, and ITS regions used in this study than in the 18S rDNA sequence and is more strongly supported in the analysis using Bayesian analysis than the NJ or ML.

*Acaulospora mellea* was reported for the first time in the rhizosphere of grasses in a tropical savanna of Colombia. In *A. mellea*, GW2 L2 is stained with light purple in Melzer’s reagent [25], whereas *A. koreana* has a stronger staining response to Melzer’s reagent. The fact that *A. koreana* is phylogenetically distant with *A. mellea* was supported by both maximum likelihood and Bayesian analysis. Rather, phylogenetic analysis showed that *A. koreana* were most close to *A. lacunosa*, which has been reported from acid soil in West Virginia. The size and color of spores and the structure of spore walls are similar to those of *A. koreana*, but *A. lacunosa* have irregular saucer-shaped pits with corn-shaped edges on the spore walls [26], while the spore wall surface of *A. koreana* was smooth and had no pit.

*Acaulospora koreana* was isolated from the rhizosphere of woody plants, *L. obtusiloba* and *S. obassia* in forests of central Korea. The central part of Korea has vegetation structure of mixed forest with coniferous and bread-leaves. AMF having different host plants coexist together in forest soil due to the complex intertwining of various woody and herbaceous roots. To date, about 90 species of AMF have been reported in various host plants in Korea [27]. However, few studies on the diversity of AMF have been conducted to date in the Korean forest ecosystem, which accounts for 70% of Korean peninsula, and, therefore, it is thought that the species diversity of AMF is hugely underestimated [28]. Accurate identification of AMF species diversity in forest soils through the collection of persistent strains remains an important topic.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

[1] Smith S, Read D. Mycorrhizal symbiosis, 3rd ed. San Diego: Academic Press; 2008.

[2] Verbruggen E, Van Der Heijden MG, Weedon JT, et al. Community assembly, species richness and nestedness of arbuscular mycorrhizal fungi in agricultural soils. Mol Ecol. 2012;21:2341–2353.

[3] van der Heijden MG, Boller T, Wiemken A, et al. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology. 1998;79:2082–2091.

[4] van der Heijden MG, Klironomos JN, Ursic M, et al. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. 1998;396:69–72.

[5] Morton JB. Variation in mycorrhizal and spore morphology of *Glomus occultum* and *Glomus diaphanum* as influenced by plant host and soil environment. Mycologia. 1985;77:192–204.

[6] Morton J. Taxonomy of VA mycorrhizal fungi: classification, nomenclature, and identification. Mycotaxon. 1988;32:267–324.

[7] Morton JB, Benny GL. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of *Glomaceae*. Mycologia. 1990;37:471–491.

[8] Morton JB. Taxonomic and phylogenetic divergence among five *Scutellospora* species based on comparative developmental sequences. Mycologia. 1995;87:127–137.

[9] Krüger M, Stockinger H, Krüger C, et al. DNA-based species level detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytologist. 2009;183:212–223.

[10] Stockinger H, Krüger M, Schüssler A. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol. 2010;187:461–474.

[11] Blaszkowski J, Chwast G, Kovács GM, et al. *Septaglomus fuscum* and *S. furcatum*, two new species of arbuscular mycorrhizal fungi (Glomeromycota). Mycologia. 2013;105:670–680.

[12] Schoch CL, Seifert KA, Huhndorf S, et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA. 2012;109:6241–6246.

[13] Lee J, Lee S, Young JPW. Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol. 2008;65:339–349.

[14] Simon L, Bousquet J, Lévesque RC, et al. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature. 1993;363(6424):67–69.

[15] Liu Y, He J, Shi G, et al. Diverse communities of arbuscular mycorrhizal fungi inhabit sites with very high altitude in Tibet Plateau. FEMS Microbiol Ecol. 2011;78:355–365.

[16] Opik M, Moora M, Zobel M, et al. High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. New Phytologist. 2008;179:867–876.

[17] Daniels BA, Skipper HA. Methods for the recovery and quantitative estimation of propagules from soil. In: Schenck NC, editor. Methods and Principles of Mycorrhizal Research. St. Paul: American Phytopathological Society; 1982. p. 29–35.

[18] Gerdemann J, Nicolson TH. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc. 1963;46:235–244.
Koske R, Gemma J. A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res. 1989;92:486–488.

Stürmer SL, Morton JB. Developmental patterns defining morphological characters in spores of four species in Glomus. Mycologia. 1997;89:72–81.

Walker C. Taxonomic concepts in the Endogonaceae: spore wall characteristics in species descriptions. Mycotaxon. 1983;18:443–455.

Tamura K, Peterson D, Peterson N, et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–2739.

Ronquist F, Teslenko M, van der Mark P, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–542.

Schüßler A, Walker C. The Glomeromycota: a species list with new families and new genera. Kew, UK: The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University; 2010.

Schenck N, Spain JL, Sieverding E, et al. Several new and unreported vesicular-arbuscular mycorrhizal fungi (Endogonaceae) from Colombia. Mycologia. 1984;76:685–699.

Morton JB. Three new species of Acaulospora (Endogonaceae) from high aluminium, low pH soils in West Virginia. Mycologia. 1986;78:641–648.

Eo J-K, Park S-H, Lee E-H, et al. Biodiversity and distribution of Arbuscular mycorrhizal fungi in Korea. Kor J Mycol. 2014;42:255–261.

Lee E-H, Eo J-K, Ka K-H, et al. Diversity of arbuscular mycorrhizal fungi and their roles in ecosystems. Mycobiology. 2013;41:121–125.