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Phylogenetic Status of an Undiscovered Zygomycete Species, *Syncephalastrum monosporum*, in Korea

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ABSTRACT : During a survey of undiscovered taxa in Korea, two zygomycete fungal isolates, EML-BT5-1 and EML-BT5-2, were isolated from the seed of a pumpkin (*Cucurbita pepo*) fruit in Korea. Based on their morphological characteristics and a sequence analysis of four genes, ITS1-5.8S-ITS2, 18S, 28S rDNA, and EF-1α, the isolates were confirmed to be *Syncephalastrum monosporum* in the family Syncephalastraceae. To our knowledge, the zygomycete fungal species *S. monosporum* has not been previously described in Korea.

KEYWORDS : Mucorales, Multigenes, Pumpkin seed, *Syncephalastrum* species, Undiscovered taxa

The genus *Syncephalastrum* with the type species *Syncephalastrum racemosum* was described by Schröter (1886) [1]. This genus currently includes two species, *S. monosporum* and *S. racemosum*, according to Index Fungorum (www.indexfungorum.org). The species belonging to this genus in the family Syncephalastraceae are characterized by the production of cylindrical merosporangium on the surface of fertile vesicles [2]. *Syncephalastrum* spp. are frequently isolated from soil, dung, plant materials, and organic substrates [2, 3]. Species of *Syncephalastrum* have been reported to have important biotechnological applications as producers of endoglucanase, xylanase, and aspartic proteinase (syncephapepsin) [4-6]. In addition, *S. racemosum* has shown great potential to produce chitosan used as film support for lipase immobilization [7]. However, some of these species have been implicated in mucormycosis in humans and animals [8, 9].

In Korea, only one species of *S. racemosum* has been recorded [10]. During studies of the diversity of fungi in the order Mucorales isolated from a pumpkin (*Cucurbita pepo*) seed sample in Korea, a zygomycete species, *Syncephalastrum* sp. was isolated.

In recent years, several multi-loci analyses of the nuclear 18S ribosomal RNA small subunit (SSU), nuclear large subunit 28S ribosomal RNA (LSU), and translation elongation factor-1α (EF-1α) have been conducted to evaluate the phylogeny of mucoralean species [11-15]. However, only a few phylogenetic analyses of the genus *Syncephalastrum* have been performed.

The objectives of the present study were to analyze the phylogenetic status of *S. monosporum*, based on a multi-loci sequence analysis and to describe the morphological characteristics of this species in detail.

C. pepo seeds were collected from pumpkin fruit collected on the campus of Chonnam National University, Gwangju, Korea. The samples were transported to the laboratory in plastic bags. Hyphal tips were transferred to potato dextrose agar (PDA) plates, using a sterile needle under a stereomicroscope. The plates were incubated at 25°C for 3~5 days in the dark until colonies could be distinguished. Pure isolates were maintained in PDA slant tubes and stored in 20% glycerol at -80°C in the Environmental Microbiology Laboratory Fungal Herbarium, Chonnam National University, Gwangju, Korea, under deposition number (EML-BT5-1). The strain was also deposited in glycerol at -80°C in the Culture Collection of National Institute of Biological Resources (NIBR), Incheon, Korea (KOSPF0000133913).

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Genomic DNA was directly extracted from mycelia, using the HiGene Genomic DNA prep kit for fungi (Biopact Corp., Daejeon, Korea). The internal transcribed spacer (ITS), small subunit of 18S rDNA and large subunit of 28S rDNA, and translation elongation factor-1α (EF-1α) sequences were amplified with the primer pairs ITS1, ITS4 [16]; NS1, NS4 [14] LROR, LR5F [17]; and MEF-11, MEF-41 [14], respectively.

The sequences were aligned using ClustalX [18] and edited manually [19]. Phylogenetic analyses were performed in MEGA 6 [20] with the default settings. Maximum likelihood (ML) phylogenetic trees were constructed from individual datasets of the ITS and 28S rDNA sequences and combined datasets of the 18S and 28S rDNA and EF-1α gene sequences. The nearest-neighbor-interchange was selected as the ML heuristic method, and the initial ML tree was set automatically. *Umbelopsis isabellina* and *U. ramanniana* sequences were used as outgroups. The ITS, 18S, 28S, and EF-1α sequences of strains EML-BT5-1 and EML-BT5-2 were deposited in the GenBank database with accession numbers KY047152, KY047155, KY 047158, and KY047154 for EML-BT5-1; and KY047143, KY047156, KY047157, and KY047153 for EML-BT5-2, respectively. A BLASTn search showed that the ITS se-

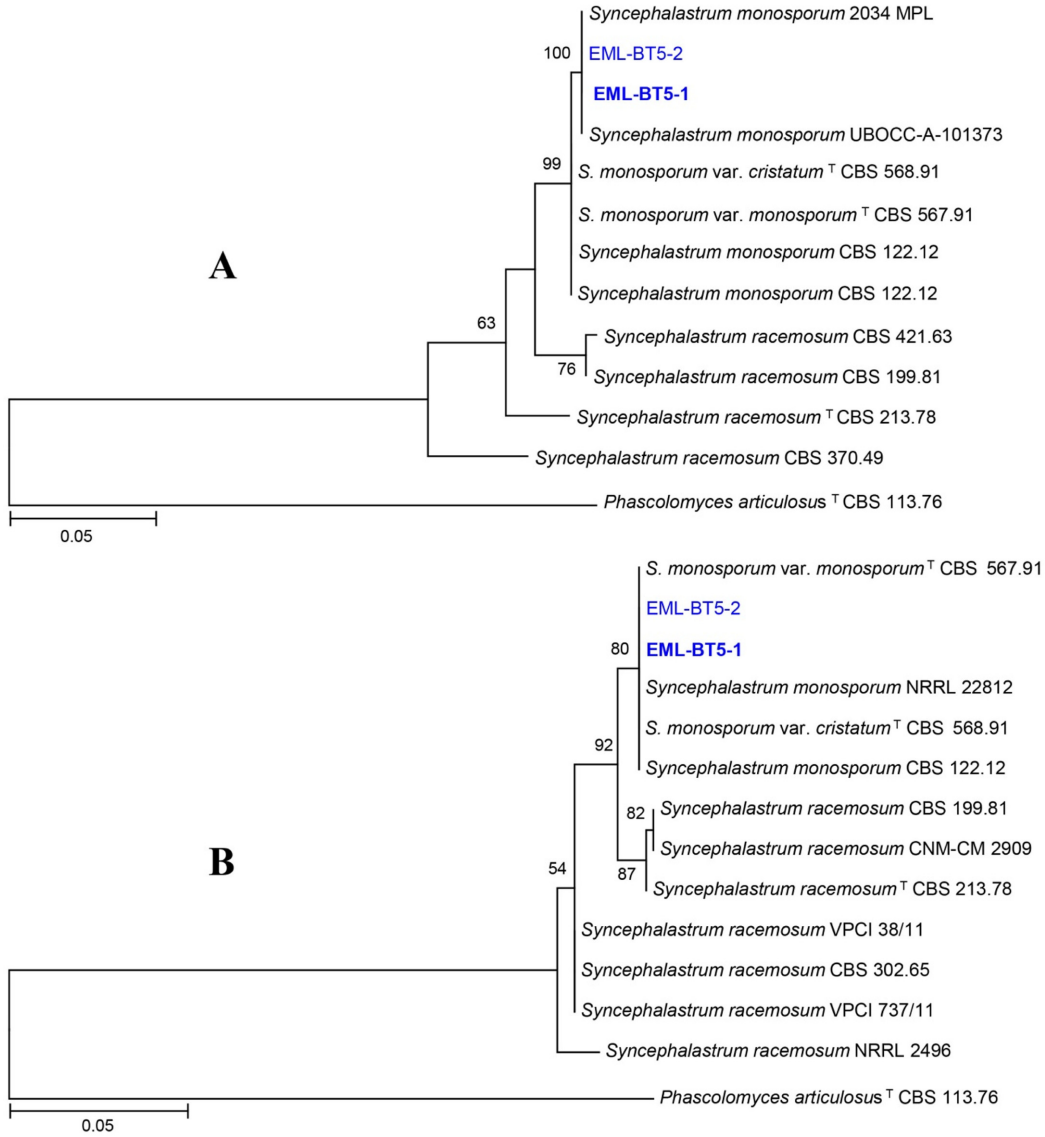

Fig. 1. Phylogenetic tree of EML-BT5-1 and EML-BT5-2 based on a maximum likelihood analysis of internal transcribed spacer (A) and 28S (B) rDNA sequences. Sequence of *Phascolomyces articulosus* was used as an outgroup. Bootstrap values with more than 50% support in 1,000 replications are shown above branches. The bar indicates the number of substitutions per nucleotide position.

quence of strains EML-BT5-1 and EML-BT5-2 were most closely related to representative *S. monosporum* (GenBank accession no. KP233744 and KF225035) with nucleotide identities of 99.5% (428/430 bp) and 98.7% (462/468 bp), respectively. Based on the 28S rDNA sequence analysis, strains EML-BT5-1 and EML-BT5-2 showed 99.8% (584/585 bp) identity value with *S. monosporum* sequence (GenBank accession no. AF157215). The 18S rDNA sequences of EML-BT5-1 and EML-BT5-2 showed 99.9% (1000/1001 bp) and 99.2% (934/942 bp) identity values with sequences of *S. monosporum* var. *pluriproliferum* (GenBank accession no. AF157161) and *S. monosporum* var. *pluriproliferum* (GenBank accession no. JX644490), respectively. The EF-1α gene sequence of strains EML-BT5-1 and EML-BT5-2 showed 99.9% (696/697 bp) and 99.8% (564/565 bp) identity values with *S. monosporum* var. *pluriproliferum* (GenBank accession no. AF157294) and *S. monosporum* var. *pluriproliferum* (GenBank accession no. JX644588), respectively. The ITS, 18S, 28S, and EF-1α phylogenetic analyses indicated that isolates EML-BT5-1 and EML-BT 5-2 were nearly identical to *S. monosporum* in the family Syncephalastraceae (Figs. 1, 2).

To examine morphological characteristics and growth rate, EML-BT5-1 was cultured in PDA (39 g in 1 L deionized water; Difco, Detroit, MI, USA), synthetic mucor agar (SMA; 40 g dextrose, 2 g asparagine, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.5 g thiamine chloride, and 15 g agar, in 1 L of deionized water), and malt extract agar (MEA; 33.6 g in 1 L deionized water; Difco), and incubated at 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C in the dark for 7 days. Samples were observed under a light microscope (DFC290; Leica Microsystems, Wetzlar, Germany) and by field emission scanning electron microscopy (SEM; Hitachi S4700; Hitachi, Tokyo, Japan). For SEM, the isolate was fixed in 2.5% paraformaldehyde-glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 hr, washed with cacodylate buffer (Junsei Chemical, Tokyo, Japan), and then fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hr. After this, the blocks were dehydrated with graded ethanol and dried in a fume hood. Finally, samples were sputter-coated with gold and observed by SEM at the Korea Basic Science Institute, Gwangju, Korea.

***Syncephalastrum monosporum* R.Y. Zheng, G.Q. Chen & F.M. Hu, Mycosystema 1: 39 (1988) (Fig. 3).**

Description: On SMA, the colonies grew rapidly and attained a diameter of 85~86 mm after 4 days at 30°C. The color of colonies was cotton-white. The reverse side of the colony was also white. Sporophores were hyaline, rarely brownish, with septae, 8.5~16.0 μm in width, and

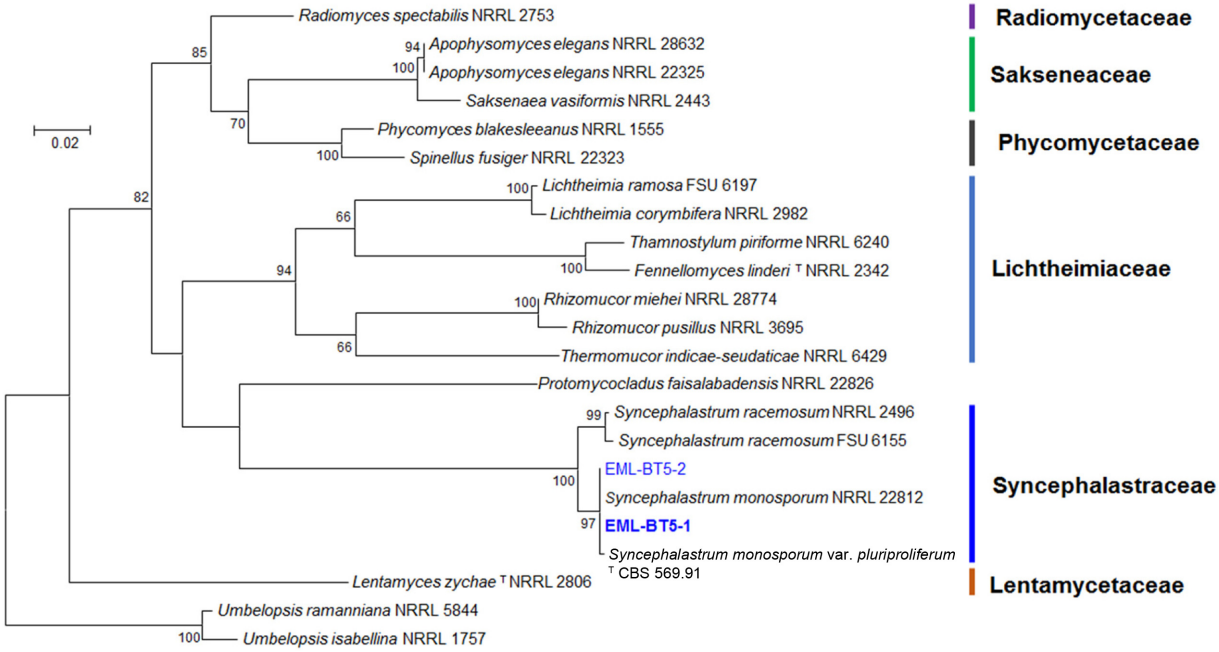

Fig. 2. Phylogenetic status of EML-BT5-1 and EML-BT5-2 in the Syncephalastraceae clade based on a maximum likelihood analysis of a combined dataset of 18S and 28S rDNA and translation elongation factor 1-alpha sequences. *Umbelopsis isabellina* and *U. ramanniana* sequences were used as outgroups. Bootstrap values with more than 50% support in 1,000 replications are shown above branches. The bar indicates the number of substitutions per nucleotide position.

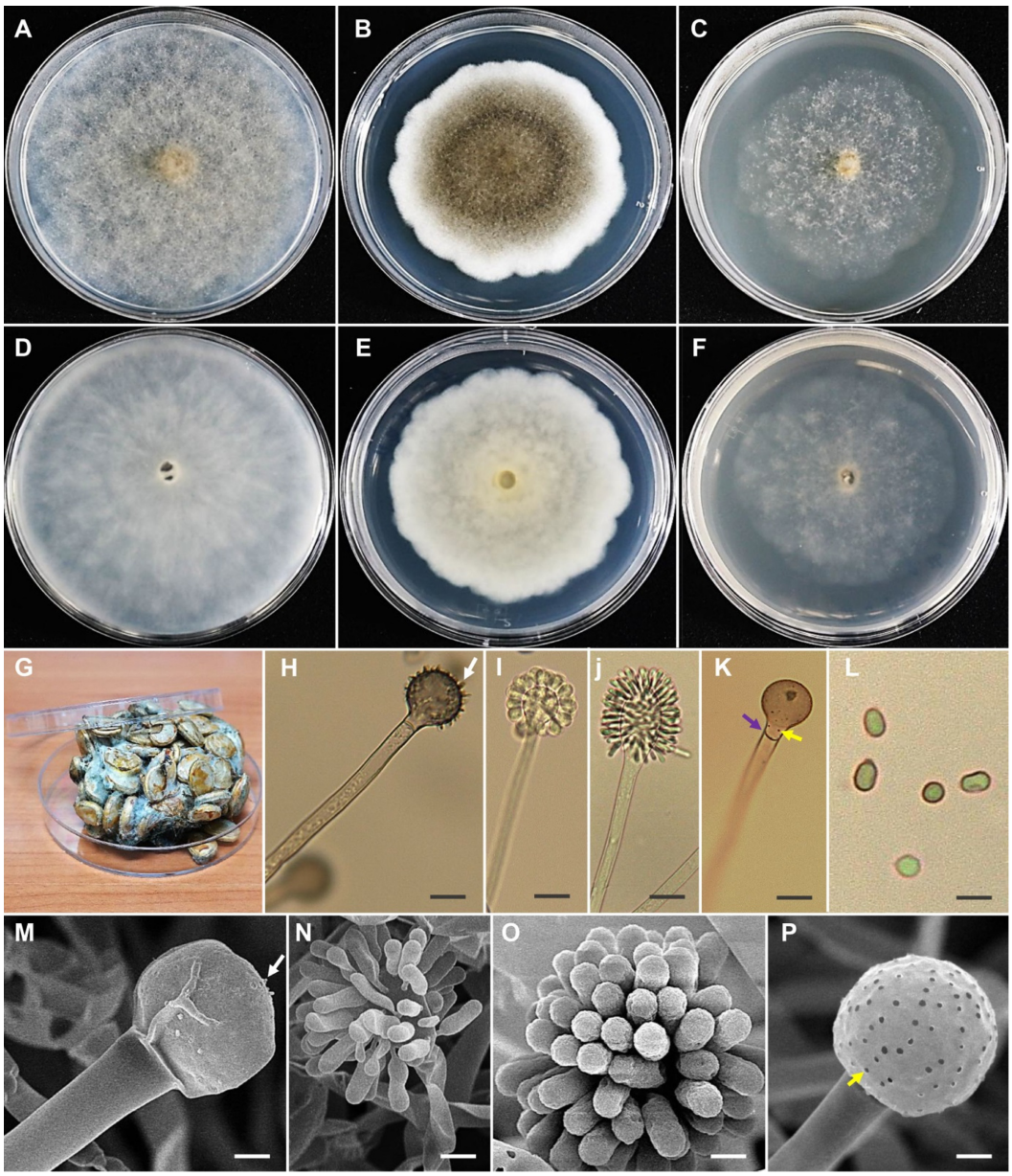

Fig. 3. Morphology of S*yncephalastrum monosporum* EML-BT5-1. A, D, colony in synthetic mucor agar; B, E, colony in potato dextrose agar; C, F, colony in malt extract agar; G, growth of mycelia on pumpkin seed surfaces; H, M, development of sporangiola on vesicles and young sporangiola (white arrow); I, J, N, O, vesicles bearing mature sporangiola; K, P, sporophores with a septa (purple arrow), old vesicles, and scars remaining after detachment of sporangiola (yellow arrow); L, sporangiospores; A~C, obverse view; D~F, reverse view; H~M, light microscopy; M~P, scanning electron microscopy (scale bars: H~K = 50 μm, L~N, O = 10 μm, P = 20 μm).

variable in length. Vesicles were globose, subglobose, ovoid, or subpyriform, sometimes rounded or papillate at the apices, measuring 15.0~34.5 μm in diameter. Sporangiola developed on vesicles and covered their entire surfaces, were rod-shaped, ovoid to subglobose, rounded at the apices, uniform in width throughout or becoming broader at the top, occasionally narrower in the middle portion, and measuring 12.5~32.0 × 4.5~7.5 μm. The sporangial wall was subpersistent, slowly dissolving or broken to release spores. Single sporangiospores were ovoid to subglobose, measuring 4.0~6.5 μm in diameter. Zygospores were not observed, and rhizoids were not well developed. The isolates grew at different rates according to the temperature and medium. The average diameters of EML-BT5-1 on SMA, PDA, and MEA were 21.5 mm, 17 mm, and 15 mm at 24 hours, respectively. The optimal temp-

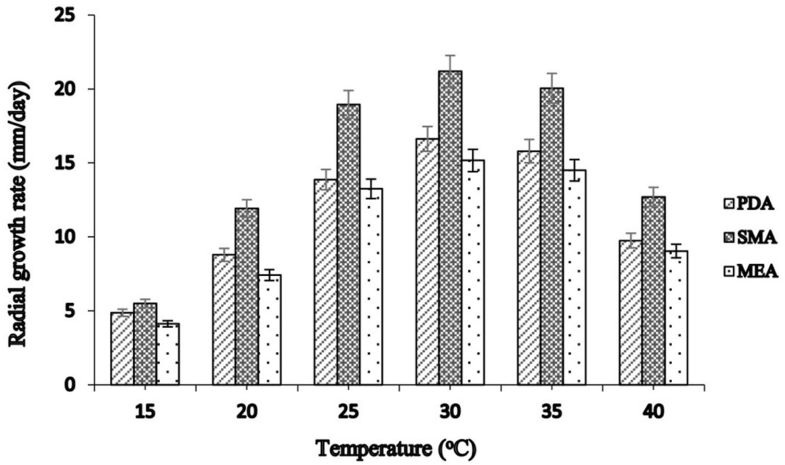

Fig. 4. Effect of temperature and culture medium on mycelial growth of S*yncephalastrum monosporum* EML-BT5-1. Mycelia were grown on synthetic mucor agar (SMA), potato dextrose agar (PDA), and malt extract agar (MEA) at different temperatures.

erature range for growth was 25~30°C. Slow growth was observed at 40°C, and no growth was observed at 5°C. SMA was the best medium for mycelial growth, followed by PDA; the growth of colonies was the slowest on MEA (Fig. 4). The morphology and physiology of the isolate was generally similar to those previously described for the species by Zheng et al. [21].

Recently, Hoffman et al. [11] have performed a phylogenetic evaluation of some species belonging to the genus *Syncephalastrum* and several species in the class Zygomycetes, using 18S and 28S rDNA, EF-1α, and actin gene sequences. This study showed that there was only a single genus, *Syncephalastrum*, with two species in the family Syncephalastraceae. In the ITS and 28S trees, our strains, EML-BT5-1 and EML-BT5-2, were not distinct from *S. monosporum*. Furthermore, based on the phylogenetic analysis using a combined dataset of 18S and 28S rDNA and EF-1α sequences (Fig. 2), EML-BT5-1 and EML-BT5-2 were located within a clade that included *S. monosporum* and *S. racemosum* in the family Syncephalastraceae.

Based on the morphological, physiological and molecular analyses, the fungus was identified as *S. monosporum*. Herein, *S. monosporum* is described as a new record of zygomycete fungi belonging to undiscovered taxa in Korea.

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