

Isolation and Characterization of *Blakeslea trispora* Isolated from Gut of Grasshopper and Soldier Fly Larva in Korea

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ABSTRACT : During a survey of fungal diversity in insect guts in Korea, two fungal strains, EML-PGH2 and EML-PUK188, were isolated from the gut of grasshopper and soldier fly larvae inhabiting the bulrush plants at a pond located in the Chonnam National University Arboretum, Gwangju, Korea. Based on their morphological characteristics and a phylogenetic analysis of the internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA sequences, the strains were identified as *Blakeslea trispora*. To our knowledge, the zygomycete species *B. trispora* has not been previously described in Korea.

KEYWORDS : *Blakeslea trispora*, Grasshopper, Mucorales, Soldier fly larva, Undiscovered taxa

The genus *Blakeslea* (Choanephoraceae, Mucorales) was established by Thaxter (1914) with the type species *B. trispora* [1]. According to Index Fungorum (www.index-fungorum.org), the genus *Blakeslea* includes only two species: *B. trispora* and *B. monospora*. The species belonging to this genus are characterized by production of both sporangia and sporangiola on sporangiophores, and appendages on the sporangiospores, and formation of zygosporangia with opposed suspensors [2]. They are commonly isolated from soil, dung, flower, and cowpeas [1, 3-6]. Several studies have shown that *B. trispora* is the model fungus useful for study of trisporic acid biosynthesis [7].

The Choanephoraceae family was first described by Schröter for only a genus *Choanephora*. However, later Kirk [2] monographed the family and added two genera, *Blakeslea* and *Poitrasia*. In the year 2013, Papp et al. [8] performed the phylogenetic analysis of *Gilbertella*, *Blakeslea*, *Choanephora*, and *Poitrasia* in Mucoraceae. This

study suggested that the *Gilbertella* (Gilbertellaceae) is located between Choanephoraceae and Mucoraceae. Later, Voigt and Olsson [9] revised the family Choanephoraceae based on a multigene (act, ref-1alpha, 18S and 28S rRNA) data set obtained from selected species of 50 genera of the Mucorales and demonstrated that *Gilbertella* could belong to the Choanephoraceae. According to these authors, four genera, including *Blakeslea*, *Choanephora*, *Gilbertella* and *Poitrasia*, classified under the Choanephoraceae.

In Korea, only one species of *Choanephora*, *C. cucurbitarum*, causing soft rot disease on eggplant (*Solanum melongena*) was recorded [10]. During a study on the Mucorales, a species of *Blakeslea* was first isolated from grasshopper and soldier fly (Stratiomyidae) larva samples in Korea. To our knowledge, there were no previously published records of this genus, which was isolated from the gut of grasshopper and soldier fly larva.

The objective of the present study was to perform the morphological and molecular analyses to characterize an unrecorded zygomycete species – *Blakeslea trispora* in Korea.

Grasshopper from weedy plant and soldier fly larvae inhabiting the bulrush (*Typha orientalis*) from an aquatic plant, were collected at CNU Arboretum located in Chonnam National University, Gwangju, Korea in 2016. The insect placed in polyethylene and kept at ambient temperature until being transported to the laboratory. The gut was removed from each insect and placed on a sterile Petri dish, cut into small pieces, and spread onto potato

Kor. J. Mycol. 2016 December, 44(4): 355-359
<https://doi.org/10.4489/KJM.2016.44.4.355>
pISSN 0253-651X • eISSN 2383-5249
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Received October 29, 2016
Revised November 18, 2016
Accepted November 21, 2016

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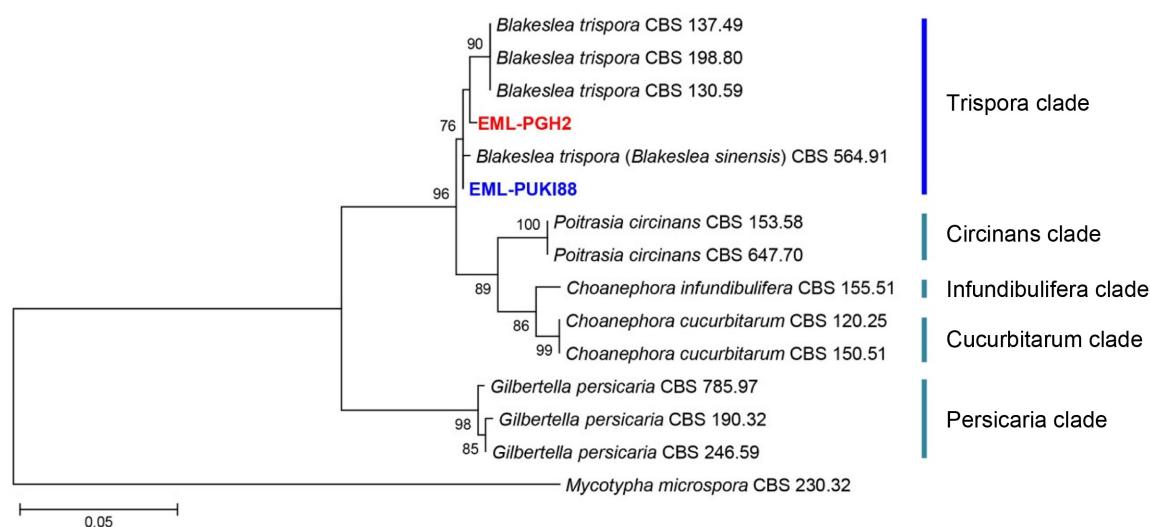


Fig. 1. Phylogenetic tree based on maximum likelihood analysis of internal transcribed rDNA sequences of *Blakeslea trispora* EML-PUKI88 and *B. trispora* EML-PGH2. *Mycotypha microspora* was used as outgroup. Bootstrap support values of $\geq 50\%$ are indicated at the nodes. The bar indicates the number of substitutions per position.

dextrose agar (PDA) amended with streptomycin (50 mg/L). Plates were incubated at 20°C for 3~7 days. Hyphal tips were transferred with a glass needle to PDA plates amended with the antibiotics mentioned above using a stereomicroscope. Pure isolates were transferred to slant tubes, and stored in 20% glycerol at -80°C at the Environmental Microbiology Laboratory Herbarium (EMLH; Chonnam National University, Gwangju, Korea) as EML-PGH2 and EML-PUKI88. Genomic DNA was directly extracted from mycelia using the HiGene Genomic DNA

prep kit for fungi (Biopact Corp., Daejeon, Korea). The internal transcribed spacers (ITS1 and ITS2) and 5.8S gene were amplified using primers ITS1 (5'- TCCGTAGGTGA ACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') following the method by White et al. [11]. The sequences were initially aligned using CLUSTAL X [12], and edited manually [13]. Phylogenetic analyses were performed using MEGA 6 [14] with the default settings. Phylogenetic trees were constructed from the data using maximum likelihood (ML). The sequences of EML-PGH2 and

Table 1. Morphological characteristics of EML-PUKI88 and the reference species, *Blakeslea trispora* on malt extract agar medium

Character	EML-PUKI88	<i>Blakeslea trispora</i> ^a
Colony color	rapid-growing, first white then yellowish	rapid-growing, first white then yellow
Sporangiophores bearing sporangia	8.0~12 μ m in width, variable in length	11~14 μ m in width, pale brown
Sporangia	35~50 \times 36~60 μ m, sub-globose, multispored, initially white, turning to yellow, dark brown with age	(30)~60~90~(180) μ m, spherical, multispored, initially white, turning to yellow and pale brown, dark brown with age
Sporangiospores from sporangia	6~9 \times 9~15 μ m, ellipsoid, reddish-brown, hyaline appendages at each pole	(6)~8~18~(22) \times (3)~4~8~(12) μ m, ellipsoid, reddish-brown, hyaline appendages at each pole
Sporangiophores bearing sporangiola	12~26 μ m in width, variable in length, bearing 2~26 enlargements	14~28 μ m in width, bearing (1)~8~16~(32) enlargements
Sporangiola	8~16 \times 11~18.5 μ m, ellipsoidal, mostly 3 sporangiospores	10~16~(20) \times 8~12~(14) μ m, ellipsoidal, containing (1)~3~(5) sporangiospores
Sporangiospores from sporangiola	11~18.5 \times 6~9 μ m, ellipsoid, reddish-brown	(8)~10~16~(20) \times (4)~5~8~(9) μ m, ellipsoid, reddish-brown
Zygospores	absent	40~80 μ m in diameter
Chlamydospores	present	present

^aFrom the description by Thaxter [1].

EML-PUKI88 strains were deposited in the GenBank database with accession numbers, KY047145 and KY047144, respectively. A BLASTn search revealed that in rDNA ITS region EML-PGH2 and EML-PUKI88 revealed sequence similarities of 98.6% (566/574 bp) and 98.7% (567/574 bp) with *B. sinensis* (current name, *B. trispora*; GenBank accession no. JN206230) and *B. trispora* (GenBank accession no. JN943005), respectively. Phylogenetic analysis of ITS region showed that the isolates EML-PGH2 and EML-PUKI88 were placed within *Trispora* clade along with *B. trispora* (Fig. 1).

To confirm the phylogenetic result, the morphology of

the isolate EML-PUKI88 was observed under a light microscope (DFC 290; Leica Microsystems, Wetzlar, Germany). The isolate was cultured on PDA, synthetic mucor agar (SMA; 40 g dextrose, 2 g asparagine, 0.5 g KH_2PO_4 , 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g thiamine chloride, and 15 g agar, in 1 L of deionized water), and malt extract agar (33.6 g MEA in 1 L of deionized water; BD, Sparks, MD, USA). Colonies were characterized after 5~7 days of cultures at 25°C.

Blakeslea trispora Thaxt., Botanical Gazette Crawfordsville 58: 353 (1914) (Table 1, Fig. 2).

Description: Colonies grew rapidly at 25°C on MEA, SMA and PDA, filling the petri dish after 2~3 days of

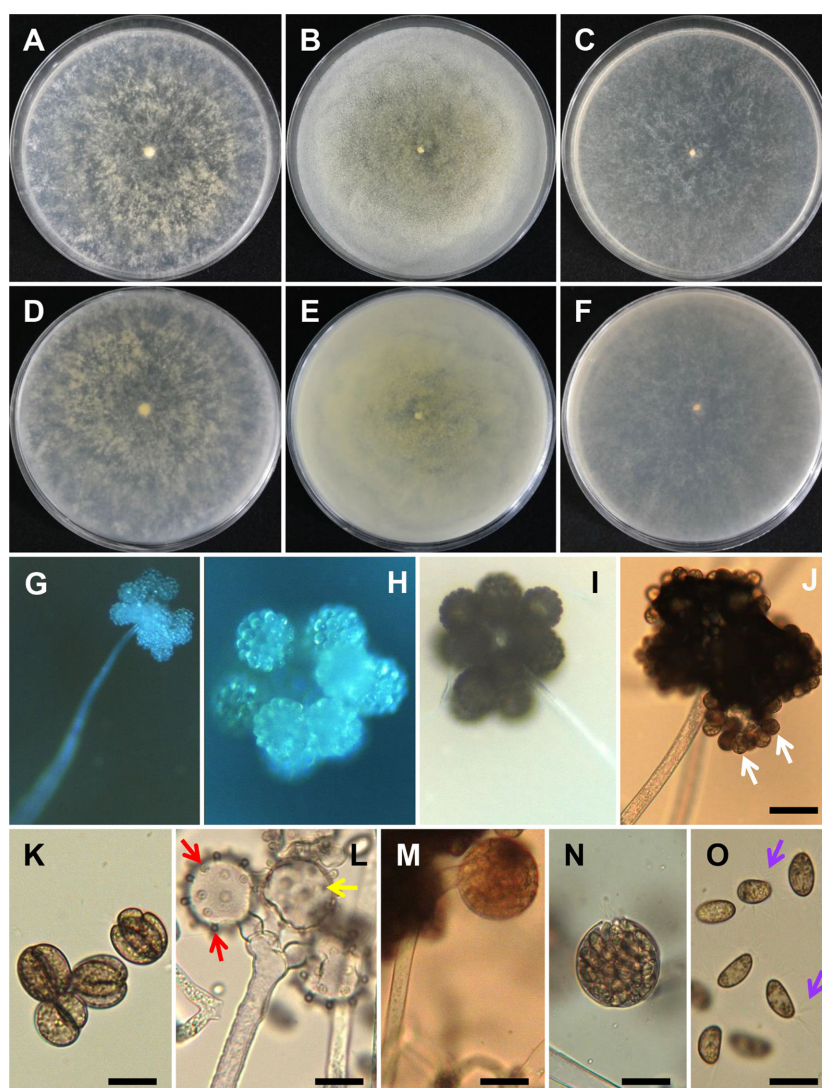


Fig. 2. Morphology of *Blakeslea trispora* EML-PUKI88. Colonies on synthetic mucor agar (A, D), potato dextrose agar (B, E), and malt extract agar (C, F). A~C, obverse view; D~F, reverse view; G~I, branching sporangiophore with apical vesicles bearing few spored sporangia (under stereo-microscope); J, sporangiophore with some sporangia (white arrows) attached on the surface of vesicles; K, three-spored sporangium; L, terminal spherical vesicle (yellow arrow) after sporangia detached, showing small spherical pedicels (red arrows) over the surface; M, sporangiophore with a developing sporangium; N, mature sporangium; O, sporangiospores from sporangium with appendages (purple arrow) (scale bars: J, M, N = 50 μm ; K, L, O = 20 μm).

incubation. The initial color of colonies was white, which later turned to yellowish. The colony reverse was also yellowish (Fig. 2). Sporangiophores bore spore arising from substrate mycelium, non-septate, unbranched, often circinate below the sporangium, 8.0–12 µm in width, variable in length. Sporangia measured 35–50 × 36–60 µm, were sub-globose, multispored, initially white and later becoming yellow to dark brown. Sporangiospores from sporangia measured 6–9 × 9–15 µm, were ellipsoid, reddish-brown, bearing a group of straight fine radiating appendages from either pole. Sporangiophores bore sporangia arising from substrate mycelium or aerial hyphae, erect, 12–26 µm in width, variable in length, bearing 2–26 enlargements. Sporangia measured 8–16 × 11–18.5 µm, were ellipsoidal, mostly 3 spore. Spore from sporangia were ellipsoidal, reddish-brown, and measured 11–18.5 × 6–9 µm, hyaline appendages at each pole. Zygospores were not observed on this medium. The isolate produces abundant mycelia on PDA agar and the sporulation is excellent on PDA agar. Comparing the colony morphology and culture characteristics of the isolate on MEA medium, with previous descriptions [1], the present isolate was generally similar to those of *B. trispora* (Table 1).

To determine the growth rate, EML-PUKI88 was cultured on MEA, SMA and PDA media. The plates were incubated at 10°C, 20°C, 25°C, 30°C, and 35°C in the dark for 7 days. The average growth rates of EML-PUKI88 on MEA, SMA, and PDA were 40.5 mm/day, 51.5 mm/day, and 59 mm/day at 25°C, respectively. The optimal growth temperature range was 25–30°C. Among the different temperatures and culture media, the best mycelial growth was found at 25°C on PDA media. On all media, the isolates grew rapidly at 25–30°C, and stopped growing at a temperature of 10°C.

The genus *Blakeslea* is considered monotypic. Three species were recognized within genus *Blakeslea* as *B. trispora*, *B. monospora*, and *B. sinensis*. However, recently, Hoffmann et al. [15] and Walther et al. [16] performed phylogenetic analysis including the sequences of *B. trispora* and *B. sinensis* which is regarded as synonym of *B. trispora*. However, the sequences of *B. monospora* was not available in GenBank. The present molecular data of this species was consistent with the phylogeny presented by Walther et al. [16]. In the ITS tree, our strains, EML-PUK882 and EML-PGH2, were clustered within *Trispora* clade containing *B. trispora* (Fig. 1). Based on the morphological, physiological and molecular analyses, the fun-

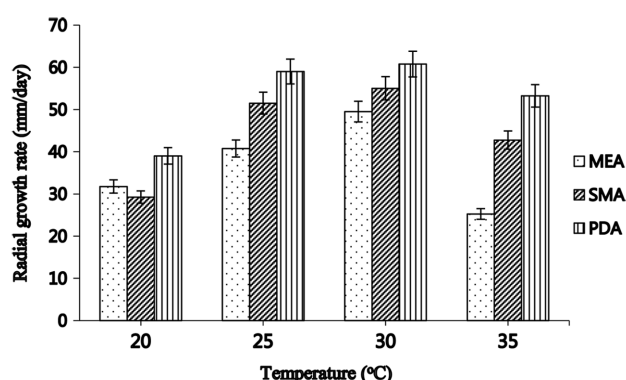


Fig. 3. Effect of temperature and culture medium on mycelial growth of *Blakeslea trispora* EML-PUKI88. Mycelia were grown on malt extract agar (MEA), synthetic mucor agar (SMA) and potato dextrose agar (PDA), at different temperatures, as indicated.

gus was identified as *B. trispora*.

Little is known about fungi inhabiting the gut of insects including grasshopper and soldier fly larvae at a pond in Korea. Herein *B. trispora* is described as a new record of zygomycete fungi belonging to undiscovered taxa in Korea.

Acknowledgements

This work was supported by the Project on Survey and Discovery of Indigenous Species of Korea funded by NIBR of the Ministry of Environment (MOE), Republic of Korea.

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