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Research Note

Characterization of Two New Records of Mucoralean Species Isolated from Gut of Soldier Fly Larva in Korea

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Abstract While surveying the diversity of fungi of the order Mucorales, two isolates, EML-PUKI12-1 and EML-PUKI06-1, were obtained from the gut of soldier fly larvae inhabiting the bulrush at a pond located in the Chonnam National University Arboretum, Gwangju, Korea. The isolates were confirmed as *Mucor irregularis* and *Mucor fragilis* species, respectively, based on the morphological characteristics and phylogenetic analysis of rDNA internal transcribed spacer region. Such mucoralean species belonging to undiscovered taxa has not previously been described in Korea.

Keywords *Mucor fragilis*, *Mucor irregularis*, Mucorales, Soldier fly larva

The genus *Mucor* was established by Micheli [1] for a single species, *M. mucedo* L. It is characterized by fast-growing colonies, simple or branched sporangiophores without basal rhizoids, non-apophysate sporangia and pigmented and ornamented zygosporangial wall [2]. Their zygospores are produced by conjugation of heterothallic gametangia. *Mucor* spp. can be easily isolated from soil, dung, water, stored grains, and plants [3-5]. There are more than 300 species name for described in literature, but only more than 50 are known and described [6, 7]. Several species of which have important economical application, including the production of enzymes, fumaric acid, fatty acid, and also antifungal agents for plants [8, 9]. Twenty-seven zygomycetous fungi which have been described in Korea were assigned in 12 genera and 8 families [4, 10, 11]. Among them, a new species, *Mucor koreanus*, was isolated from a tangerine fruit of Korea in 2015 [4]. To our knowledge, literature record of this genus from gut of soldier fly (Stratiomyidae) larva has been not published.

The objective of the present study was to perform the morphological and molecular analyses to characterize two unrecorded mucoralean species—*Mucor irregularis* and *Mucor fragilis* in Korea.

Soldier fly larvae inhabiting the bulrush (*Typha orientalis*) which is a kind of aquatic plant were collected at CNU Arboretum located in Chonnam National University, Gwangju, Korea in 2016. Soldier fly larva samples were placed in polyethylene bag and kept at ambient temperature until being transported to the laboratory. The entire gut was removed from each insect and placed on a sterile Petri dish, cut, and spread onto potato dextrose agar (PDA; Becton, Dickinson and Co., Sparks, MD, USA) amended with streptomycin (50 mg/L). Plates were incubated at 20°C for 3~7 days. Hyphal tips were transferred to new PDA plates amended with the antibiotics under a stereomicroscope. To isolate pure cultures, individual colonies of varied morphologies were transferred to PDA plates. Pure isolates were maintained in PDA slant tubes and stored in 20% glycerol at −80°C at the Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, Korea.

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′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) following the method by White *et al.* [12]. The sequences were initially aligned using CLUSTAL X [13], and edited manually [14]. Phylogenetic analyses were performed using MEGA 6 [15] with the default

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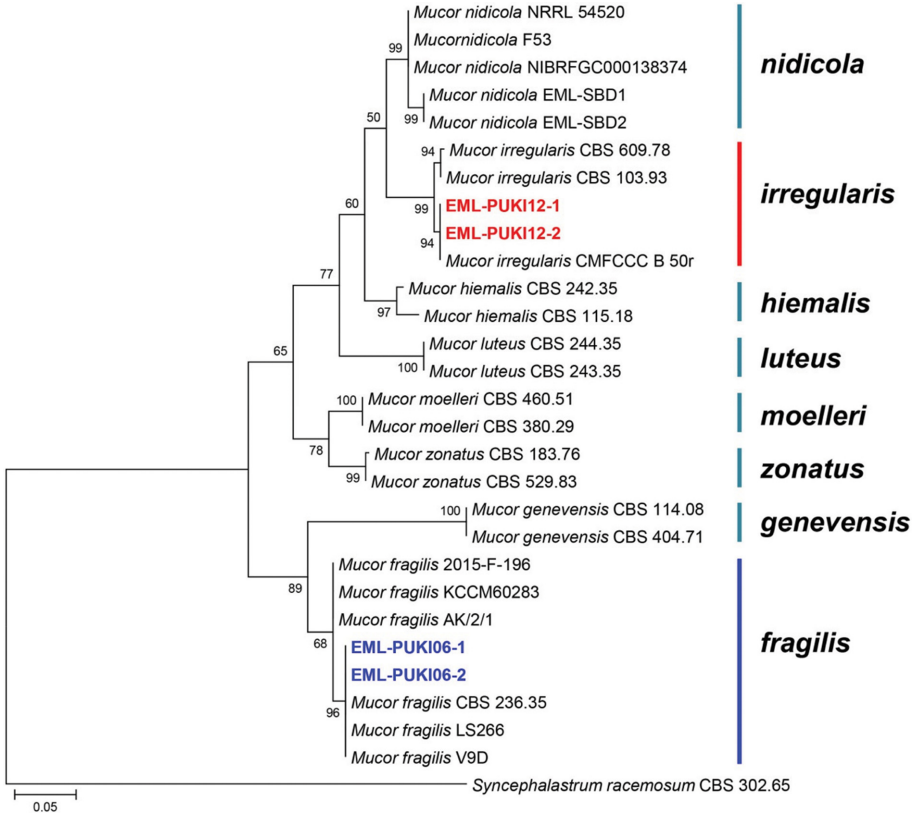

Fig. 1. Phylogenetic tree based on maximum likelihood analysis of internal transcribed rDNA sequences for *Mucor irregularis* EML-PUK12-1, *M. irregularis* EML-PUK12-2, *M. fragilis* EML-PUKI06-1, and *M. fragilis* EML-PUKI06-2. *Syncephalastrum racemosum* was used as outgroup. Bootstrap support values of ≥ 50% are indicated at the nodes. The bar indicates the number of substitutions per position.

settings. Phylogenetic trees were constructed from the data using maximum likelihood. The ITS sequences of EML-PUKI12-1, EML-PUKI12-2 and EML-PUKI06-1, EML-PUKI06-2 strains were deposited in the GenBank database with accession numbers (KY047151, KY047146, KY047147, and KY047150, respectively). A BLASTn search revealed that the rDNA ITS homology of EML-PUKI12-1 and EML-PUKI06-1 represented 99.1% (572/577 bp) and 99.2% (610/615 bp) sequence identity values with *M. irregularis* (GenBank accession No. JX975255) and *M. fragilis* (GenBank accession No. FN650655). The phylogenetic tree of the ITS region with a high bootstrap value (Fig. 1), indicated that the isolates EML-PUKI12-1 and EML-PUKI06-1 were identical to *M. irregularis* and *M. fragilis*.

To confirm the molecular phylogenetic result, the morphology and growth rate of the isolates EML-PUKI12-1 and EML-PUKI06-1 were determined. Cultural features were observed on PDA, 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 (33.6 g MEA in 1 L of deionized water; Becton, Dickinson and Co.). The plates were incubated at 10°C, 20°C, 25°C, 30°C, and 35°C in the dark for 7 days. Samples were mounted in lactophenol solution (Junsei Chemical Co. Ltd., Tokyo, Japan) and observed using an Olympus BX51 microscope with DIC optics (Olympus, Tokyo, Japan).

Mucor irregularis Stchigel, Cano, Guarro & Ed. Álvarez: 71 (2011) (Fig. 2).

Description. Colonies grew rapidly at 30°C on MEA, attained a diameter of 65~67 mm after 2 days. The initial color of colonies was whitish, which later turned to light yellow. The colony reverse was light yellow. Sporangiophores mostly sympodially branched, grew to width of 6~12 µm and a variable length. Sporangia measured 24.5~49.5 × 22.5~48 µm, were globose to subglobose, light yellow, and were multispored. The columellae measured 17.5~30 × 16~29.5 µm, and globose to ellipsoidal, with a collarette. The sporangiospores were variable, mostly ellipsoidal and measured 3.0~8.5 × 2.5~7.0 µm in diameter. Chlamydospores were present on the aerial mycelia. Zygospores were not observed. On PDA and SMA, the colonies grew slowly than on MEA, attained a diameter of 62~64 mm and 57~59 mm after 2 days at 30°C, respectively. The color of the colonies was cotton yellow. The colony reverse color was pale yellow. Zygospores were not observed in this media. The colony morphology and culture characteristics of the EML-PUKI12-1

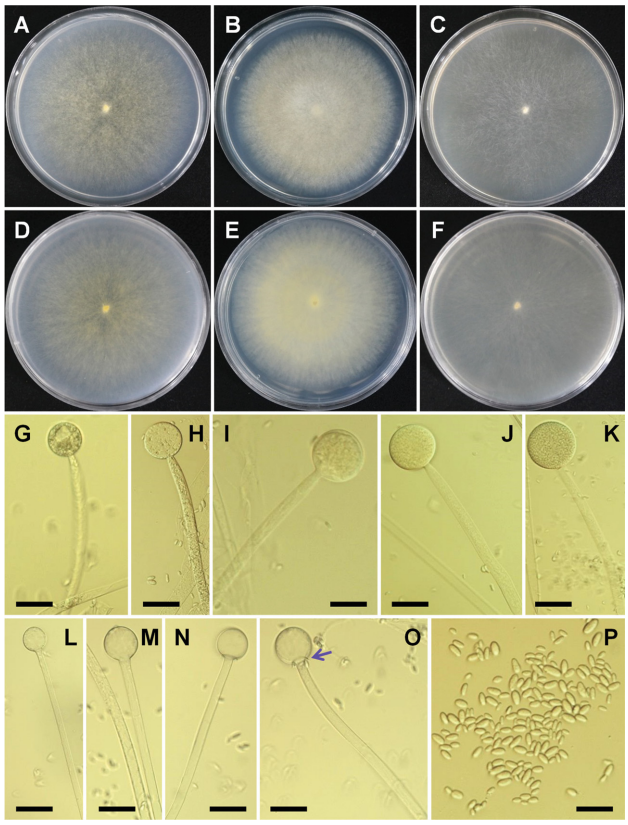

Fig. 2. Morphology of *Mucor irregularis* EML-PUKI12-1. A~F, Colonies on synthetic mucor agar (A, D), potato dextrose agar (B, E), and malt extract agar (C, F) (A~C, obverse view; D~F, reverse view); G~K, Young and mature sporangia; L~O, Columella with collarette (purple arrow); P, Sporangiospores (scale bars: G~P = 20 μm).

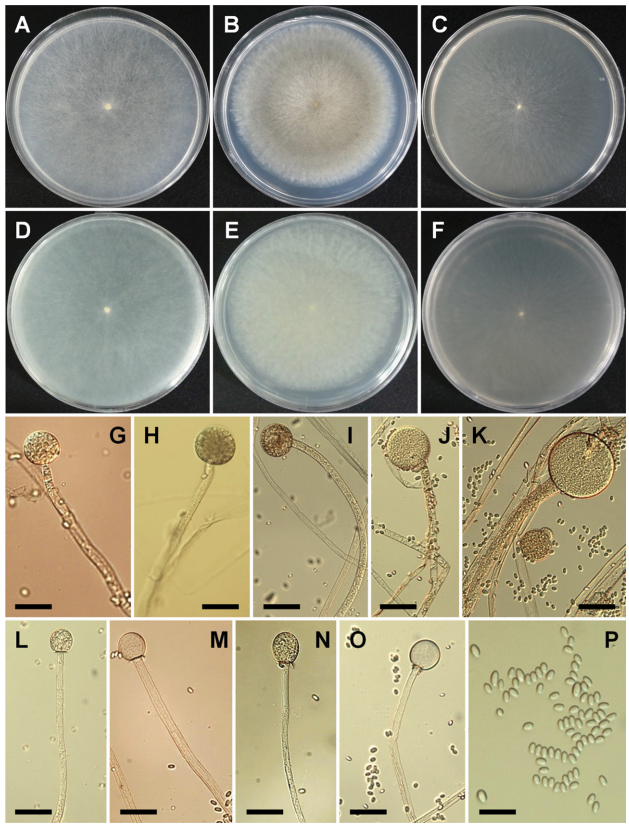

Fig. 3. Morphology of *Mucor fragilis* EML-PUKI06-1. A~F, Colonies on synthetic mucor agar (A, D), potato dextrose agar (B, E), and malt extract agar (C, F) (A~C, obverse view; D~F reverse view.); G~K, Young and mature sporangia; L~O, Columella with collarette; P, Sporangiospores (scale bars: G~P = 20 μm).

isolate on MEA was compared with the previous description [16]. Morphology of the present isolate was generally similar to the previous description of *M. irregularis*.

Mucor fragilis Bainier, Ann. Sci. Nat. Bot. 19: 208 (1884) (Fig. 3).

Colonies grew rapidly at 25°C on MEA, filling the Petri dish after 4 days of incubation. The initial color of colonies was white, which later turned to gray. The colony reverse was whitish. Sporangiophores grew to width of 5.5~10.5 μm and a variable length. Sporangia measured 25~65 × 23.5~60 μm, were globose to subglobose, light yellow, and multispored. The columellae measured 17~29 × 15.5~26.5 μm, and ellipsoidal to conical, with a collarette. Sporangiospores were ellipsoidal, and measured 4.0~9.5 × 3~7 μm. Zygospores were not observed. The isolate produced abundant mycelia on PDA agar; and the sporulation was excellent on PDA agar and SMA agar, respectively. Comparing the colony morphology and culture characteristics of the isolate on MEA medium, with previous descriptions [17, 18], the present isolate was generally similar to those of *M. fragilis*.

The isolates were observed to grow over a wide range of temperatures with varying growth rates on MEA, SMA, and PDA (Fig. 4). The average growth rates of EML-PUKI12-1 and EML-PUKI06-1 on MEA, SMA, and PDA were 28 and 27 mm/day, 25.5 and 32 mm/day, 26 and 27 mm/day at 25°C, respectively. The optimal growth temperature range was 25~30°C. Among the different temperature and culture media, the best mycelial growth was found at 30°C on MEA media for EML-PUKI12-1 and 25°C on SMA media for EML-PUKI06-1. On all media, the isolates grew slowly below 10°C, rapidly at 25~30°C, and could grow well at temperature 35°C.

Despite the wide intraspecific variation found among some taxa, the rDNA ITS region has been used as a critical barcode marker for identification of mucoralean fungi at the level of species. The results of our molecular data analysis of the two mucoralean species were consistent with the phylogeny presented by Walther *et al.* [7]. In the ITS tree, our strains: EML-PUKI12-1, EML-PUKI12-2, EML-PUKI06-1, and EML-PUKI06-2 completely matched the *irregularis* and *fragilis* clade, respectively.

Although some kinds of new and undescribed zygomycetous fungi in Korea have been reported in several recent studies,

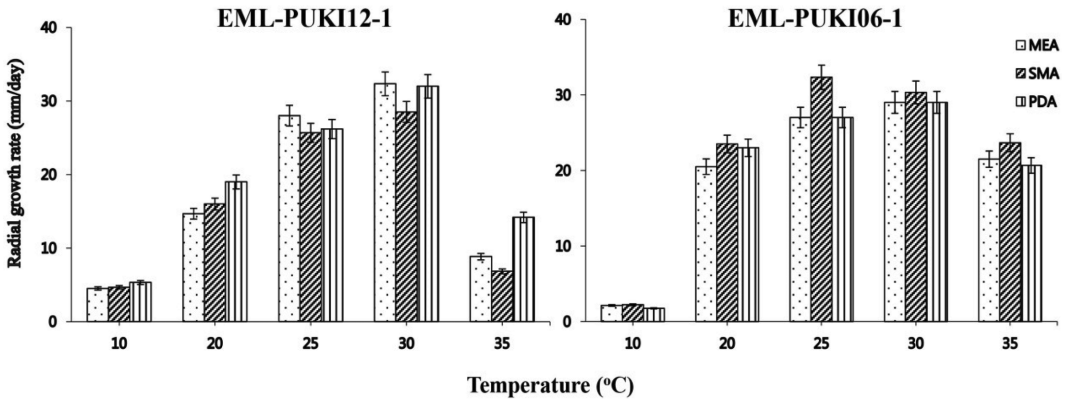

Fig. 4. Effect of temperature and culture medium on mycelial growth of *Mucor irregularis* EML-PUKI12-1 and *M. fragilis* EML-PUKI06-1. Mycelia were grown on malt extract agar (MEA), synthetic mucor agar (SMA), and potato dextrose agar (PDA), at different temperatures, as indicated.

data relating to the diversity of zygomycetous fungi in Korea are still lacking. Therefore, *M. irregularis* and *M. fragilis* isolated from the gut of insects and described as new record can contribute to the knowledge of diversity of zygomycetous fungi in Korea.

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