

## *Mariannaea samuelsii* Isolated from a Bark Beetle-Infested Elm Tree in Korea

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During an investigation of fungi from an elm tree infested with bark beetles in Korea, one isolate, DUCC401, was isolated from elm wood. Based on morphological characteristics and phylogenetic analysis of the internal transcribed spacer and 28S rDNA (large subunit) sequences, the isolate, DUCC401, was identified as *Mariannaea samuelsii*. Mycelia of the fungus grew faster on malt extract agar than on potato dextrose agar and oatmeal agar media. Temperature and pH for optimal growth of fungal mycelia were 25°C and pH 7.0, respectively. The fungus demonstrated the capacity to degrade cellobiose, starch, and xylan. This is the first report on isolation of *Mariannaea samuelsii* in Korea.

**KEYWORDS** : Elm Tree, ITS rDNA, LSU rDNA, *Mariannaea samuelsii*

### Introduction

*Mariannaea* species are widespread and have been isolated from leaves of conifer trees, decaying wood, submerged wood in freshwater streams, insect larva, and soil [1-6]. *M. elegans*, the type species of the *Mariannaea* genus, was described by Samson in 1974 [1], and its teleomorphic stage of *Nectria mariannaeae* was collected in Jamaica and Venezuela in 1991 by Samuels and Seifert [2]. Eight species of the genus *Mariannaea* have been reported. Among these eight species, *M. pruinosa* was also reported from China as having teleomorphic stage as *Cordyceps pruinosa*, an insect pathogen [3]. In Korea, *M. elegans* was reported in 2004 as the anamorphic name of *C. pruinosa* [7]. However, *M. pruinosa* has recently been accepted as the anamorphic name of *C. pruinosa* [8]. To further explore *Mariannaea* fungi in Korea, we investigated an elm tree infested with bark beetles and isolated two morphologically similar *Mariannaea* species. One species isolated from a bark beetle larva was identified as *M. elegans* var. *elegans* in a previous work [9]. In the present study, based on morphology and molecular analysis of rDNA sequences, we identified another species isolated from elm wood as *M. samuelsii*. Growth properties and

ability to degrade polymeric substrates against *M. samuelsii* were also described. This is a newly discovered species in Korea.

### Materials and Methods

**Fungal isolation.** In September, 2010, an elm tree with branches infested with unidentified bark beetles was sampled in Asan City in Chungchungnam-do (province) of Korea. The sampled elm wood branches were cut into pieces. For fungal isolation, several pieces of elm wood were surface sterilized with 70% ethanol for two minutes and washed with sterile water. After drying for a few minutes, the prepared pieces of elm wood were placed on 1% malt extract agar (MEA; Difco, Detroit, MI, USA) supplemented with streptomycin (200 µg/mL) and incubated at 25°C for 3~7 days. Mycelia grown out from the elm wood pieces were transferred to new MEA plates, followed by incubation. Subsequently, single spore isolation was performed on MEA. Pure cultures of the isolates were maintained on MEA and stocked in 10% glycerol at -20°C. After comparison of colony pattern on MEA and micromorphological characteristics of the pure cultures, one isolate with morphology resembling that of *Mariannaea*

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was coded as DUCC401 and used for subsequent study. The present isolate, DUCC401, was deposited in the Dankook University Culture Collection (DUCC), Cheonan, Korea.

**Morphological observation.** Colony patterns were observed on MEA, potato dextrose agar (PDA), and oatmeal agar (OA) at 25°C for 7~14 days. Color charts for R-colors by Glynn EF (<http://research.stowers-institute.org/efg/R/Color/Chart>) were used for determination of colony color. A phase-contrast microscope (Axioskop 40; Carl Zeiss, Jena, Germany) and a scanning electron microscope (SEM, Hitachi S-4300; Hitachi, Tokyo, Japan) were used for observation of micromorphological characteristics of isolate DUCC401. Examination of fungal structures was performed using fresh materials prepared on MEA at 25°C for 7~14 days. The "Agar block smear preparation" method for light microscopic observation, described by Woo *et al.* [10], was used for cutting and preparation of malt extract agar blocks containing mycelia. For SEM observations, several samples were prepared according to the method described by Tang *et al.* [9]. The prepared samples were dried using a Hitachi critical point drier, coated with platinum palladium for 50 sec using a Hitachi E-1030 ion sputter, and observed using a SEM operating at 15.0 kV.

**Molecular analysis.** Mycelia of isolate DUCC401, freshly grown on PDA for seven days, were harvested for DNA extraction using the drilling method described by Kim *et al.* [11]. Fungal specific primer pairs, internal transcribed spacer (ITS)1F-ITS4 and LROR-NL4, were used in performance of PCR amplification of the ITS region and large subunit (LSU) of ribosomal DNA (rDNA), respectively [12, 13]. The PCR reaction mixture (50 µL final volume) contained 100 ng of fungal genomic DNA, 20 pmol of each primer, 10 mM of the four deoxynucleotide triphosphates (dNTPs), 1× PCR buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM MgCl<sub>2</sub>, 50 mM KCl), and 1.0 unit Taq DNA polymerase (Promega, Madison, WI, USA). PCR conditions were: denaturation at 94°C for 10 min with one cycle, followed by 30 cycles of denaturation at 94°C for one min, annealing at 56°C for the ITS gene and 52°C for the LSU gene for one min, and extension at 72°C for one min, and final extension at 72°C for 10 min. PCR products were run in a 1.5% agarose gel, and the target DNA bands were cut and purified using a PCR clean-up kit (Qiagen, Hilden, Germany). The purified target DNA was ligated into T&A cloning vectors (RBC, Taipei, Taiwan), and subsequently transformed into competent *Escherichia coli* DH5α cells, following the manufacturer's instructions (RBC, Korea). An ABI 3700 automated sequencer (Perkin-Elmer Inc, Waltham, MA, USA) was used at Macrogen Inc. (Seoul, Korea) for performance of DNA sequencing.

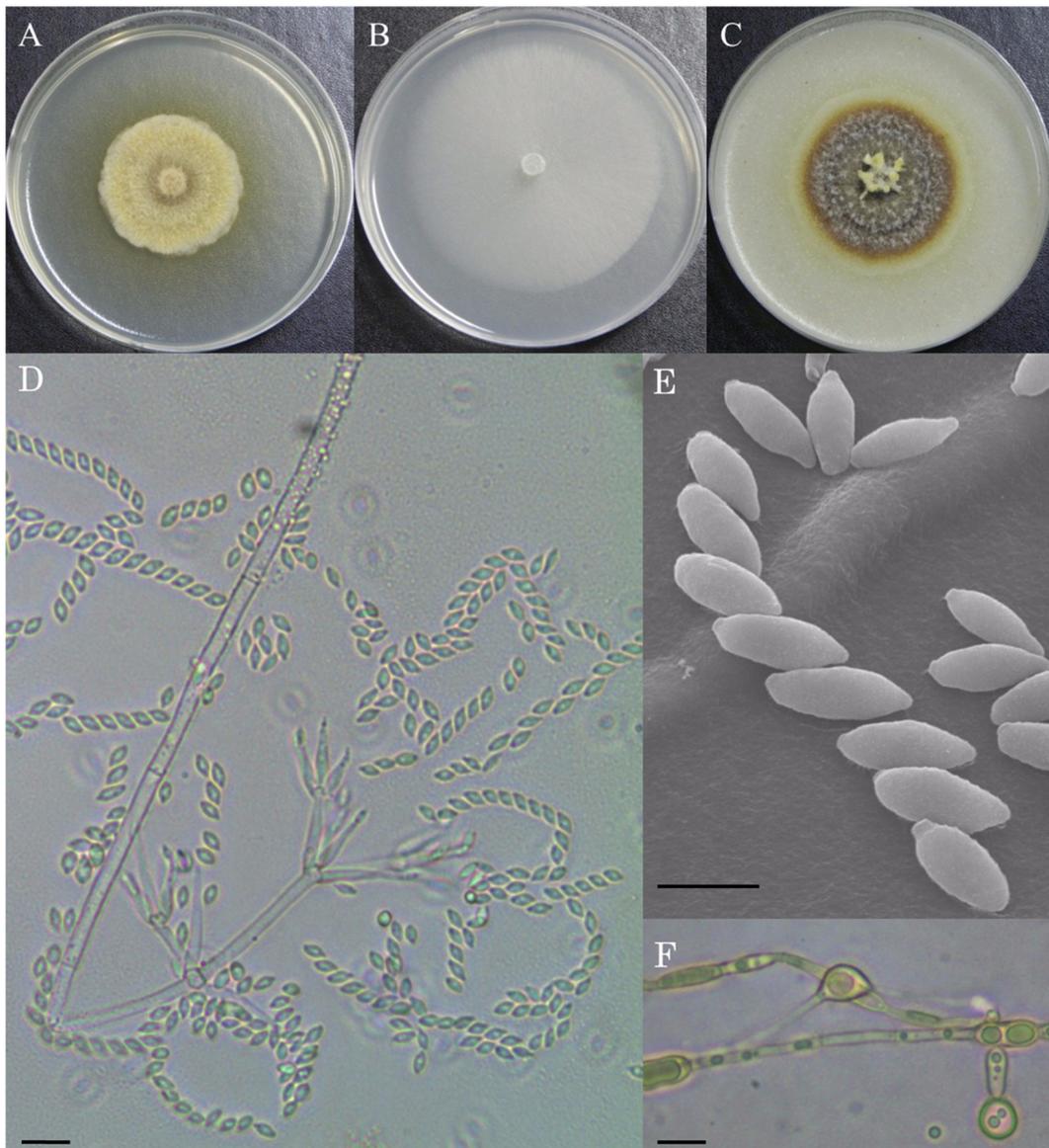
The obtained ITS and LSU sequences were blasted in the

GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). The reference sequences used in this study were also obtained from the GenBank database. Phylogenetic analyses based on ITS and LSU sequences were performed using the MrBayes ver. 3.2 program, according to the manual [14]. Bayesian analysis was set with the GTR substitution model with gamma-distributed rate, running over 100,000 generations, and stopped when the average standard deviation fell below 0.01. *Neonectria radicola* and *Neonectria lucida* were used as out-groups for ITS and LSU phylogenetic analysis, respectively [15].

**Growth and extracellular enzyme tests.** PDA, MEA, and OA were used for mycelial growth on solid media at 25°C for 14 days. MEA was used for measurement of mycelial growth at different temperatures (20~35°C) and pH (4.0~10.0). The ability to produce extracellular enzymes with the capacity to degrade polymeric carbon sources was estimated on chromogenic reaction medium. The chromogenic medium contained 0.5% of each carbon source, including: CM-cellulose (Sigma-Aldrich, St. Louis, MO, USA), D-cellobiose (Sigma-Aldrich), polygalacturonic acid (MP Biomedicals, Sanata Ana, CA, USA), starch (Sigma-Aldrich), xylan (Sigma-Aldrich), and avicel (Fluka, Cork, Ireland), 0.1% yeast nitrogen base without amino acid (BD, Franklin Lakes, NJ, USA) as a nitrogen source, 0.05% Congo Red (Sigma-Aldrich) as a chromo dye, and 1.5% agar. The ability to degrade protein was measured on 2% agar with 10% skim milk powder (Fluka, Buchs, Switzerland) [16]. After cultivation at 25°C for 14 days, the colony diameter and the clear zone caused by fungal growth were measured for evaluation of relative extracellular enzyme activity.

## Results and Discussion

**Morphology.** Colonies of isolate DUCC401 formed on MEA were white first and later became yellowish with time (Fig. 1B). Colony color was yellow on PDA and yellow brown on OA (Fig. 1A and 1C). Colonies were zonate on PDA and OA, but weakly on MEA. Colon reverse appeared whiter on MEA, and yellow to brown on PDA and OA. Vegetative hyphae were hyaline and thin on MEA. The conidiophore of isolate DUCC401 was long and thin, having a stalk up to 300 µm in length and 4~6 µm in width. The isolate showed morphological similarities to *Mariannaea elegans* [1], *M. aquaticolar* [4], and *M. samuelsii* [6] in production of verticillate conidiophores and imbricate chains of fusiform conidia. Conidiophore structures of the isolate were irregular and complex with 2~3 level verticillate branches and formed verruca at the base and apex (Fig. 1D). Each verticillate branch could sometimes repeat at the second branch and rarely at the third branch, with whorls of 3~6 phialides.

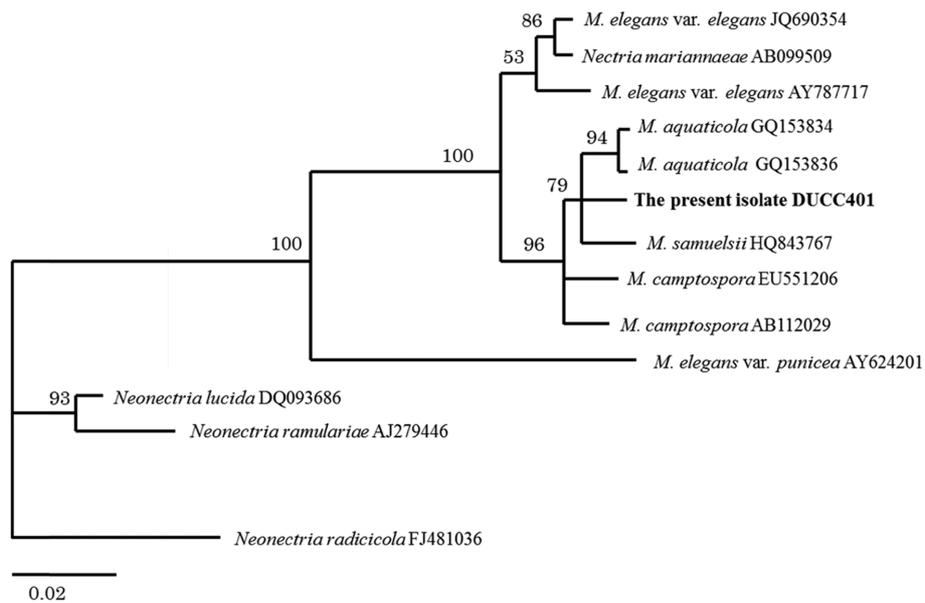


**Fig. 1.** Colony morphology (A~C) and micromorphological characteristics (D~F) of isolate DUCC401. Colony patterns shown on potato dextrose agar (A), malt extract agar (B), and oat meal agar (C) media after growth at 25°C for 14 days. D, conidiophores with imbricate conidial chains observed using a light microscope; E, conidia observed using a scanning electron microscope; F, chlamydospores (scale bars: D, F = 10  $\mu$ m, E = 5  $\mu$ m).

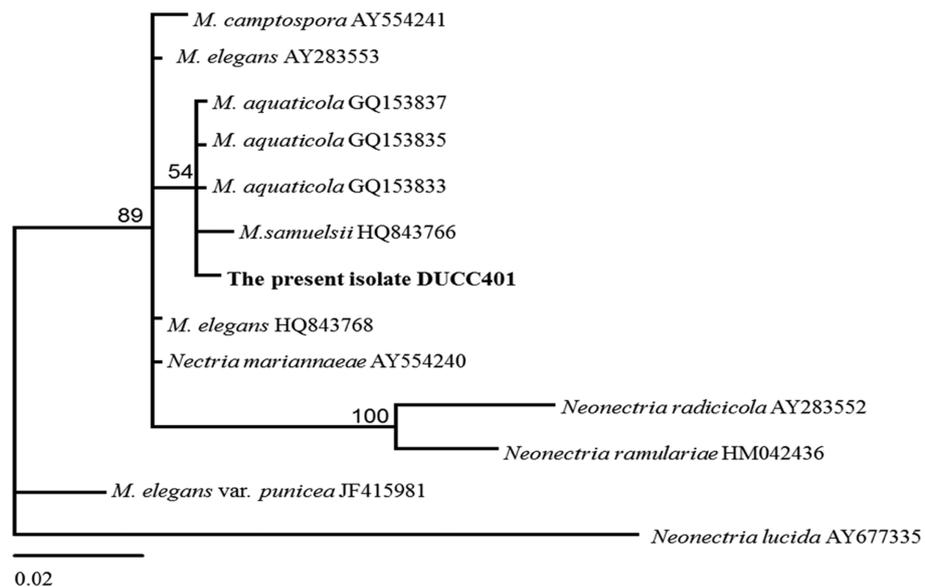
The length between two branch nodes was 20~40  $\mu$ m. The phialides measured 12~25  $\times$  2~4  $\mu$ m in size, with a slender flask shape, tapering toward the apex, hyaline, and smooth walled, but sometime bearing verruca (Fig. 1D). Conidia measured 4.5~7  $\times$  2.5~3.5  $\mu$ m in size and formed in imbricate chains, which were attached with phialides. Conidia showed greater uniformity in shape and size, were hyaline fusiform, widest in the 1/3 or 1/4 part of conidia, and usually formed a neck at one end of conidia, which was clearly observed by SEM (Fig. 1E). Chlamydospores measuring 10~17  $\times$  6~8  $\mu$ m in size were observed at intermediate and terminal mycelium, and were globose or ellipsoidal, and brown in color (Fig. 1F). No microstructure

of sexual stage was observed, indicating that isolate DUCC401 was in anamorphic stage.

**Molecular analysis.** Isolate DUCC401 had 599 bp in its ITS sequence and 619 bp in its LSU sequence. The ITS and LSU sequences were deposited in GenBank with accession Nos. JX125048 and JX125049, respectively. Isolate DUCC401 was grouped with *Mariannaea aquatica* and *Mariannaea samuelsii* in the phylogenetic trees, which were generated based on ITS and LSU sequences (Figs. 2 and 3). In the ITS-based phylogenetic tree, the isolate was separated from *M. aquatica* in a clade with *M. samuelsii*. In the LSU-based phylogenetic tree, the isolate was not



**Fig. 2.** Phylogenetic analysis of isolate DUCC401 based on internal transcribed spacer rDNA sequences. Using the MrBayes program, Bayesian analysis was performed by setting with the evolutionary model to the GTR substitution model with gamma-distributed rate. Bayesian posterior probabilities were given at the nodes. *Neonectria radicola* was used as an out-group. The bar indicates the number of nucleotide substitutions per site. The present, isolate DUCC401, is shown in bold.



**Fig. 3.** Phylogenetic analysis of isolate DUCC401 based on large subunit rDNA sequences. Using the MrBayes program, Bayesian analysis was performed by setting with the evolutionary model to the GTR substitution model with gamma-distributed rate. Bayesian posterior probabilities were given at the nodes. *Neonectria lucida* was used as an out-group. The bar indicates the number of nucleotide substitutions per site. The present isolate, DUCC401, is shown in bold.

separated from *M. aquaticola* and *M. samuelsii*. ITS sequences of fungi are known to show more variability than LSU sequences; therefore, the ITS-based phylogenetic tree little better resolved phylogenetic relationships of the isolate to other species. The two closely related species, *M. aquaticola* and *M. samuelsii*, showed morphologic similarities, and differed only by four substitutions in ITS sequence and three substitutions in LSU sequence.

*M. aquaticola* and *M. samuelsii* differed slightly in conidia size and the presence of chlamydospores. *M. aquaticola* produced large conidia (5~10 × 2~4.5 μm) and *M. samuelsii* produced medium-sized conidia (3.5~7.5 × 2.5~3.5 μm) (Table 1). In addition, chlamydospores were observed in *M. samuelsii*, but were absent in *M. aquaticola* [4, 6]. Morphologically, isolate DUCC401 showed greater similarity to *M. samuelsii* than *M. aquaticola*

**Table 1.** Morphological characteristics of isolate DUCC401 and three *Mariannaea* species

Characteristics	DUCC401 (present study)	<i>M. samuelsii</i> <sup>a</sup>	<i>M. aquaticola</i> <sup>b</sup>	<i>M. elegans</i> var. <i>elegans</i> <sup>c</sup>
Colony color on malt extract agar	White to yellowish	Brownish orange	Yellowish to dark brown	Yellow-brown
Conidia size ( $\mu\text{m}$ )	4.5~7 $\times$ 2.5~3.5	3.5~7.5 $\times$ 2.5~3.5	5~10 $\times$ 2~4.5	4~6 $\times$ 1.5~2.5
Conidia shape	Ellipsoidal to fusiform, uniformity in size and shape, widest at the 1/3 or 1/4 part of the cell	Broadly fusiform or ellipsoidal widest at one part of the cell	Ellipsoidal to fusiform	Ellipsoidal to fusiform, widest in the middle of the cell
Chlamydo-spore	Present	Present	Absent	Present

<sup>a</sup>Data from Gräfenhan *et al.* [6].

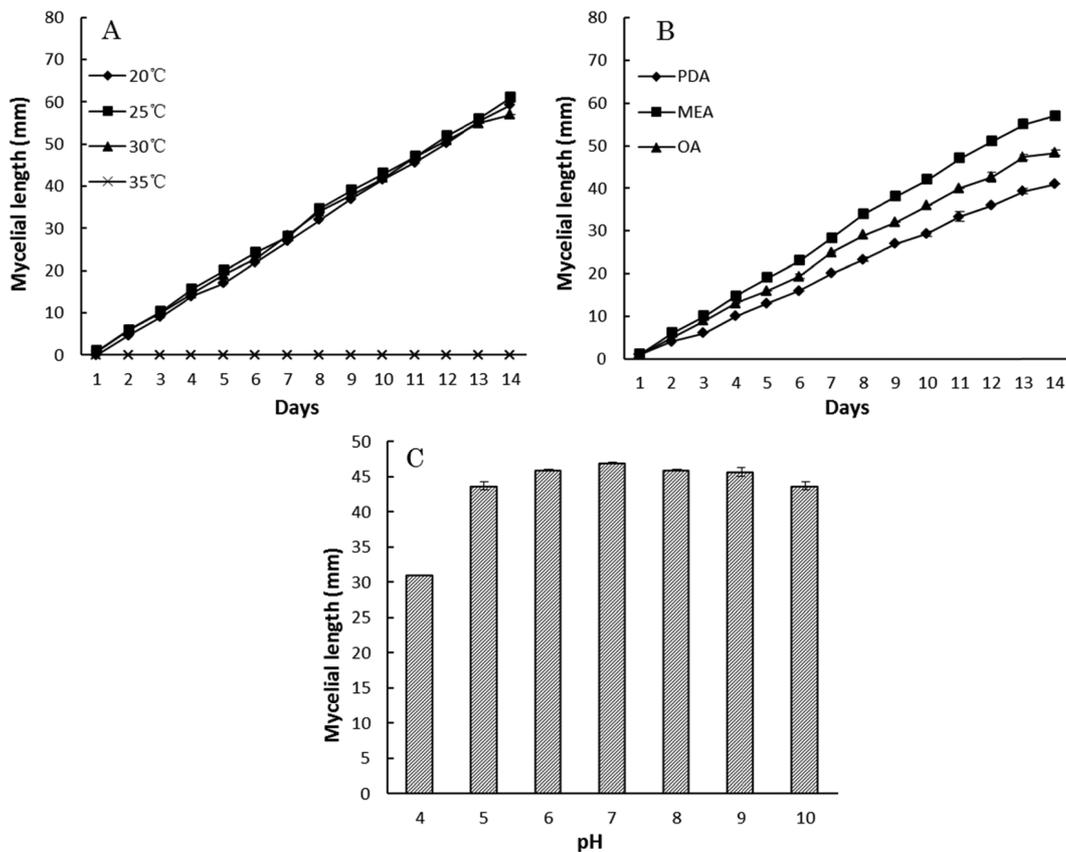
<sup>b</sup>Data from Cai *et al.* [4].

<sup>c</sup>Data from Samson [1] in Mycobank (<http://www.mycobank.org>).

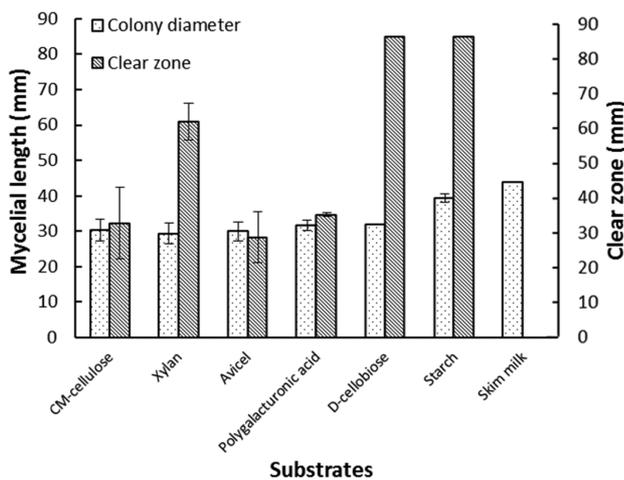
in that it has a medium range of conidia size (4.5~7  $\times$  2.5~3.5  $\mu\text{m}$ ) and chlamydo-spores. Thus, we identified isolate DUCC401 as *M. smuelsii*. This species was recently described as a new species and was isolated from soil under a coniferous tree species, *Podocarpus* sp. [6].

**Growth and biochemical properties.** Mycelia of isolate DUCC401 grew faster on MEA than PDA and OA (Fig. 4A~4C). They grew well at a wide range of temperatures,

20~30°C, with no significant difference between temperatures. However, they did not grow at 35°C. This growth property at 35°C is comparable to that of *M. elegans* var. *elegans* DUCC400, which is able to grow at 35°C [9]. Mycelia of isolate DUCC401 grew well at pH 6, 7, and 8. At pH 4, they showed a slight reduction in growth. Growth of the isolate was observed on all of the substrates tested, as shown in Fig. 5. The ability to degrade xylan, cellobiose, and starch, which are present in wood cells as component



**Fig. 4.** Mycelial growth of isolate DUCC401 on malt extract agar (MEA) at different temperatures (A), on different media at a temperature of 25°C (B), and on MEA at different pH at a temperature of 25°C for 10 days (C). PDA, potato dextrose agar; OA, oatmeal agar.



**Fig. 5.** Mycelial length of isolate DUCC401 grown on chromogenic reaction medium containing each enzymatic substrate and on skim milk agar and the size of the clear zone resulting from fungal growth on the media. Clear zone formed at the tips of fungal mycelia was measured to evaluate the ability of substrate degradation.

compounds, was clearly demonstrated. Therefore, we did not rule out the possibility that isolate DUCC401 could function as a wood degrader on elm wood. In addition, isolate DUCC401 did not show a clear zone on skim milk agar, indicating that it does not have the capacity for significant production of protein degrading enzymes.

In conclusion, according to morphological characteristics and molecular analysis of ITS and LSU sequences, we identified isolate DUCC401 as *M. samuelsii*. This is the first report to describe isolation of *M. samuelsii* from an elm tree in Korea. In a previous work, we identified *M. elegans* var. *elegans*, isolated from a bark beetle larva, which was captured from elm wood, where we isolated *M. samuelsii* DUCC 401. Thus, we assume that *M. samuelsii* DUCC 401 might have been introduced to the wood of the elm tree by bark beetles. Due to the resemblance of *M. elegans* var. *elegans* to *M. samuelsii*, this study should help us to differentiate these two species according to both morphological and molecular characteristics.

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