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Identification and Characterization of *Eurotium rubrum* Isolated from Meju in Korea

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We isolated and identified a strain of *Eurotium rubrum* from Meju that has not been reported in Korea. This fungus is yellowish brown; reverse dark brown on CYA and PDA while yellow on 2% MEA at 25°C. Cleistothecia are first bright yellow and gradually turned brown. Mycerial growth on CYA attained a diameter of 30 mm at 20°C, 37 mm at 25°C and 32 mm at 30°C after 15 days. The isolate grew slower on 2% MEA (< 20 mm 15 days at 25°C) compared to CYA and PDA (< 40 mm 15 days at 25°C). Cleistothecia are superficial, yellow to light brown, globose to subglobose, 40~75 μm in diameter. Asci are 8-spored and globose to subglobose 8~11 μm. Ascospores are disciform, 4.0~5.0 μm in length and 4.2~4.5 μm in width. Conidia are ovate or bacillar, finely roughened to densely spinulose, 4.6~6.0 μm in length and 3.0~4.3 μm in width. Compared to known *Eurotium rubrum*, the Korean isolate showed 99% sequence similarity in ITS rDNA (554 bp) and calmodulin (750 bp) gene and 100% in β-tubulin (1016 bp) gene. The *E. rubrum* isolate also had weak β-glucosidase and protease activities.

KEYWORDS : β-tubulin gene, Calmodulin gene, *Eurotium rubrum*, ITS rDNA, Meju

Soybean and its products are a major protein source in Asian countries. In Korea, the most important soybean products are soy paste and soy sauce (Lee, 1995). They are derived from Meju, a brick of dried fermented soybeans, and have been manufactured for centuries at home using traditional methods in which natural microflora, especially *Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., and *Bacillus* sp., are used in the fermentation process (Park *et al*., 2003). These microorganisms convert carbohydrates into amino acids, sugars, organic acids, alcohol, and esters, creating the characteristic flavor of Meju (Kim *et al*., 2001). In spite of their roles in fermentation, fungi are also one of the potential problems in the quality of Meju production. Currently, many fungal species have been known to be involved in the contamination of Meju production. In Korea, consumers have expressed concern regarding the manufacture of foods like Meju using traditional methods because of possible contamination with mycotoxins, especially aflatoxins. Since fungal species such as *Aspergillus flavus* Gourama, H. & Bullerman, L. B. and *A. parasiticus* Gourama, H. & Bullerman, L. B. are known to produce aflatoxins (Gourama and Bullerman, 1995). Meju is always carefully examined for the presence of *Aspegillus* spp.

In this study, we isolated and identified *Eurotium rubrum* from improperly fermented Meju from a factory in Korea. This fungus was found to be a new Meju contaminant in Korea. Morphological characters including anamorph and telemorph of the isolated fungus were described with its physiological and molecular features.

Materials and Methods

Fungal isolation and culture conditions. Improperly fermented, factory made meju were obtained in Ulsan, Korea, in the spring of 2008 (Fig. 1). The Meju were obtained in dried form with fully grown fungi. Meju samples were broken into small pieces and put on potato dextrose agar (PDA) or Czapek yeast extract agar (CYA) and incubated for several days at 25°C. Mycelia grown out from the small pieces of Meju were transferred to new PDA and single spore isolates were obtained from the PDA-grown fungi. The obtained fungal isolates were

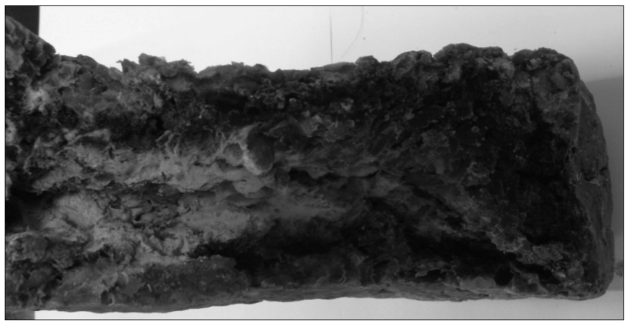

Fig. 1. The internal view of an improperly fermented Meju used in this study. Fungal colonization is shown with uneven distribution.

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maintained on PDA for the duration of the experiment and stored either at –80°C in 10% glycerol for long-term storage or in water at 4°C for short-term storage.

Observation of cultural and morphological characteristics. To identify optimal growth media, pre-cultured isolates were transferred to the center of 2% malt dextrose agar (MEA) or CYA and grown for 15 days at 25°C. Mycelial growth was recorded by measuring the diameters of the colonies. Light microscopic images of the morphological features of the isolates were examined under a phase-contrast microscope (Karl Zeiss, Axioskop 40) after growing the culture on CYA at 25°C for 5~10 days. For scanning electron microscopic observations (SEM), fungal isolates were grown for 5~10 days at 25°C on CYA. Agar blocks were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 16 h and 1% osmium tetroxide in 0.1 M phosphate for 1 h. Samples were subsequently washed with 0.05 M cacodylate buffer, dehydrated in a series of ethanol washes (50% for 20 min, 75% for 20 min, 90% for 20 min, 95% for 20 min and 100% for 20 min), passed through ethanol-isoamylacetate, dried with a Hitachi critical point dryer and coated with platinum-palladium at 25 nm using an Hitachi E-1030 ion sputter. The prepared specimens were examined by a Hitachi S-4300 scanning electron microscope operating at 10 kV.

Nucleic acid preparation, PCR amplification and nucleotide sequencing. Fungi were grown for 5~7 days on PDA at 25°C and fungal genomic DNA for PCR was obtained from the mycelia of the PDA-grown cultures using the drilling method described by Kim *et al.* (1999). The ITS ribosomal DNA regions were amplified by PCR using the universal primer pairs, ITS1 and ITS4 (White *et al.*, 1990). The β-tubulin gene was amplified using the primers T10 (O'Donnell and Cigelnik, 1997) and BT12 (Kim *et al.*, 2003), and the calmodulin gene was amplified using the primers CL1 and CL2A (O'Donnell *et al.*, 2000). PCR reaction mixture (a total volume of 50 μl) contained 100 ng fungal genomic DNA, 20 pmol of each primer, 10 mM (each) of the four deoxynucleotide triphosphates (dNTPs), 1 × PCR buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM $MgCl_2$, 50 mM KCl), 1 unit Thermostable polymerase (Solgent Corp.). Amplification was performed in a Gene Amp-950 thermal cycler (ABI, USA). PCR conditions were programmed as follows: one cycle of denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and final one cycle of extension at 72°C for 10 min. The PCR products were purified with a PCR clean-up kit, ligated into T&A cloning vectors, and the ligated vectors were transformed into competent *Escherichia coli* DH5α cells according to the manufacturer's instructions (RBC, Korea). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). The obtained nucleotide sequences were searched through BLASTN at GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Molecular phylogenetic analysis. The determined nucleotide sequences were manually edited using the Chromas v2.31 program and aligned using the ClustalW2 program. Reference sequences of related taxa were obtained from the GenBank database. The aligned sequences were analyzed with PAUP 4.0 program (Swofford, 2002). Phylograms based on β-tubulin and calmodulin gene sequences were constructed by the neighbor-joining method (Kimura, 1980). Bootstrap values were generated with 1000 replicates through heuristic searches. *Penicillium steckii* was used as an outgroup.

Extracellular enzyme activity test. Fungal isolate was precultured on PDA at 25°C for 5 days. For the observation of extracellular enzyme activity, the precultured isolate was transferred onto the media containing each of 0.5% CM-cellulose (Sigma, USA), D-cellobiose (Sigma, USA), pectin (MP Biomedicals, USA), starch (Sigma, USA), skim milk (Sigma, USA), xylan (Sigma, USA) as enzymatic carbon source, 0.1% yeast nitrogen base without amino acid (Difco, USA) as fundamental nitrogen source, 0.5% dyes (Congo Red, Sigma, USA) for chromogenic reaction, and 1.5% agar powder (Yoon *et al.*, 2007). After 7 days of culturing at 25°C, enzymatic activity was evaluated by observing clear zone (plaque) formation around the fungal colony by reaction between the enzymes secreted by the fungus and chromogenic substrates.

Results and Discussion

Several fungal isolates were obtained from improperly fermented, factory produced Meju. Among the isolates, some of them showed slow growing property with pigment on PDA. These slow growers produced very similar colony morphology and microscopic features of aspergillum (asexual spore-forming structure common to all Aspergilli) and cleistothecia, implying that they were the same species. Therefore, one of the isolates coded as DKU001 was identified and its mycological characters described in this study.

When DKU001 was grown at 25°C for 2 weeks, the colony color of this fungus was orange red on CYA, yellowish brown on PDA and yellow on 2% MEA (Fig. 2). The colony color was clearer on CYA than on MEA and PDA while fungal growth was faster on CYA and PDA (< 40 mm) than on 2% MEA (< 20 mm) (Fig. 3). After 14

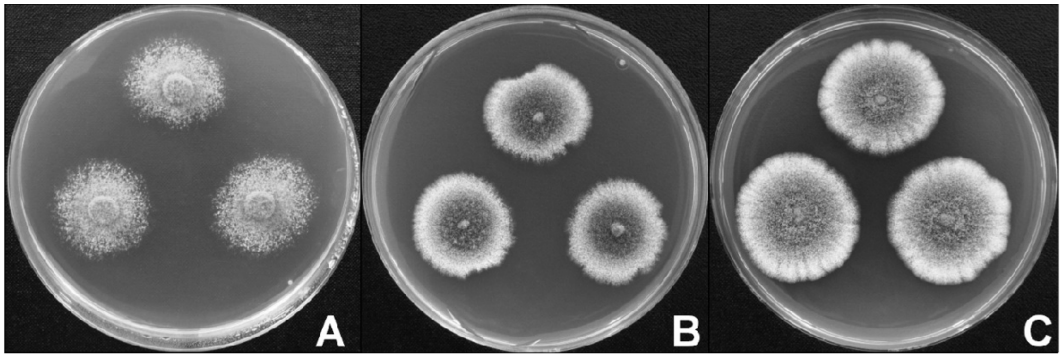

Fig. 2. Colonies of the DKU001 isolate cultured on 2% MEA (A), PDA (B) and CYA (C) at 25°C for 14 days. MEA: malt extract agar, PDA: potato dextrose agar, and CYA: Czapek yeast agar.

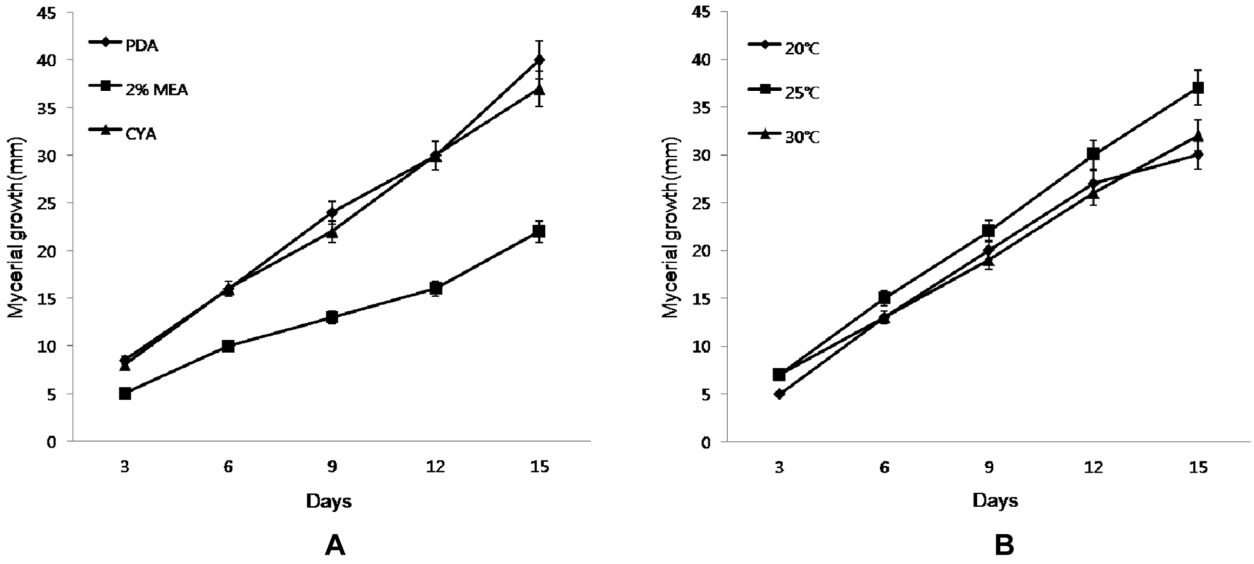

Fig. 3. Variations in mycerial growth of the isolate DKU001 on different media (A) at 25°C and different temperatures (B) on PDA. PDA: potato dextrose agar, MEA: malt extract agar, and CYA: Czapek yeast agar.

days of incubation on CYA, mycelia of the fungus grew to a diameter of 30 mm at 20°C, 37 mm at 25°C and 32 mm at 30°C. Similarly, DKU001 grew on PDA better at 25°C than 20 or 30 (Fig. 3).

Anamorph and telemorph of DKU001 as observed by light and scanning electron microscopy are shown in Figs. 4 and 5. Conidia were formed and borne on aspergillum indicating that this fungus is an *Aspergillus* species (Figs. 4A~C, 5A~F). Conidia of the fungus was ovate or bacillar, finely roughened to densely spinulose, 4.6~6.0 μm in length and 3.0~4.3 μm in width (Figs. 5G~I.). Cleistothecia were easily produced on CYA. Their color was first bright yellow and gradually turned brown. The microscopic analysis showed that the fungal cleistothecia were superficial, yellow to light brown, globose to subglobose, 40~75 μm in diameter (Figs. 4D, 5J~L). Ascus of the fungus was 8-spored, globose to subglobose 8~11 μm in diameter (Fig. 4E). Ascospores were disciform, 4.0~5.0 μm in length and 4.2~4.5 μm in width with a narrow to broad furrow, and two indistinct ridges, with smooth convex surfaced walls (Figs. 5M~O).

The observed morphological characters of DKU001 are summarized at Table 1. Based on the results of growth properties and morphological characteristics found in anamorphic *Aspergillus* species and telemorphic *Eurotium* species, DKU001 was identified as *Eurotium rubrum*. This species has been known to have several synonyms such as *Aspergillus sejunctus* Bainier & Sartory, *Aspergillus ruber* Thom & Church, *Eurotium repens* de Bary, *Aspergillus rubrum* (Jos. König *et al.*) Thom & Church. It is also considered as the perfect stage of *Aspergillus rubrobrunneus* Samson & W. Gams. These indicate that identification of *E. rubrum* is not easy based on morphology. Therefore, we further analyzed DKU001 using molecular methods.

We amplified the ITS rDNA region, calmolulin and β-

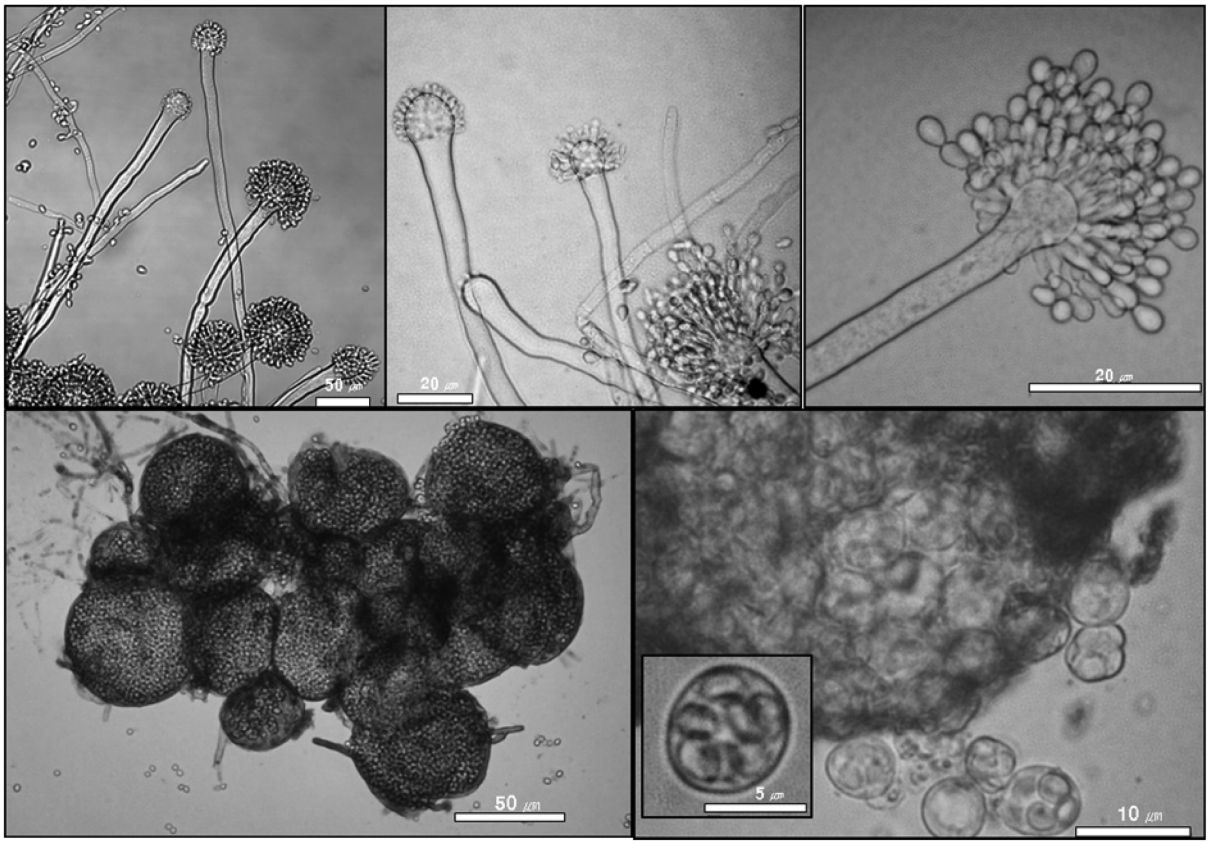

Fig. 4. Morphological features of DKU001 by light microscopy. A~C, conidial heads; D, cleistothecia; E, asci with ascospores.

Table 1. Morphological characters of *Eurotium rubrum* and the isolate DKU001

Characteristics	*Eurotium rubrum*[a]	Present study
Colony color	orange-red to ferruginous	orange-red
Cleistothecia		
color	orange-red, yellow	yellow to light brown
shape	spherical	globose to subglobose
size	80~120 μm	40~75 μm
Ascus		
shape	globose to subglobose	globose to subglobose
size	10~12 μm	8~11 μm
Ascospore		
color	orange-red, yellow	yellow
shape	ellipsoidal, lenticular	disciform
size	5.2~6.0 × 4.4~4.8 μm	4.0~5.0 × 4.2~4.5 μm
Conidia		
shape	ovate, barrel	ovate, bacillar
size	5.0~7.5 μm long	4.6~6.0 × 3.0~4.3 μm

[a]Data from IMI descriptions of fungi and bacteria (Kozakiewicz, 1995) and Mycobank (www.mycobank.org/MycoTaxo.aspx).

tubulin genes by PCR, and determined the nucleotide sequences of the PCR amplicons. The determined size of PCR amplicons are shown in Table 2. The three determined sequences of DKU001 were deposited in GenBank under accession numbers GQ354133~354135 (Table 2). When homologous sequences for the determined sequences were searched in the GenBank DNA database, sequence similarity between DKU001 and known *E. rubrum* were 99% in genes for ITS (accession no. GQ354133, 554 bp) and calmodulin (accession no. EF652011, 750 bp) and 100% for the β-tubulin (accession no. EF651921, 1016 bp) gene sequence. Phyloge-

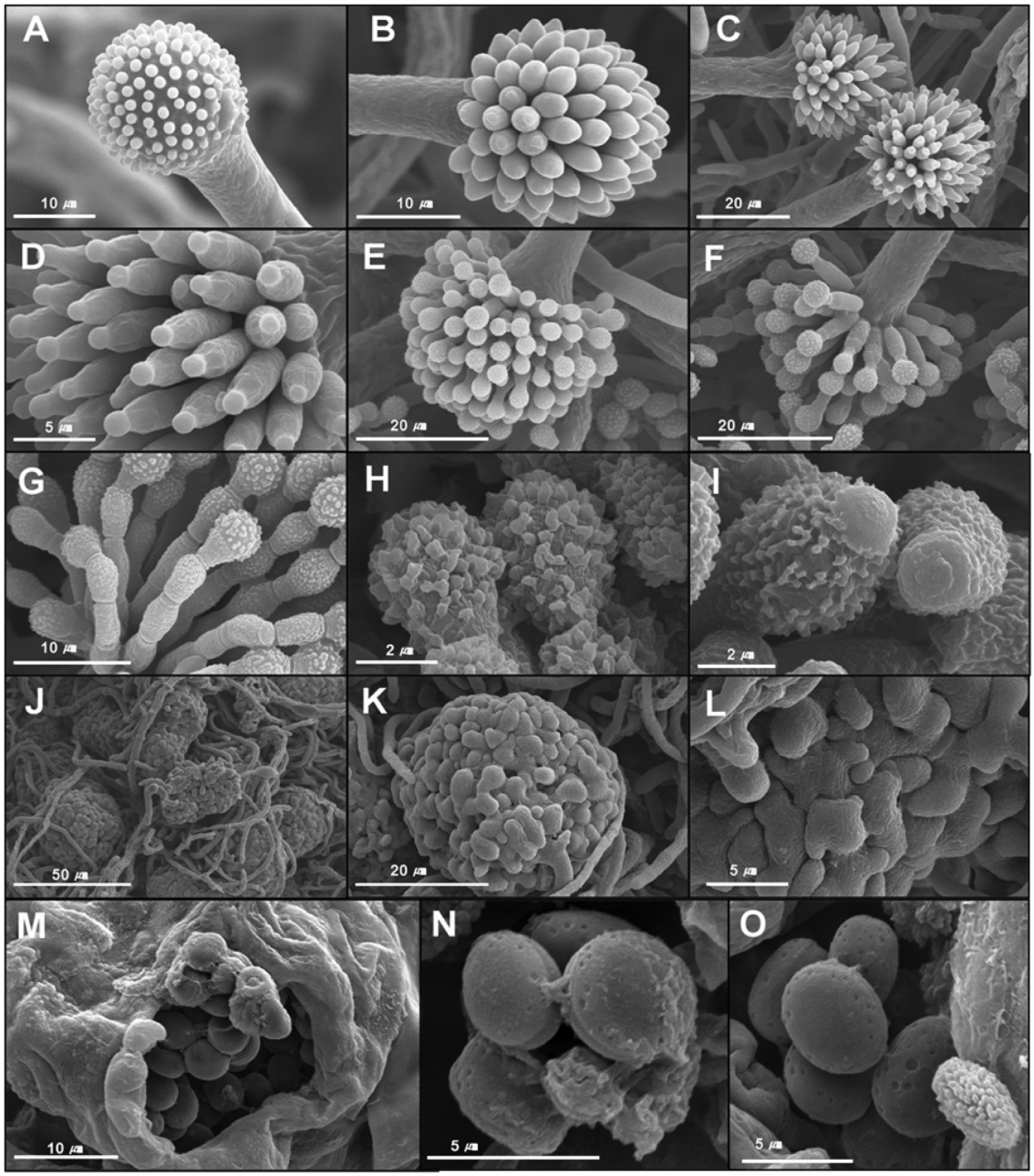

Fig. 5. Morphological features of DKU001 as observed by scanning electron microscopy. A~F, conidial heads; G~I, conidia. J~L; cleistothecia; M~O, ascospores.

Table 2. The determined size and GenBank accession numbers of the PCR amplified target genes in the isolate DKU001

Target genes	Size	GenBank accession no.
ITS	554 bp	GQ354133
Calmodulin	750 bp	GQ354134
β-tubulin	1016 bp	GQ354135

netic analysis based on the calmodulin (Fig. 6A) and β-tubulin (Fig. 6B) gene sequences also revealed that DKU001 was placed with *E. rubrum*. These molecular results strongly support the morphological identification of DKU001 as *E. rubrum*.

E. rubrum is a species that is frequently encountered in tropical and subtropical regions. This species is not known to be a pathogen of animals or humans, but the species has been previously isolated from human nails (Smith, 1989). It has been reported as an endophyte in the mangrove plant *Hibiscus tiliaceus* (Li *et al*., 2008), and is frequently reported from soils and dried or concentrated food products, leather goods, cotton, seeds, and other dried products (Wheeler *et al*., 1986). In Korea, the description

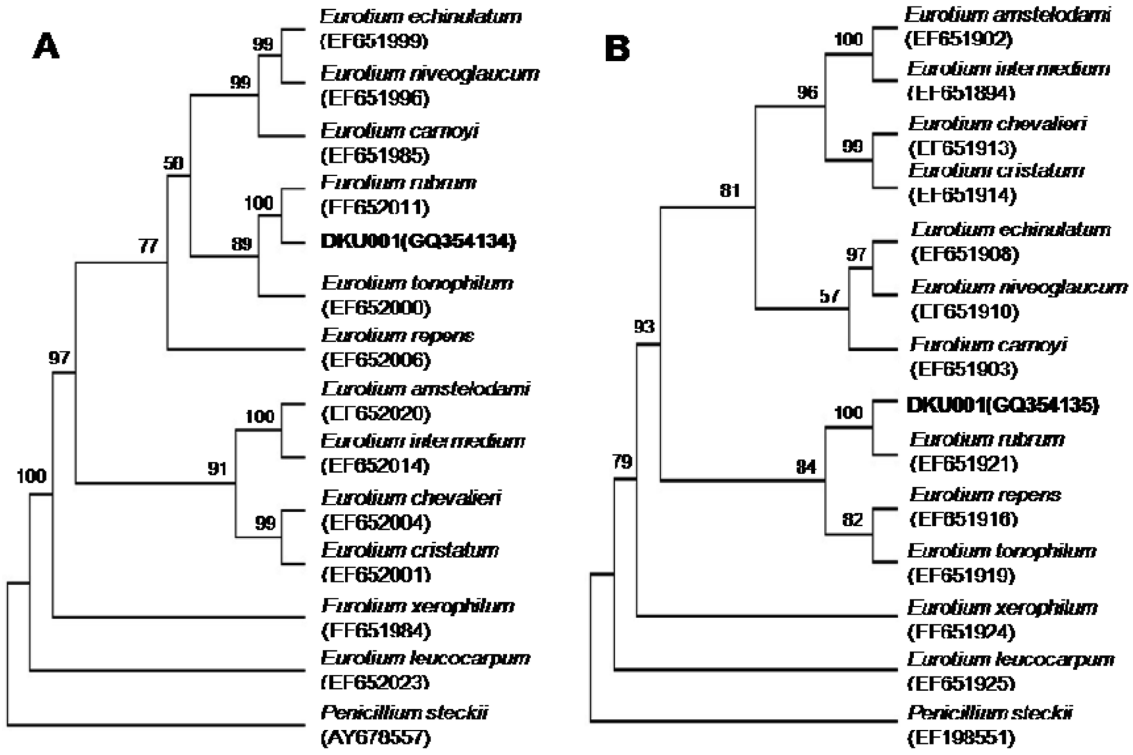

Fig. 6. Phylogenetic relationships of DKU001 to other *Eurotium* species. Cladogram based on the analysis of partial nucleotide sequence of the calmodulin (A) and β-tubulin (B) gene was generated by the neightor-joining analysis. Numbers at nodes represent percentage of bootstrap resampling base on 1000 replicates. *Penicillium steckii* was used as an outgroup.

Table 3. Extracellular enzyme activities in the isolate DKU001

Isolate	Enzymes						
	Amylase	Avicelase	CM-cellulase	β-glucosidase	Pectinase	Protease	Xylanase
DKU001	–	–	–	+	–	+	–

+: activity detection, –: no activity detection. Enzyme activity was determined by observing clear zone formed around the fungal colony in the chromogenic substrate medium containing Congo Red.

of *E. rubrum* has not been officially reported. However, two isolates of *E. rubrum* (KACC43556, 43557) from soil and hay were deposited to Korean Agriculture Culture Collection (KACC) in 2007 by S. H. Yu (Chungnam National University). Therefore, we compared the ITS rDNA sequence of two isolates of *E. rubrum* (KACC43556 and 43557) with the Meju isolate DKU001 from this study. ITS rDNA sequence information on KACC43556 and 43557 was downloaded from the Website of Korean Agriculture Culture Collection (http://kacc.rda.go.kr/). The compared isolates shared 100% sequence identity (data not shown), confirming that the Meju isolate and the two isolates from soils or hay are the same species.

In Meju, several fungal species have been previously reported. Among them, fast-growing species such as *Aspergillus oryzae* Kundu, A. K. & Manna, S., *Rhizopus oryzae* Kumar *et al*. and *Mucor* sp. have been reported to be involved in Meju fermentation. These species are known to produce protease and alpha-amylase (Kumar *et al*., 2005; Kundu and Manna, 1975; Thompson and Eribo, 1984). Thus, we examined whether DKU001 could also produce not only protease and alpha-amylase but also other extracelluar enzymes. This slow-growing species showed only weak β-glucosidase and protease activity (Table 3), indicating that the *E. rubrum* DKU001 is a lesser contributor in the fermentation of Meju. At this point, we do not know what the role of *E. rubrum* DKU001 is in the improperly fermented Meju. Recently, the ability of producing benzaldehyde derivatives and secondary metabolites was reported in *E. rubrum* (Li *et al*., 2008; Slack *et al*., 2009). Because benzaldehyde could be easily oxidized in air, it cannot be ruled out that the species' metabolites may hamper the proper fermentation process.

So far, no toxic effects of *E. rubrum* have been reported. This means the species is not considered to be a

food toxic mold at this point. Since this species is considered to be a xerophile, the species may able to colonize Meju which will eventually be dried after the fermentation process. Considering that *E. rubrum* is present in soils and hay in Korea, it can be assumed that Meju contamination may have occurred through the environment.

Overall, we identified the presence of *E. rubrum* in improperly fermented Meju and described its morphological characters and molecular and physiological properties. This is the first description of *E. rubrum* isolated from fermented soybeans in Korea.

Acknowledgements

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