RESEARCH ARTICLE

Isolation and Identification of Two Unreported Fungi in Korea: *Dothidea insculpta* and *Metarhizium rileyi*

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ABSTRACT

In this study, the fungal strains KNU-Gunwi 2B and KNU-SOT5 were isolated from root-soil in a hillside and the cherry tree bark (*Prunus serrulata*), respectively from Gyeongbuk province in Korea. The strain KNU-SOT5 produced dark brown chlamydospores that were smooth to lightly rough-walled, globose to ellipsoidal, and the conidia were aseptate, guttulate, mostly fusiform with a diameter of 5.3-17.6×4.2-7.0 µm. Strain KNU-Gunwi 2B produced phialides that were smooth-walled, cylindrical with semi-papillate apices and the conidia were pale-green, broadly ellipsoid, and sometimes cylindrical with a diameter of 4.4-8.0×2.3-4.0 µm. The strain KNU-SOT5 and KNU-Gunwi 2B were resolved based on cultural and morphological characteristics, along with the phylogenetic analysis using the small subunit (SSU), large subunit (LSU), and internal transcribed spacers (ITS) regions. The fungal strains KNU-SOT5 and KNU-Gunwi 2B were identified as *Dothidea insculpta* and *Metarhizium rileyi*, which have not been reported in Korea.

Keywords: Cherry tree, *Dothidea insculpta*, *Metarhizium rileyi*, Phylogeny, Soil-inhabiting fungi

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INTRODUCTION

The class Dothideomycetes is the largest and most diverse Ascomycota containing 11 orders, 90 families, 1,300 genera, and over 19,000 known species [1,2]. The order *Dothideales*, that belongs to the class Dothideomycetes, was introduced by Lindau (1897) to provide a single-family of *Dothideaceae*. Since then, the family *Dothideaceae* had numerous changes in its genera concept and inclusion from 1896 to early 2000, including ascomata developing nonostiolate loculi in stromata, opening by apical fissure or dehiscence, displaying eight-or many-spored asci at the base of the locules with one or many-septate, a different hyaline or brown appearance, and presenting guttulate ascospores [3]. *Dothidea insculpta* is a fungal species in the family *Dothideaceae*. The family *Dothideaceae* is composed of fungi that are biotrophic, necrotrophic, or saprobic. These fungi infect twigs and other plant parts, as well as sometimes be found on leaves as a plant pathogen that causes serious crop loss [2]. *Metarhizium rileyi* (Clavicipitaceae,

Hypocreales, Ascomycota) under the class Sordariomycetes is widespread, and commonly known as an important entomopathogenic fungus [4]. Yeast-like hyphal bodies and a true filamentous growth phase are distinct characteristics of *M. rileyi* [5]. *M. rileyi* was described as *Botrytis rileyi* Farlow in 1883 originally, and later as *Spicaria rileyi* Farlow. In 1974, this fungus was re-described and transferred it to the genus *Nomuraea* Maublanc by Kish, Samson, and Allen [6]. However, it was proposed as a new combination by Kepler, Humber, Bischoff, and Rehner in 2014, moving it to the genus *Metarhizium*. It is also known as *Nomuraea rileyi* Farlow [7], as it is a key mortality factor for caterpillar populations in various crops under specific environmental conditions, including subtropical and tropical soybeans [8]. This study aimed to identify fungi isolated from soil and cherry tree (*Prunus serrulata*) bark. We present the morphological and molecular characteristics of two unreported fungal species namely, *Dothidea insculpta* and *Metarhizium rileyi* in Korea.

MATERIALS AND METHODS

Sample collection and fungal isolation

The soil sample from hillside root-soil and cherry tree (*Prunus serrulata*) bark were collected from Gyeongsangbuk-do (36°11'45.9"N, 128°34'10.8"E; 35°38'36.6"N, 128°37'31.2"E) in Korea. Bark samples were collected from the cherry tree, placed in sterile plastic bags, and taken to the laboratory for fungal isolation. A small piece of bark was cut and put onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates and incubated at 25℃. Soil samples were collected from between the surface and 20 cm below the surface, stored in a plastic bag, brought to the laboratory, and stored at 4℃ until analyzed. Then, the soil serial dilution was performed and roughly, 1 g of soil was weighed and suspended in 10 mL of sterile distilled water to make serial dilutions (10^{-1} to 10^{-5}). Each soil serial dilution was vortexed, and 0.1 mL of each dilution was spread on PDA plates. Well-developed individual colonies were isolated and cultured again on fresh PDA plates and incubated at 25℃ until mycelium development. The pure cultures were preserved in 20% glycerol at -80℃ to produce a stock for future studies.

Morphological characterization

The morphological characteristics of the isolated strains were studied by growing on PDA, and malt extract agar (MEA; Difco, Detroit, MI, USA) media. The KNU-SOT5 strain was studied after 5 days at 20℃ [3]. The KNU-Gunwi 2B strain was studied after 20 days at 28℃ [4]. The cultural characteristics, colony color, texture, growth, shape, and size were studied and mycological characteristics were observed using a light microscope (BX-50; Olympus, Tokyo, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

Fungal strains were grown on PDA plates and scraped mycelia was used for the genomic DNA extraction.

Total genomic DNA was extracted using a HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) following the manufacturer's instructions. The polymerase chain reaction (PCR) was carried out using three partial gene portions in this study. NS1 and NS4 primers were used to amplify a region spanning the small subunit (SSU) rDNA [9]. LROR and LR5 primer pairs were used to amplify a segment of the large subunit (LSU) rDNA [10] and internal transcribed spacers (ITS) were amplified by using the primer pairs ITS1F and ITS4 [9,11]. The amplified PCR products were purified using EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Solgent Co., Ltd. (Daejeon, Korea). The PCR purification products were deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

Phylogenetic analysis

To make the phylogenetic tree, we analyzed the LSU, SSU, and ITS sequences for the strain KNU-SOT5, and ITS sequences for the strain KNU-Gunwi 2B and also retrieved the allied sequences from the NCBI GenBank database (Table 1). The LSU, SSU, and ITS datasets were first analyzed separately and then the individual datasets were concatenated into a combined dataset. The phylogenetic tree was constructed based on the maximum likelihood algorithm using the Kimura model and the MEGA 7.0 software to bootstrap for 1,000 replications [12,13].

Table 1. GenBank accession numbers used in this study for phylogenetic analyses.

Species	Strain numbers	GenBank accession numbers		
		ITS	SSU	LSU
Dothidea berberidis	CBS 186.58	EU167601	EU167601	EU167601
D. hippophaeos	CBS 188.58	MH857750	U42475	DQ678048
D. insculpta	**KNU-SOT5**	**LC591852**	**LC591853**	**LC591854**
D. insculpta	CBS 189.58	AF027764	DQ247810	DQ247802
D. muelleri	CBS 191.58	EU167593	EU167593	EU167593
D. ribesia	MFLUCC 13-0670	KM388545	KM388550	KM388553
D. sambuci	CBS 198.58 (=DAOM 231303)	AY883094	NG_012432	AF382387
D. sambuci	AFTOL-ID 274	DQ491505	AY544722	AY544681
Metarhizium anisopliae	ARSEF 7487	HQ331446	-	-
M. anisopliae	ARSEF 7450	HQ331464	-	-
M. flavoviride var. flavoviride	FI-1170 (ARSEF 2025)	AF138269	-	-
M. flavoviride var. flavoviride	ARSEF1184	AY646383	-	-
M. guizhouense	ARSEF 5714	JN049829	-	-
M. majus	ARSEF1946	HM055450	-	-
M. pemphigi	FI-72	AF139850	-	-
M. pemphigi	qc1401	KT371489	-	-
M. rileyi	BUM9.24	MH143805	-	-
M. rileyi	AMGSPA 1319	MG637450	-	-
M. rileyi	**KNU-Gunwi 2B**	**LC591851**	-	-
M. koreanum	BCC16762	MN781905	-	-
M. koreanum	BCC30455	MN781904	-	-
Dothiora viburnicola	CBS 274.72	NR155058	KU728515	NG059134
Saccharomycopsis vini	CBS 4110^T	NR163535	-	-

ITS, Internal transcribed spacer; SSU, Small subunit rRNA; LSU, Large subunit rRNA.

Bold letters indicate fungal strains isolated in this study.

RESULTS AND DISCUSSION

Dothidea insculpta Wallr., Flora Cryptogamica Germaniae 2: 864 (1833) [MB#173197] (Fig. 1)

Specimen examined: Cheongdo-gun, Gyeongbuk province (35°38'36.6"N, 128°37'31.2"E), isolated from cherry tree (*Prunus serrulata*) bark. The stock culture (NREFFGC000000241) was deposited in the National Institute of Biological Resources (NIBR) as a metabolically inactive culture.

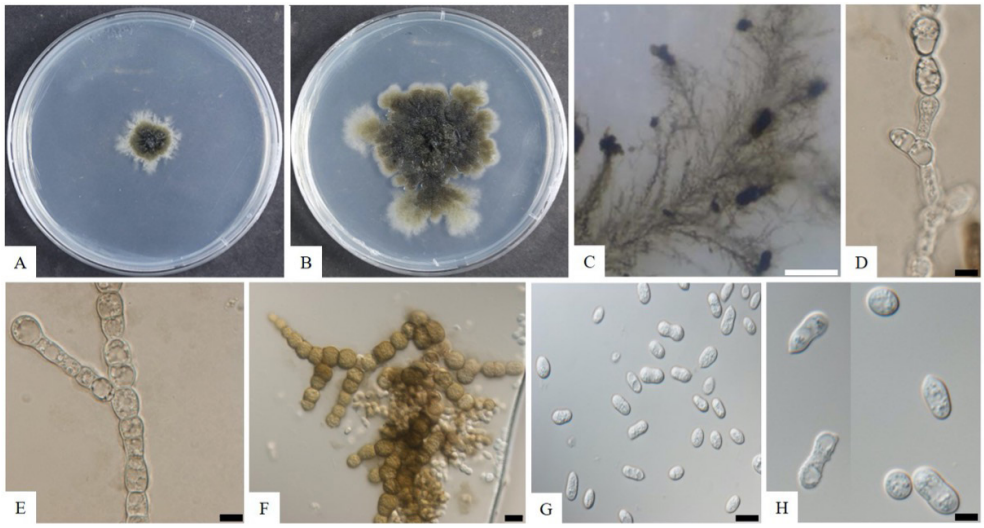

Fig. 1. Morphological characteristics of *Dothidea insculpta* KNU-SOT5. A, colonies on potato dextrose agar (PDA) after 5 days; B, large colonies on PDA after 2 wks; C, feathery mycelium and blackish conidiomata-like structures observed under a stereomicroscope; D, E, conidiophores; F, melanized hyphae developing into chlamydospores; G, conidia. Scale bars: C=50 μm; F=20 μm, D, E=10 μm, G=5 μm.

Morphological characteristics of KNU-SOT5

The colonies were rhizoid, flat, rough-surfaced, and had sparse to moderate aerial mycelium with feathery rhizoid margins. Colony diameter reached 24 mm on PDA and 11 mm on MEA media after 5 days at 20°C. On PDA, the colony upper surface was white around the margin, which turned to greenish-black as approaching the center; the back color was black (Fig. 1A). The rhizoid form colony was more clearly observed after 2 weeks of incubation (Fig. 1B). Hyphae were sub-hyaline to brown, branched, thin-walled, septate. The strain also produced feathery mycelium and blackish conidiomata-like structures on the agar surface (Fig. 1C). Conidiophores were observed, which arise directly from the mycelia and short side-branches with top swollen, smooth-walled, hyaline (Fig. 1D and 1E). Chlamydospores were dark brown, smooth to lightly rough-walled, and globose to ellipsoidal (Fig. 1F). Conidia were aseptate, guttulate, variable in shape, but mostly fusiform, and 5.3-17.6×4.2-7.0 μm in size (Fig. 1G). The culture and morphological characteristics of the isolated strain KNU-SOT5 suggested that the fungus was most closely related to *Dothidea insculpta*.

Molecular phylogeny of the KNU-SOT5

Sequences containing 582, 991, and 829 bp were obtained which corresponded to the ITS, SSU, and LSU, accordingly. According to result of Basic Local Alignment Search Tool (BLAST) in NCBI, the obtained sequence showed 99.4, 99.9, and 99.9% similarities with the strain of *Dothidea insculpta* CBS 189.58 from the sequences of ITS, SSU, and LSU, accordingly. A phylogenetic tree was constructed based on the combined sequences of ITS, SSU, and LSU regions by using a maximum likelihood method. The strain KNU-SOT5 was clustered together with *D. insculpta* CBS 189.58 showing 87% bootstrap values (Fig. 2).

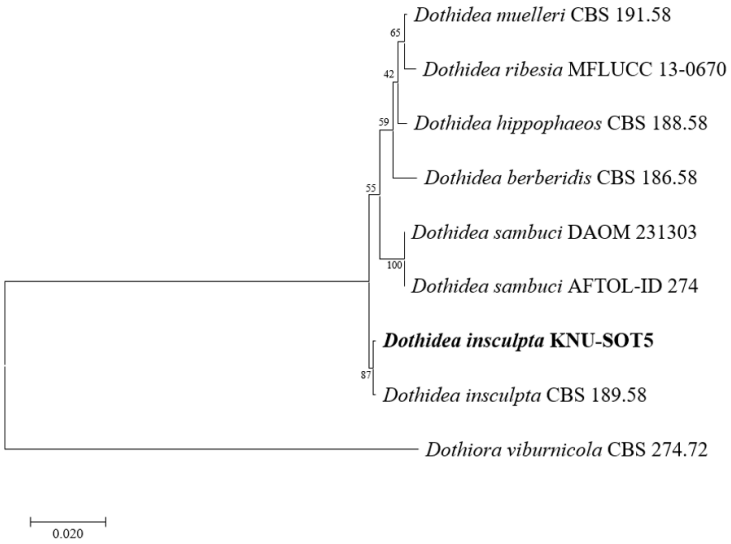

Fig. 2. Maximum likelihood phylogenetic tree based on a combined dataset of partial sequences of internal transcribed spacer (ITS) region, large subunit rRNA (LSU) and small subunit rRNA (SSU) gene. *Dothiora viburnicola* CBS 274.72 was used as an out-group. The strain isolated in this study is in bold, and the bootstrap values are based on 1,000. Bar, 0.02 substitutions per nucleotide position.

In the previous study, *D. insculpta* was isolated from a dead branch of *Clematis vitalba* tree which is under the family *Ranunculaceae* from Italy [3]. *Dothidea* species are widely distributed around the world, especially in tropical areas [14]. And also, another species belongs to the genus *Dothidea,* namely *D. cladonema* (=*Clypeococcum cladonema*) found on volcanic rocks from France [15], *D. eucalypti* on leaves of *Eucalyptus dalrympleana* (Myrtaceae) in Australia [16], *D. sambuci* on *Sambucus nigra* (Adoxaceae) in Austria [3]. Though, there are so many *Dothidea* species isolated from different countries, but still now, there is no study in Korea. However, during fungal diversity surveys, there were two fungal strains, namely *Paecilomyces variotii* and *Talaromyces amestolkiae* were isolated from samples of rat dung and fig tree leaf collected at a garden located in Gwangju in Korea [17]. A disease also called "Brown rot" was discovered to be present on cherry fruits (*Prunus avium* L.) in the city of Hwaseong, Korea [18]. In this study, the strain KNU-SOT5 was collected from Gyeongsangbuk-do associated with the cherry tree (*Prunus serrulata*) bark

in Korea. Thus, further research is being required for this species in future, not only a better understanding of the fungal disease of cherry tree but also the classification and ecology that are mostly important in Korean environmental conditions. To our knowledge, this is the first report of *Dothidea insculpta* in Korea.

Metarhizium rileyi (Farl.) Kepler, S.A. Rehner & Humber, Mycologia 106 (4): 824 (2014) [MB#807862] (Fig. 3)

Specimen examined: Gunwi-gun, Gyeongbuk province (36°11'45.9"N, 128°34'10.8"E), isolated from hillside root-soil. The stock culture (NREFFGC000000235) was deposited in the National Institute of Biological Resources (NIBR) as a metabolically inactive culture.

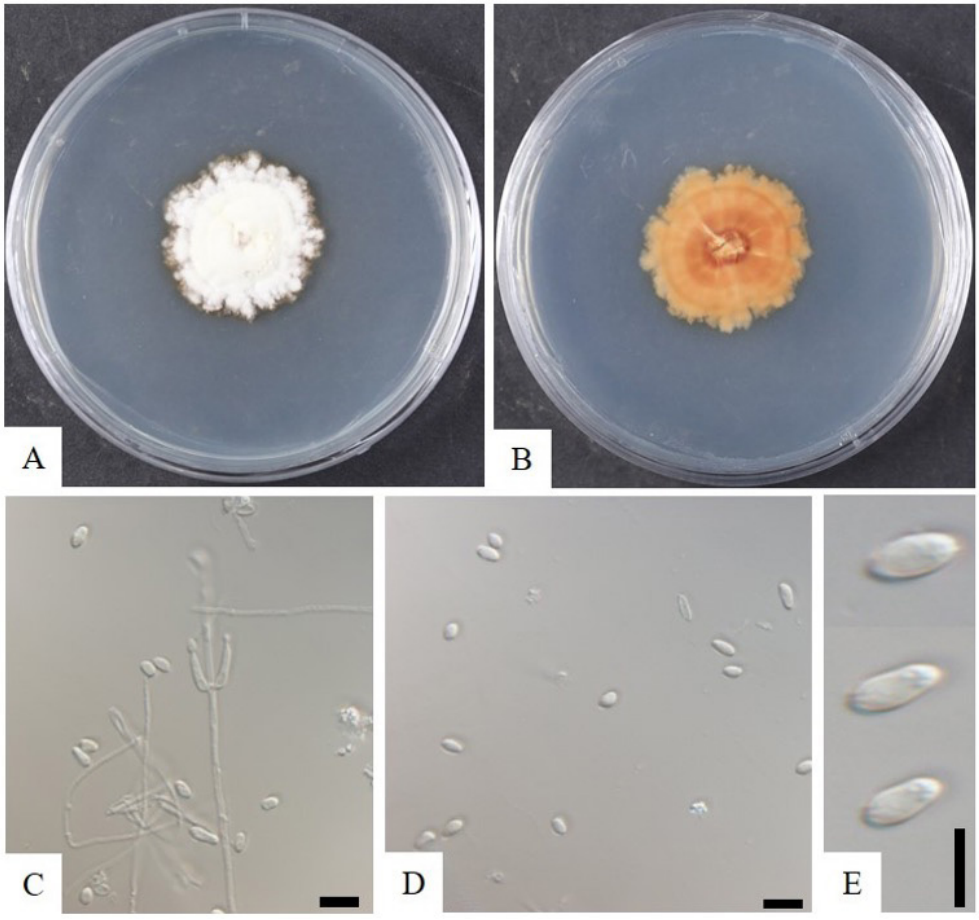

Fig. 3. Morphological characteristics of *Metarhizium rileyi* KNU-Gunwi 2B grown for 20 days on potato dextrose agar (PDA). A, front colony; B, reverse colony; C, simple microscopic images of conidiophores; D, E, conidia. Scale bars: C, D=10 μm, E=5 μm.

Morphological characteristics of KNU-Gunwi 2B

The strain KNU-Gunwi 2B was cultured on PDA media for 20 days at 28℃ to study the cultural and morphological characteristics (Fig. 3A and 3B). The colony diameter reached 24 mm on PDA plates with

the slow growth rate, and the color was white with irregular borders that turned to pale-green; the back color was pale yellow. The hyphae were up to 2.4 μm in width, hyaline, septate, and smooth. The conidiophores were hyaline, erect, septate, originating from hyphae with two phialides (Fig. 3C). Phialides were smooth-walled, cylindrical with semi-papillate apices, and 10.0-12.0×2.0-3.0 μm in size. The conidia were broadly ellipsoid, sometimes cylindrical, and with the diameter of 4.4-8.0×2.3-4.0 μm (Fig. 3D and 3E).

Molecular phylogeny of the KNU-Gunwi 2B

From the sequence analysis, a 632 bp sequence was obtained from ITS regions. The ITS sequence had 99.8% similarities with the different strains of *Metarhizium rileyi* (MH143805, AB268359, and MG637450). The ITS regions sequences were used to construct a phylogenetic tree by using the Maximum Likelihood method with the retrieved allied species sequences from the NCBI database. The phylogenetic tree revealed that the strain KNU-Gunwi 2B was clustered with different strains of *M. rileyi*

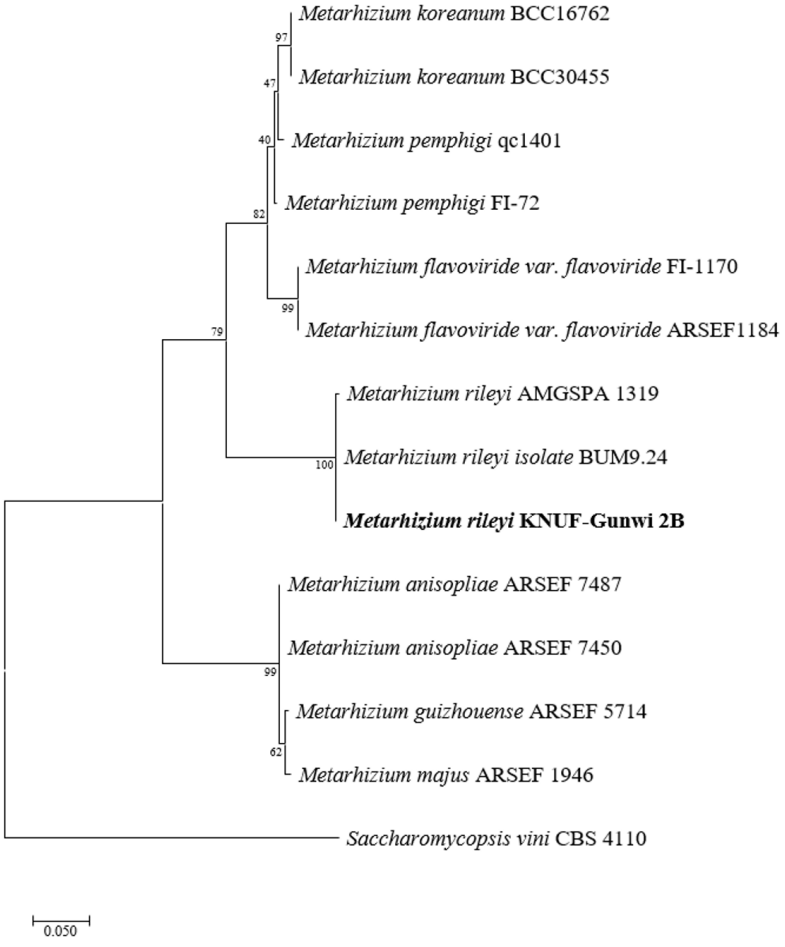

Fig. 4. Maximum likelihood phylogenetic tree analysis based on *Metarhizium rileyi* KNU-Gunwi 2B internal transcribed spacer rDNA sequences. *Saccharomycopsis vini* CBS 4110 was used as an out-group. The strain isolated in this study is in bold, and the bootstrap values are based on 1000 replications. Bar, 0.05 substitutions per nucleotide position.

BUM 9.24 and *M. rileyi* AMGSPA 1919 with 100% bootstrap values (Fig. 4).

There are many new species were added to *Metarhizium* in the last five years [19-24]. Most well-known *Metarhizium* species were identified as entomopathogens. Some were also reported as endophytes (soil and rhizosphere inhabitants) [25-27], resulting in increased plant growth and providing increased pest and disease tolerance [28,29]. *Metarhizium* were also reported in China, Japan, and Thailand mainly from insects, soils, and plant roots [28,30]. Moreover, there are some species belonging to the genus *Metarhizium* isolated from diversified hosts and habitats from different countries such as *M. anisopliae* (insects from Orthoptera, Coleoptera-Brazil, India, Eritrea, Eastern Africa), *M. flavoviride* var. *flavoviride* (insects from Coleoptera, Agricultural Soil-France, Germany, Netherlands), *M. flavoviride* var. *minus* (insects from Homoptera-Philippines, Solomon Islands), *M. frigidum* (insects from Coleoptera, Soil, Termite mound-Australia) [31]. However, the fungal strains were also isolated from environmental soil that was found in various agricultural areas, namely *Penicillium raphiae* [32], *Metarhizium guizhouense* and *Mortierella oligospora* in Korea [33]. In the present study, the strain KNU-Gunwi 2B was isolated from hillside root-soil in Gyeongsangbuk-do also and identified as *Materhizium rileyi*.

In conclusion, further research is being required for these two species in the future not only to have a better understanding of soil and cherry tree fungal diseases but also about these species classification and ecology that are mostly important in agricultural production in Korean environmental conditions. To our knowledge these two species, namely *Dothidea insculpta* KNU-SOT5 and *Metarhizium rileyi* KNU-Gunwi 2B, are the first reports in Korea.

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