RESEARCH ARTICLE

New Korean Records of Three Fungal Species Isolated from the Hyphosphere Soil of Mushrooms on Jeju Island

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ABSTRACT

Jeju Island hosts a unique ecosystem that serves as a biodiversity hotspot for numerous species and rare organisms. During an investigation of fungal diversity in the hyphosphere soil of *Pseudotulostoma japonicum* in Tamna Valley and *Armillaria mellea* in the Gyorae Gotjawal Forest, we identified three species belonging to Ascomycota fungi that had not been previously recorded in Korea. To perform taxonomic study on these species, we observed morphology and analyzed the nucleotide sequences of the internal transcribed spacer regions, the large subunit of rDNA, the β-tubulin gene, and the translation elongation factor 1-α gene. Consequently, *Mariannaea humicola*, *Nigrograna hydei*, and *Trichoderma pubescens* were listed as newly recorded species in Korea.

Keywords: Hyphosphere soil, Jeju Island, *Mariannaea humicola*, *Nigrograna hydei*, *Trichoderma pubescens*

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INTRODUCTION

Jeju Island in Korea, with its volcanic origin and unique temperate and subtropical climate, supports rich biodiversity, particularly within the rhizosphere where microorganisms thrive and engage in complex interactions [1]. This biodiversity is enhanced by high rainfall and diverse ecosystems, such as the Tamna Valley and Gyorae Gotjawal Forest, both of which are known for their fungal diversity [2,3]. These areas serve as prime locations for investigating fungal species associated with the rhizosphere. Gotjawal forests have unique volcanic soils that host diverse microbial communities shaped by environmental factors such as soil pH, organic matter content, and mineral composition [3,4]. Research in these areas has highlighted the distinctness of microbial communities in lava-formed soils and their ecological roles [3,5]. Furthermore, studies on endophytic fungi on Jeju Island have emphasized their role in nutrient cycling, environmental resilience, and their potential applications in agriculture and biotechnology [2,5]. This indicates the broad ecological significance of fungi on ecosystems of Jeju Island and underscores the importance of preserving these unique environments.

Mushrooms on Jeju Island have been surveyed in biodiversity study [1]. However, the hyphosphere

soils of wild mushrooms have not been extensively studied. Recently, we identified 11 unrecorded fungal species from the hyphosphere soil of wild mushrooms in the Gotjawal Forest by exploring the biodiversity and species distribution of this ecologically and economically important taxon [6]. We further investigated the fungi in the hyphosphere soil of wild mushrooms grown in the Gyorae Gotjawal Forest and Tamna Valley. Four additional species of Ascomycota fungi that have not been previously reported in Korea were identified. In this study, we report the molecular and morphological identification and characterization of these species.

MATERIAL AND METHODS

Collecting the hyphosphere soil associated with mushrooms and isolation of fungi

Sampling of mushroom hyphosphere soil on Jeju Island was performed for *Pseudotulostoma japonicum* in the Tamna Valley at 33.400035°N, 126.540828°E and for *Armillaria mellea* in Gyorae Gotjawal Forest at 33.28027°N, 126.26333°E on Jeju Island. Soils 10 cm deep beneath these two mushroom fruiting bodies were collected using a long seedling shovel, then placed in a zipper bag, and transported to the laboratory for analysis. Two grams of the collected wild mushroom hyphosphere soil sample and 20 mL of sterilized water were placed in a 50 mL plastic tube. The plastic tube was vortexed for 10 min to separate fungal spores or mycelia from the soil and allowed to float in sterilized water. The suspended solution was diluted to 10^{-3} using a step dilution method, and 100 μL of each diluted solution was spread on dichloran glycerol 18% (DG18; Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) medium supplemented with the antibiotic chloramphenicol (50 μg/mL) using a smear plate method. After culturing the inoculated medium in a 25°C incubator for 7 d, the growing fungal colonies were confirmed by naked eye. Fungal colonies that appeared to have different morphological features were purified on potato dextrose agar (PDA; BD) and cultured in an incubator at 25°C for 7 d. Single-spore isolation was performed with sporulating isolates, and pure cultures were obtained and assigned Dankook University Culture Collection (DUCC) numbers. Representative strains among the assigned DUCC numbers were deposited at the National Institute of Biological Resources (NIBR), Incheon, Korea, and assigned the corresponding NIBRFGC accession numbers.

Observation of colony morphology and microstructures

For detailed morphological analyses, the fungal plates were incubated at 25°C in the dark for 7–14 d on PDA, malt extract agar (MEA; MBcell, KisanBio, Seoul, Korea), Czapek yeast extract agar (CYA; MBcell, KisanBio), oatmeal agar (OA; BD), synthetic nutrient poor agar (SNA; MBcell, KisanBio), and corn meal agar (CMA; Difco, Detroit, MI, USA). Using colonies grown on PDA, microscopic features were observed under an optical microscope (BX53; Olympus, Tokyo, Japan) at 400× magnification. The lengths and thicknesses of the microstructures were measured 30 times each.

DNA extraction and phylogenetic analysis

Genomic DNA was extracted directly from the mycelia of fungal isolates using a NaviBiotech Direct DNA Prep Kit (NaviBiotech Corp., Cheonan, Korea). Using the extracted DNA as a template, the sequences of the internal transcribed spacer (ITS) regions, the large subunit (LSU) of rDNA, the β-tubulin gene (*TUB2*), and the translation elongation factor 1-α gene (*tef1-α*) were amplified by PCR using the primers and reaction conditions given in Table 1. The amplified PCR products were verified using electrophoresis on a 1% (w/v) agarose gel. The PCR products were purified using a silica gel column and 80% ethanol solution. DNA sequencing was performed by Bionics Corp. (Seoul, Korea). The determined nucleotide sequences were edited using Chromas 2.6 program (https://chromas.software.informer.com/), and the edited sequences were searched for similar sequences using the Basic Local Alignment Search Tool (BLASTN) of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The reference fungal nucleotide sequences of the ITS, LSU rDNA, *TUB2*, and *tef1-α* were downloaded from the GenBank DNA database of NCBI and given in Tables 2–4. For phylogenetic analysis, the sequences of the DUCC strains were aligned with the reference sequences using the CLUSTALW alignment tool in the MEGA 11.0 software program [7]. Maximum likelihood (ML) trees were constructed with aligned sequences using the Kimura 2-parameter model, and the reliability of phylogenetic tree branches was analyzed using 1,000 bootstrap replicates [8,9].

Table 1. Conditions of each primer set used for PCR

Target DNA region	Primers	Primer sequences	PCR condition	Reference
ITS	ITS1	5′ GAAGTAAAAGTCGTAACAAGG 3′	95°C 5min; 35 cycles: 95°C 30s, 56°C 30s, 72°C 1min; 72°C 5min	[10]
	ITS4	5′ TCCTCCGCTATTGATATGC 3′		
LSU rDNA	LR0R	5′ ACCCGCTGAACTTAAGC 3′	95°C 5min; 35 cycles: 95°C 30s, 56°C 30s, 72°C 1min; 72°C 5min	[11]
	LR5	5′ TCCTGAGGGAAACTTCG 3′		[12]
TUB2	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	95°C 10min; 35 cycles: 95°C 50s, 52°C 50s, 72°C 1min; 72°C 5min	[28]
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		
tef1-α	TEF728	5′ CATCGAGAAGTTCGAGAAGG 3′	95°C 10min; 35 cycles: 95°C 50s, 56°C 50s, 72°C 1min; 72°C 5min	[13]
	TEF1	5′ GCCATCCTTGGAGATACCAGC 3′		

ITS: internal transcribed spacer, LSU rDNA: 28S ribosomal RNA gene; *TUB2*: β-tubulin gene; *tef1-α*: translation elongation factor 1-α gene.

Table 2. Sequence information on the related species used for the phylogenetic analysis of *Nigrograna hydei* NIBRFGC000510007

Species	Strain	Source	Country	GenBank accession number	
				ITS	LSU rDNA
Nigrograna fuscidula	CBS 254.39	-	United Kingdom	MH856004	MH867504
Nigrograna obliqua	BW4	*Sambucus racemosa*	Austria	KX650557	KX650557
Nigrograna locuta-pollinis	LC11691	Bee bread	China	MF939602	MF939585
Nigrograna locuta-pollinis	LC11690	Bee bread	China	MF939603	MF939584
Nigrograna mycophila	MF5^{T}	*Acer campestre*	Austria	KX650553	KX650553
Nigrograna mycophila	TDK	*Acer pseudoplatanus*	Denmark	KX650555	KX650555
Nigrograna magnoliae	HKAS 107027	Dead branch	China	OM293746	OM293740
Nigrograna magnoliae	HKAS 107035	Dead branch	China	OM293747	OM293741
Nigrograna antibiotica	CCF 4378^{T}	Healthy phloem, *Ulmus laevis*	Czech Republic	NR 158296	NG 058663
Nigrograna impatientis	MFLU 18-2072^{T}	Dead branch of *Impatiens* sp.	Thailand	NR 172416	MN387228
Nigrograna chromolaenae	MFLUCC 17-2079	*Clematis fulvicoma*	Thailand	MT310613	MT214568
Nigrograna chromolaenae	MFLUCC 17-1437^{T}	Dead aerial stems	Thailand	NR 168877	MT214473
Nigrograna fuscidula	HF2S32	Mediterranean Sea	France	OP179042	OP179208
Nigrograna mackinnonii	CBS 110022	Mycetoma, Homo sapiens; patient	Mexico	KF015653	GQ387614
Nigrograna hydei	MFLU 18-2073^{T}	Dead branch of unidentified host	Thailand	NR 172415	MN387227
Nigrograna hydei	ELS1-2022	*Elaeagnus angustifolia*	India	OP741100	OP741109
Nigrograna hydei	**NIBRFGC000510007**	**Soil**	**Korea**	**ON911614**	**ON911618**
Nigrograna rhizophorae	MFLUCC 18-0397^{T}	*Rhizophora sp.*	Thailand	MN047085	MN420686
Occultibambusa pustula	MFLUCC 11-0502^{T}	bamboo	Thailand	KU940126	KU863115

ITS: internal transcribed spacer, LSU rDNA: 28S ribosomal RNA gene.

^{T}type strain. Strain identified in this study and its GenBank accession numbers are indicated in bold.

Table 3. Sequence information on the related species used for the phylogenetic analysis of *Mariannaea humicola* NIBRFGC000510020

Species	Strain	Source	Country	GenBank accession number		
				ITS	LSU rDNA	*TUB2*
Mariannaea atlantica	URM 8146^{T}	Soil	Brazil	MN151372	MN151398	-
Mariannaea aquaticola	MFU090224	-	-	GQ153836	GQ153835	-
Mariannaea aquaticola	MFU090223^{T}	-	-	GQ153834	GQ153833	-
Mariannaea camelliae	CMU 329^{T}	-	Thailand	NR 175622	NG 088070	-
Mariannaea camptospora	CBS 209.73^{T}	Forest soil	Netherlands	MH860663	MH872365	AY624245
Mariannaea catenulata	CBS 491.92^{T}	-	Venezuela	MH862371	MH874034	-
Mariannaea chlamydospora	LC1715^{T}	Submerged wood	China	KX986134	KX986141	KX986147
Mariannaea cinerea	LC1766^{T}	Submerged wood	China	KX986135	KX986142	KX986148
Mariannaea dimorpha	HMAS 266564^{T}	Rotten bark	China	KF767353	KJ002443	-
Mariannaea elegans	CBS 217.73A^{T}	*Pinus sylvestris*, decayed bark	Netherlands	KX986132	KX986139	KX986145
Mariannaea pinicola	CBS 745.88^{T}	*Pinus* sp.	Venezuela	MH862152	MH873845	KM232011
Mariannaea punicea	CBS 239.56^{T}		Zaire	MH857604	MH869152	AY624244
Mariannaea fusiformis	LC1701^{T}	Submerged wood	China	KX986133	KX986140	KX986146
Mariannaea humicola	CBS 740.95^{T}	Soil	Brazil	NR 148078	NG 069229	KM232012
Mariannaea humicola	CBS 102628	Decaying wood	Spain	KM231756	KM231620	KM232012
Mariannaea humicola	**NIBRFGC000510020**	**Soil**	**Korea**	**OP919499**	**OP905586**	**PX578375**
Mariannaea koreensis	DUCC 15688	Soil	Korea	OR835254	OR835270	OR841123
Mariannaea koreensis	DUCC 15750	Soil	Korea	PQ533829	PQ533831	PP111899
Mariannaea lignicola	LC1791^{T}	Submerged wood	China	KX986136	KX986143	KX986149
Mariannaea lignicola	LC1792	Submerged wood	China	KX986137	KX986144	KX986150
Mariannaea macrochlamydospora	FKI-4735^{T}	Soil	Japan	AB855777	AB855782	-
Mariannaea samuelsii	CBS 746.88^{T}	Bark	Venezuela	KM231757	KM231621	KM232014
Mariannaea samuelsii	CBS 125515^{T}	Soil	Guatemala	KM231758	MH875139	KM232015
Mariannaea submersa	MFLU 19-0549	Submerged wood	Thailand	MT496744	MT496752	-
Mariannaea submersa	MFLU 19-0542^{T}	Submerged wood	Thailand	MT496743	MT496751	-
Mariannaea superimposita	CBS 124559	Soil	Japan	AB855781	AB855786	-
Mariannaea superimposita	CBS 113472	Soil	Japan	AB855780	AB855785	-
Mariannaea terricola	URM 92163^{T}	Soil	Brazil	MK101011	MK101012	-
Nectria balansae	A.R. 4446^{T}	*Coronilla* sp.	France	HM484552	GQ505996	HM484607

ITS: internal transcribed spacer, LSU rDNA: 28S ribosomal RNA gene, *TUB2*: β-tubulin gene.

^{T}type strain. Strain identified in this study and its GenBank accession numbers are indicated in bold.

Table 4. Sequence information on the related species used for the phylogenetic analysis of *Trichoderma pubescens* NIBRFGC000510021

Species	Strain	Source	Country	GenBank accession number	
				ITS	*tef1-α*
Trichoderma pubescens	DAOM 166162[T]	-	USA	EU280121	EU279963
Trichoderma pubescens	**NIBRFGC000510021**	**Soil**	**Korea**	**OP919501**	**OP973121**
Trichoderma hamatum	DAOM 167057[T]	Spruce forest soil	Canada	EU280124	EU279965
Trichoderma atroviride	CBS 142.95[T]	Ambrosia beetle	Slovenia	MH862505	AY376051
Trichoderma evansii	Dis 282d	*Lophira alata*	Cameroon	EU856294	EU856319
Trichoderma evansii	Dis 380a	Stem, *Cola verticillata*	Cameroon	EU856295	EU856320
Trichoderma evansii	DIS 341HI[T]	*Theobroma gileri*	Ecuador	EU883568	EU883566
Trichoderma anisohamatum	YMF1.00215	-	China	MH262583	MH236494
Trichoderma anisohamatum	YMF1.00253	Soil of tobacco rhizosphere	China	MH262586	MH236495
Trichoderma longibrachiatum	PPRC-ET17	-	Ethiopia	FJ461550	FJ763157
Trichoderma hainanense	HMAS 248837[T]	-	-	NR 154568	KY688033
Trichoderma breve	HMAS 248845	-	-	KY687928	KY688046
Trichoderma afroharzianum	GJS 04-186[T]	*Moniliophthora roreri*	Peru	FJ442265	FJ463301
Trichoderma alni	CPK2494	-	-	EU518652	EU498313
Trichoderma inhamatum	CBS 274.78[T]	Maize-field soil	Colombia	KY687905	KY688019
Cladosporium cladosporioides	CBS 145.35	*Pisum sativum*	Germany	HM148013	HM148254

ITS: internal transcribed spacer, *tef1-α*: translation elongation factor 1-α gene.
[T]type strain. Strain identified in this study and its GenBank accession numbers are indicated in bold.

RESULTS AND DISCUSSION

Nigrograna hydei J.F. Zhang, J.K. Liu & Z.Y. Liu, Mycol. Progr. 19: 1369 (2020) [MB#556749]

Strains examined. Tamna Valley on Jeju Island, isolated from the mushroom hyphosphere soil of *Pseudotulostoma japonicum* in 2022, strain *N. hydei* DUCC 15293 (NIBRFGC000510007).

Phylogenetic analysis (Fig. 1)

The determined ITS sequence of strain NIBRFGC000510007 was 536 bp in size and showed 99.81% sequence similarity with that of the type strain *N. hydei* MFLU 18-2073 (GenBank accession OP741100). The LSU rDNA sequence of this strain was 908 bp and had a sequence similarity of 99.88% with that of the type strain (GenBank accession OP741109). The results of constructing an ML phylogenetic tree based on the 1,268 bp concatenated ITS and LSU rDNA sequences, including gaps, showed that the strain NIBRFGC000510007 formed an independent clade with *N. hydei* MFLU 18-2073 and *N. hydei* ELS1-2022.

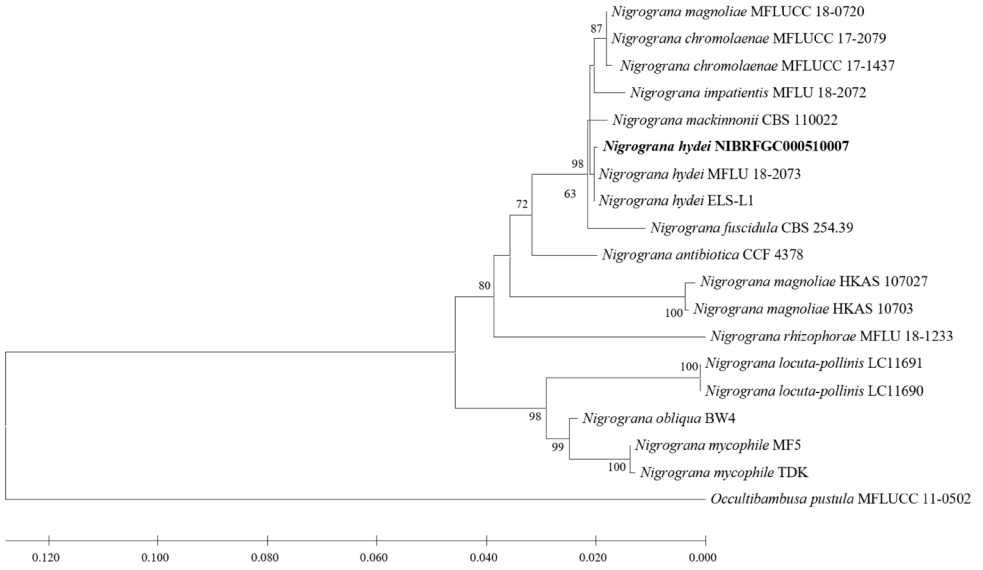

Fig. 1. Maximum likelihood (ML) phylogram constructed based on the concatenated ITS-LSU rDNA sequences. The number of nodes represents the reliability value (>60%) through 1,000 bootstrap replicates. Fungal strain isolated in this study is indicated in bold. *Occultibambusa pustula* MFLUCC 11-0502 is used as an outgroup. The reference sequences used to construct the ML phylogenetic tree are listed in Table 2.

Morphological characteristics (Fig. 2)

Description. On PDA, 15–16 mm diameter in 14 d at 25°C, aerial mycelia dense, floccose, raised at the center, circular, white at the margin, forming several concentric rings on the surface, white, reverse white at the margin, dark olive towards inside. On MEA, 22–23 mm diameter in 14 d at 25°C, aerial mycelia dense, floccose, raised at the center, circular, radial, white at the margin, forming several concentric rings on the surface, white, reverse light pinkish at the margin, dark brown towards inside. On CYA, 22–23 mm diameter in 14 d at 25°C, aerial mycelia dense, floccose, raised at the center, circular, white at the margin, forming several concentric rings on the surface, white, reverse white at the margin, dark olive

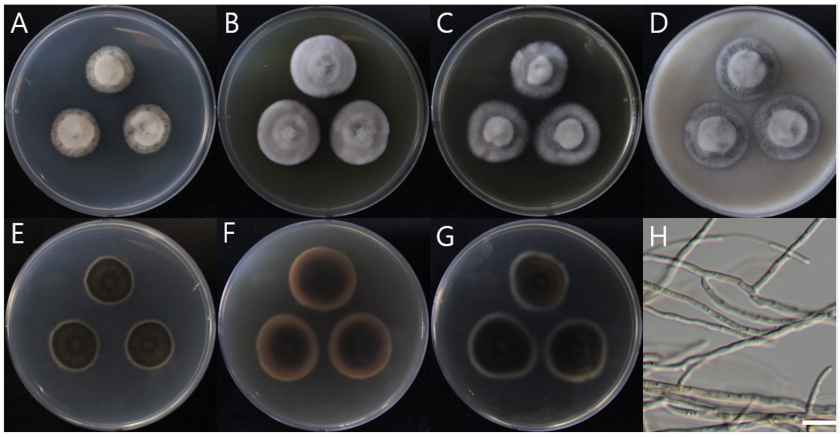

Fig. 2. Colony morphology and light microscopic image of *Nigrograna hydei* NIBRFGC000510007 grown on different media at 25°C for 14 d. A, E: PDA. B, F: MEA. C, G: CYA. D: OA. H: sterile mycelia. Obverse: A–D. Reverse: E–G. Scale bar = 10 μm.

towards inside. On OA, 32–33 mm diameter in 14 d at 25℃, aerial mycelia dense, floccose, raised at the center, circular, white at the margin, forming several concentric rings on the surface. Hyphae were hyaline, branched, septate, thin-walled, width 2–3 μm (mean ± SD: 2.5 ± 0.3 μm, n = 30), sterile. No asexual or sexual reproductive structures are observed.

Notes

The strain NIBRFGC000510007 did not exhibit sporulation on any of the test media (PDA and MEA). It appears that host substrates, such as woody plants, are required for the sexual stage. The morphological characteristics of the type of strain *N. hydei* MFLU 18-2073 from Thailand were described using ascomata and ascospores produced in woody branches of an unknown species [14]. Its germinated ascospores formed colonies on PDA after 35 d at 25℃, reaching up to 33 mm. Its colony morphology includes white at the margin, forming several concentric rings on the surface, white, reverse white at the margin, circular, dense aerial mycelia, grey from above, brown to dark pigmented in reverse, similar to that of NIBRFGC000510007. However, no asexual morphs were observed in this strain. Attempts were made to observe spores on PDA, MEA, CYA, and OA, but no reproductive structures, including conidia or chlamydospores, were observed on any of these media. Interestingly, similar to the strain NIBRFGC000510007, the strain *N. hydei* ELS1-2022 isolated from healthy leaves of *Elaeagnus angustifolia* in India did not sporulate on growth media such as PDA and MEA [15]. The anamorphic characteristics of colony morphology described for strain *N. hydei* ELS1-2022 were also similar to those of strain NIBRFGC000510007 regarding aerial mycelia, floccose, dome-shaped (circular), slightly heaped at the center (raised at the center), and periphery turning white (white at the margin and forming several concentric rings on the surface). Sterile mycelia were also common features of strains NIBRFGC000510007 and *N. hydei* ELS1-2022. *N. hydei* ELS1-2022, intercalary chlamydospores in the mycelia were not observed in strain NIBRFGC000510007. In conclusion, based on the phylogenetic and morphological analysis results, we identified strain NIBRFGC000510007 as *N. hydei*.

Mariannaea humicola L. Lombard & Crous, Stud. Mycol. 80: 213 (2015) [MB#810165]

Strains examined. Tamna Valley on Jeju Island, isolated from the mushroom hyphosphere soil of *Pseudotulostoma japonicum* in 2022, strain *M. humicola* DUCC 21466 (NIBRFGC000510020).

Phylogenetic analysis (Fig. 3)

A ML phylogenetic tree was constructed based on concatenated ITS, LSU rDNA, and *TUB2* sequences. The size of the ITS region of strain NIBRFGC000510020 was 484 bp, displaying 100% sequence similarity with that of the type strain *M. humicola* CBS 740.95 (MW340821). The size of its LSU rDNA was 865 bp, showing 99.88% sequence similarity with that of the type strain (PP380805). The size of *TUB2* was 318 bp, showing 100% sequence similarity to that of the type strain (KM232013). As a result of constructing an ML phylogenetic tree based on the 1,850 bp concatenated sequence including gaps, the strain NIBRFGC000510020 was clustered with *M. humicola* CBS 740.95, and CBS 102628 with sufficient node value and separated from *M. aquaticola*. .

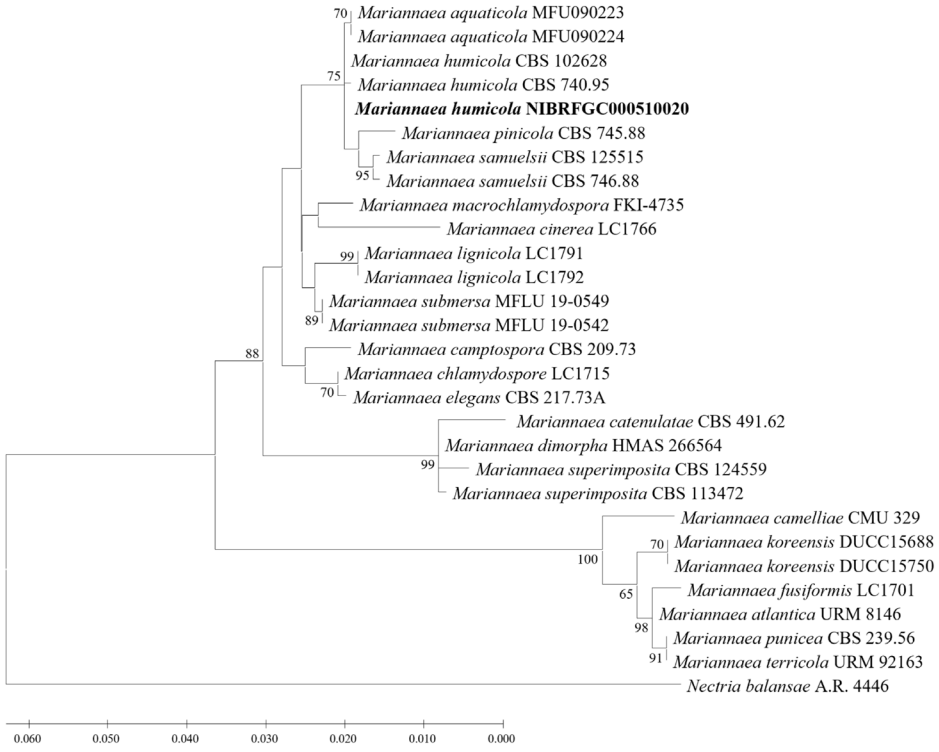

Fig. 3. Maximum likelihood (ML) phylogram constructed based on the concatenated ITS-LSU-*TUB2* sequences. The number of nodes represents the reliability value (>60%) through 1,000 bootstrap replicates. Fungal strain isolated in this study is indicated in bold. *Nectria balansae* A.R. 4446 is used as an outgroup. The reference sequences used to construct the ML phylogenetic tree are listed in Table 3.

Morphological characteristics (Fig. 4)

Description. On PDA, 26–27 mm diameter in 7 d at 25°C, surface dirty white in the center becoming tan to light yellow brown towards the margins with dirty white, irregularly distributed tuffs of fascicles, aerial mycelium abundant, forming several concentric rings on the surface, which are reverse dark brown in color. On MEA, 18–19 mm diameter in 7 d at 25°C, surface white in the center, irregularly distributed to fascicles, aerial mycelium abundant, reverse dark reddish pigment. On CYA, 26–27 mm diameter in 7 d at 25°C, surface white in the center becoming light yellow towards the margins with white, irregularly distributed to fascicles, aerial mycelium abundant, forming several concentric rings on the surface, and a reverse yellow-to-light yellowish pigment fascicles, aerial mycelium abundant, forming several concentric rings on the surface, which are reverse dark brown in color. On OA, 27–28 mm diameter in 7 d at 25°C, surface dirty white in the center becoming tan to light yellow towards the margins with dirty white, irregularly distributed tuffs of fascicles, reverse dark brown. Conidiophores arising from the agar surface from aerial hyphae or fascicles, 10.63–23.25 × 2.12–3.63 μm (mean ± SD: 16.28 ± 3.73 × 2.74 ± 0.39 μm, n = 30), hyaline, branched verticillately at 2–3 levels, with a terminal whorl of 1–8 phialides, and 1–2 lower nodes of 1–4 phialides, septate. Phialide hyaline, thin-walled, subulate, 7.81–13.67 × 3.04–3.88 μm (mean ± SD: 11.20 ± 1.83 × 3.61 ± 0.28 μm, n = 30) at the broadest part with periclinal thickening. Conidia fusiform

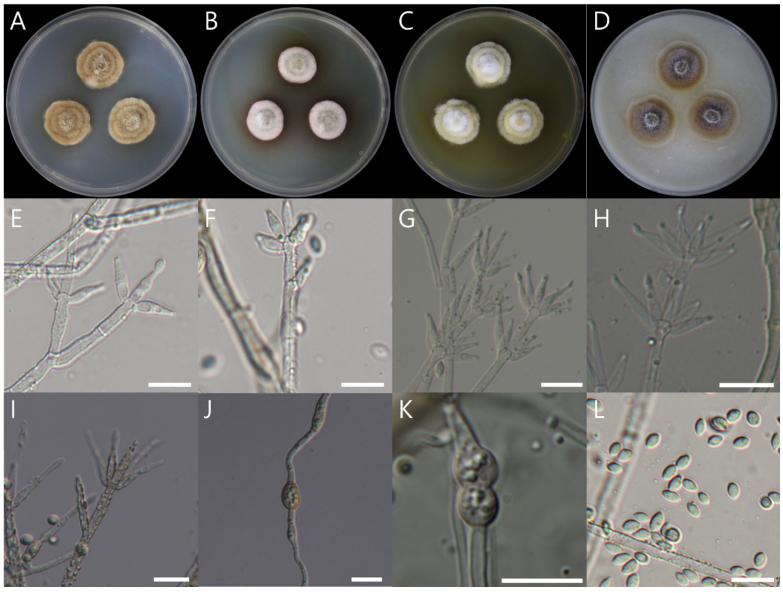

Fig. 4. Colony morphology and light microscopic images of *Mariannaea humicola* NIBRFGC000510020 grown on different media at 25°C for 7 d. A: PDA. B: MEA. C: CYA. D: OA. E–I: conidiophores with verticillate phialides. J–K: chlamydospores. L: conidia. Scale bar = 10 μm.

to ellipsoidal to obovoid, hyaline, smooth, 3.80–6.15 × 2.18–3.88 μm (mean ± SD: 5.23 ± 0.54 × 3.07 ± 0.41 μm, n = 30), with a distinct hilum at both or at one end. Chlamydospores seen. No ascomatal state was observed.

Notes

The strain NIBRFGC000510020 shows similar morphological characteristics of cultural and mycelial structures compared to those of the type strain *M. humicola* CBS 740.95 [16]. In the original description of the type strain, conidia are described as fusiform, ellipsoidal, or obovoid, hyaline, and smooth, with sizes ranging from 4.0–6.0 × 2.0–3.0 μm (mean ± SD: 5.0 ± 0.6 × 3.0 ± 0.3 μm, n = 30) which are similarly seen in the conidia of strain NIBRFGC000510020. The arising conidiophores from the agar surface from aerial hyphae or fascicles also like those of the strain NIBRFGC000510020, regarding size (80–100 μm long, axis 3–7 μm wide) and morphology of phialides which formed verticillately at 2–3 levels, with a terminal whorl of 1–5 phialides, and 1–2 lower nodes of 1–3 phialides. The ascomatal state was not observed in either strain. Unlike *M. humicola* CBS 740.95, chlamydospores were observed in the mycelium of NIBRFGC000510020. In conclusion, based on the phylogenetic and morphological analysis results, we identified strain NIBRFGC000510020 as *M. humicola*.

Trichoderma pubescens Bissett, Canad. J. Bot. 69 (11): 2405 (1992) [MB#359086]

Strains examined. Gyorae Gotjawal Forest on Jeju Island, isolated from the mushroom hyphosphere soil of *Armillaria mellea* in 2022, strain *T. pubescens* DUCC21469 (NIBRFGC000510021).

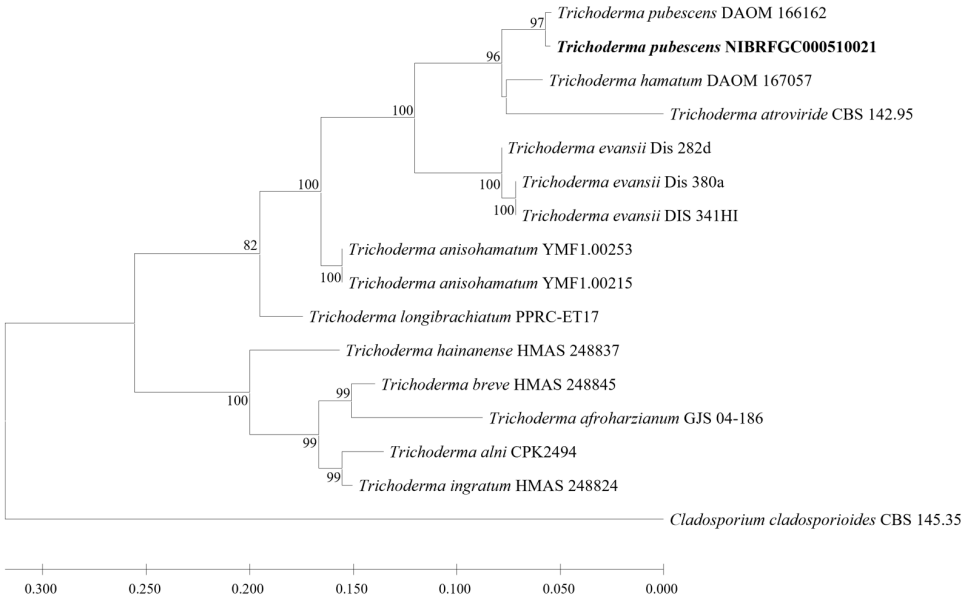

Fig. 5. Maximum likelihood (ML) phylogram constructed based on the concatenated ITS-*tef1*-α sequences. The number of nodes represents the reliability value (>70%) through 1,000 bootstrap replicates. Fungal strain isolated in this study is indicated in bold. *Cladosporium cladosporioides* CBS 145.35 is used as an outgroup. The reference sequences used to construct the ML phylogenetic tree are listed in Table 4.

Phylogenetic analysis (Fig. 5)

A ML phylogenetic tree was constructed based on the concatenated ITS and *tef1-α* sequences of *Trichoderma* spp., including those of the strain NIBRFGC000510021. The size of the ITS region of strain NIBRFGC000510021 was 364 bp, displaying 100% sequence similarity to that of the type strain *Trichoderma pubescens* DAOM 166162 (MN562094). The size of *tef1-α* was 573 bp, showing 99.18% sequence similarity to that of the type strain (LN897315). As a result of constructing an ML phylogenetic tree based on the 1,587 bp concatenated sequence including gaps, the strain NIBRFGC000510021 was grouped together with *T. pubescens* DAOM 166162 with a high node value. The most closely related species is *T. hamatum*.

Morphological characteristics (Fig. 6)

Description. On PDA, 89–90 mm diameter in 7 d at 25°C, floccose, circular, sometimes concentric, white, aerial mycelium limited, surface appearing downy, conidiation in numerous, white at first gradually turning bright green shades, yellowish, and exudate is not observed. On SNA, 89–90 mm diameter in 7 d at 25°C, floccose, circular, white in the center, sparse hyphae, aerial mycelium limited, and exudate is not observed. On CMA, 79–80 mm diameter in 7 d at 25°C, flat, circular, radial, white, aerial mycelium limited, and exudate is not observed. Hyphae were hyaline, branched, septate, thin-walled, 1–5 μm (mean ± SD: 3.05 ± 1.30 μm, n = 30). Conidiophore were hyaline, thin walled, coarse, developed at the branch of the hyphae, 8.42–11.44 × 2.30–4.60 μm (mean ± SD: 9.80 ± 1.28 × 3.14 ± 0.94 μm, n = 30), branched sterile conidiophore apices, usually at right angles with branches reflexed slightly towards the apex. Phialides were hyaline, thin walled, ampulliform to lageniform, 1–3 developed at the end or middle of

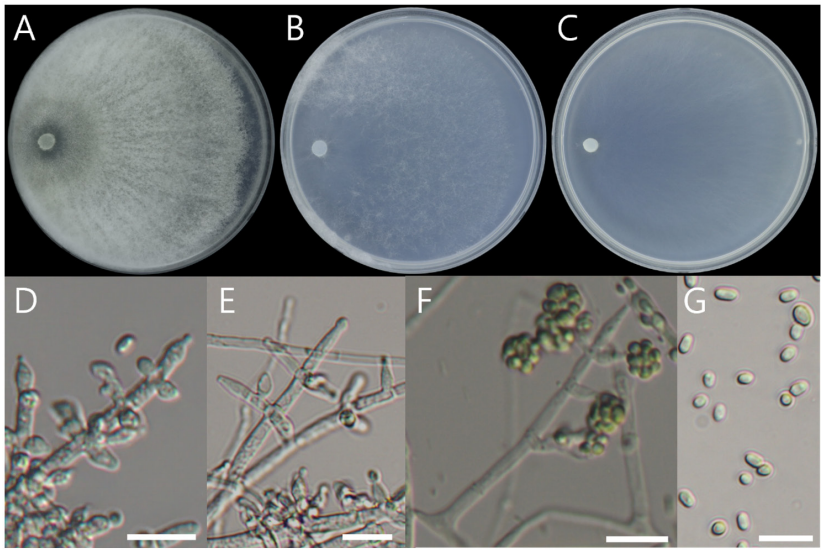

Fig. 6. Colony morphology and light microscopic images of *Trichoderma pubescens* NIBRFGC000510021 grown on different media at 25°C for 7 d. A: PDA. B: SNA. C: CMA. D–F: conidiophores and phialides. G: conidia. Scale bar = 10 μm.

the conidiophore, 6.10–16.09 × 2.11–3.93 μm (mean ± SD: 10.18 ± 3.03 × 3.18 ± 0.52 μm, n = 30). Conidia had smooth surface, without septum, dilute green, ellipsoidal, 3.55–5.31 × 2.43–3.51 μm (mean ± SD: 4.44 ± 0.39 × 2.96 ± 0.27 μm, n = 30), both ends broadly rounded, aggregated in a persistent mucilage. No sexual state was observed.

Notes

The strain NIBRFGC000510021 showed similar morphological characteristics of cultural and mycelial structures compared to those of the type strain *T. pubescens* DAOM 166162 (T) [17]. *T. hamatum* which is a morphologically similar species and phylogenetically closely related species (Fig. 5) to *T. pubescens* has compact, bluish-green, hemispherical conidiogenous pustules and ellipsoidal conidia of moderate size [17]. *T. pubescens* can be distinguished from *T. hamatum* by its branching conidiophore apices, which give the surface of the conidiogenous pustules a cottony appearance. Thus, we identified the strain NIBRFGC000510021 as *T. pubescens*.

DISCUSSION

Nigrograna belongs to the family Nigrograniaceae of the order Pleosporales and is found in various habitats, including terrestrial, estuarine, and marine environments [18]. Recently, this genus included *Nigrograna mackinnonii* as the agent responsible for most cases of black-grain eumycetoma in Latin America [19]. *N. hydei* was initially reported as a saprophyte in Thailand [14] and was later identified as an endophyte of *Elaeagnus angustifolia,* a plant species that grows in the arid and cold regions of India [15]. Importantly, a recent report on deep cutaneous fungal infection indicated that *N. hydei* can infect both immunocompromised and immunocompetent individuals [20]. This study reports the first isolation of *N. hydei* from the hyphosphere soil under *A. mellea* in Korea, suggesting a wide range of habitats for this

species. The ecological role of the isolated strain in the hyphosphere environment of mushrooms remains unknown; thus, further studies are required.

Mariannaea belongs to the family Nectriaceae of the order Hypocreales. Most *Mariannaea* species are terrestrial, with some newly discovered species found in freshwater habitats, suggesting ecological diversity [21]. *M. humicola* has been reported in decaying wood, in rhizosphere soil under *Araucaria angustifolia*, and marine environments [16,22]. *M. humicola* NIBRFGC000510020, isolated from the hyphosphere soil under *A. mellea* in the Gyorae Gotjawal Forest, represents a new ecological record for this species. This strain expanded the known habitat range of *M. humicola* and highlighted its adaptability to diverse ecological niches. Furthermore, *M. humicola* produces bioactive secondary metabolites with antifungal properties, suggesting its potential as a natural antifungal or biological control agents with applications in agriculture and pharmaceuticals [22].

Trichoderma belongs to the family Hypocreaceae of the order Hypocreales [23]. *Trichoderma* spp. are predominant over wide geographic regions in all climatic zones and are significant decomposers of woody and herbaceous materials [24]. They are also found in marine environment [25]. *T. pubescens* was first isolated in 1992 and has been reported in diverse habitats, including hardwood forest soils, mangrove soils, and tomatoes [18,26,27]. *T. pubescens* has demonstrated potential as an effective biocontrol agent, particularly for mitigating the effects of *Rhizoctonia solani*, a major cause of root rot in tomato plants [26]. Furthermore, from an environmental engineering perspective, its ability to degrade organic pollutants highlights its potential role in soil ecosystem restoration and sustainable agricultural management [27]. *T. pubescens* isolation from the hyphosphere soil under *A. mellea* in the Tamna Valley is the first report of this species in Korea, highlighting its ecological importance and adaptability to unique mushroom habitats on Jeju Island.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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