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Article

Isolation and Characterization of Two Rare Mucoralean Species with Specific Habitats

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Abstract: The order Mucorales, the largest in number of species within the Mucoromycotina, comprises typically fast-growing saprotrophic fungi. During a study of the fungal diversity of undiscovered taxa in Korea, two novel mucoralean strains, CNUFC-GWD3-9 and CNUFC-EGF1-4, were isolated from specific habitats including freshwater and fecal samples, respectively, in Korea. On the basis of their morphological characteristics and sequence analyses of internal transcribed spacer (ITS) and large subunit (LSU) of 28S ribosomal DNA regions, the CNUFC-GWD3-9 and CNUFC-EGF1-4 isolates were confirmed to be *Gilbertella persicaria* and *Pilobolus crystallinus*, respectively. It is ecologically, pathologically and mycologically significant to find such rare zygomycetous fungi in such specific habitats.

Keywords: Mucorales; phylogeny; rare fungi; undiscovered taxa

1. Introduction

Previously, taxa of the former phylum Zygomycota were distributed among the phylum Glomeromycota and four subphyla *incertae sedis*, including Mucoromycotina, Kickxellomycotina, Zoopagomycotina, and Entomophthoromycotina [1]. Recently, Spatafora et al. [2] proposed two new phyla, Mucoromycota and Zoopagomycota, on the basis of phylogenetic analyses of a genome-scale data set for 46 taxa, including 25 zygomycetes and 192 proteins. According to these results, Mucoromycota and Zoopagomycota were newly formalized phyla of fungi and comprised six subphyla. The phylum Mucoromycota comprises the subphyla Mucoromycotina, Mortierellomycotina, and Glomeromycotina, whereas the phylum Zoopagomycota comprises the subphyla Entomophthoromycotina, Zoopagomycotina, and Kickxellomycotina.

Mucorales is the largest order within the Mucoromycotina and comprises 15 families, 57 genera, and approximately 334 species [3]. Most mucoralean species are saprotrophic and grow on different organic substrates, such as fruits, soil, dung, and plants [4, 5]. Several species are parasites or pathogens of animals, plants, and fungi [4, 5]. Among these, a few species cause human and animal diseases called mucormycosis, as well as allergic reactions [6]. The traditional classification of Mucorales has been determined on the basis of morphological characteristics, such as the size and shape of the sporangium, sporangiophore, sporangiospore (asexual reproduction), and zygospore (sexual reproduction) [4, 5]. Recently, several molecular studies evaluating mucoralean species had indicated that some of the genera may be polyphyletic [4, 5].

The genus *Gilbertella* belongs to the subphylum Mucoromycotina, order Mucorales, family Choanephoraceae. It was named *Choanephora persicaria* by E.D. Eddy in 1925 [7] and then renamed as the genus *Gilbertella* by C.W. Hesseltine in 1960 [8]. Species of this genus are characterized as having sporangia with a persistent wall dehiscing via a longitudinal suture; sporangiospores with apical, hyaline appendages; and Mucor-type zygospores [9]. Previously, the genus *Gilbertella* was

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assigned within the Choanephoraceae because it had not been seen since its original description [7]. Hesseltine placed the genus within the Mucoraceae because the zygospores are of Mucor-type [8]. Later, *Gilbertella* was confirmed through studies of DNA sequence data as in fact belonging to the family Choanephoraceae [10].

Gilbertella persicaria, which is heterothallic, has a sporangial wall that splits into hemispheres at maturity, and sporangiospores that bear long filamentous appendages on the ends. This species has been reported as a plant pathogen of peach, pear, tomato, and some tropical fruits [7, 11-15]. In Index Fungorum (2018; http://www.indexfungorum.org), the genus *Gilbertella* contains only one species, named *Gilbertella persicaria* (E.D. Eddy) Hesselt.

Genus *Pilobolus* Tode (Pilobolaceae, Mucorales) is characterized by positive phototropism and its method of spore dispersal; that is, through the ballistic discharge caused by the elevated pressure generated by subsporangial swelling of the sporangiophore [16, 17]. *Pilobolus* species are attached to the substrate by an absorptive structure, the swollen trophocyst, which is semi-immersed in the substrate [17]. The trophocysts are generally ovoid to globose, whereas the rhizoidal extension is long and cylindrical [17]. The sporangiophores are straight, unbranched, and positively phototropic, with two rings of orange pigment at the base and near the subsporangial vesicle [17]. The sporangia are hemispherical and contain the spores, which are globose or ellipsoidal depending on the species [17]. Zygospores are formed in the substrate and have apposed suspensors [18].

Pilobolus species are coprophilous and have typically been detected on herbivore dung and are frequently observed sporulating on this substrate [19, 20]. Coprophilous fungi are an important part of the ecosystem, participating in the recycling of nutrients in animal dung [21]. In Index Fungorum 2018, the genus *Pilobolus* contains 15 species; namely, *P. crystallinus* (F.H. Wigg.) Tode, *P. hallierii* Rivolta, *P. kleinii* Tiegh., *P. lentiger* Corda, *P. longipes* Tiegh., *P. microsporus* Bref., *P. minutus* Speg., *P. nanus* Tiegh., *P. oedipus* Mont., *P. pestis-bovinae* Hallier, *P. pirottianus* Morini, *P. proliferens* McVickar, *P. pullus* Massee, *P. roridus* (Bolton) Pers., and *P. umbonatus* Buller.

Until now, 8 new species have been registered in Index Fungorum and dozens of unreported species have been discovered in Korea, but information about the species diversity of mucoralean fungi is still lacking. In Korea, within the Choanephoraceae, only 3 species have been described, whereas species belonging to the Pilobolaceae have not yet been described.

The aim of the present study was to perform molecular and morphological analyses to characterize two novel mucoralean species from specific habitats such as freshwater and animal feces in Korea: *Gilbertella persicaria* and *Pilobolus crystallinus*.

2. Results

2.1. Molecular Phylogenetic Status

A molecular phylogenetic analysis was generated using two sequence datasets (ITS and 28S rDNA). Phylogenetic analyses showed that the strains CNUFC-GWD3-9 and CNUFC-EGF1-4 were placed within the same clade with species of *Gilbertella* and *Pilobolus* (Figure 1, 2, and 3). A BLASTn search showed that the ITS rDNA sequences of CNUFC-GWD3-9 and CNUFC-EGF1-4 have high sequence identities of 99.7% (490/491 bp) and 99.3% (572/576 bp) with *G. persicaria* (GenBank Accession No. NR111692) and *P. crystallinus* (GenBank Accession No. FJ160958), respectively. In the BLASTn analysis of the 28S rDNA sequences, CNUFC- GWD3-9 and CNUFC-EGF1-4 strains revealed 100% (653/653 bp) and 100% (538/538 bp) identity values with *G. persicaria* (GenBank Accession No. JN939197) and *P. crystallinus* (GenBank Accession No. JN982939), respectively.

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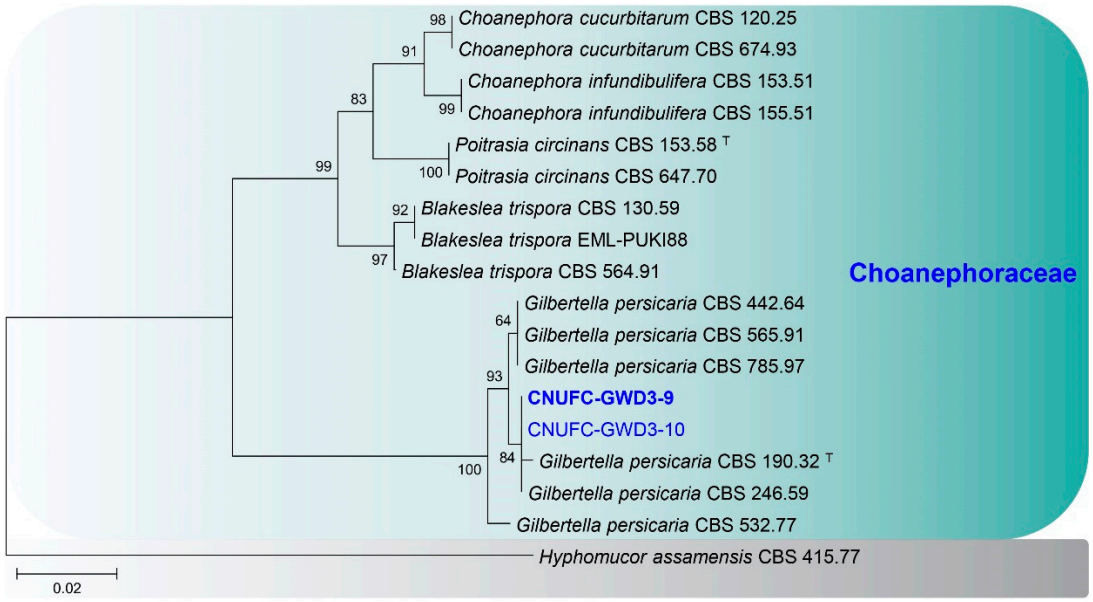

Figure 1. Phylogenetic tree based on neighbor-joining analysis of internal transcribed spacer rDNA sequences for *Gilbertella persicaria* CNUFC-GWD3-9 and *G. persicaria* CNUFC-GWD3-10. *Hyphomucor assamensis* was used as an outgroup. Bootstrap support values of ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

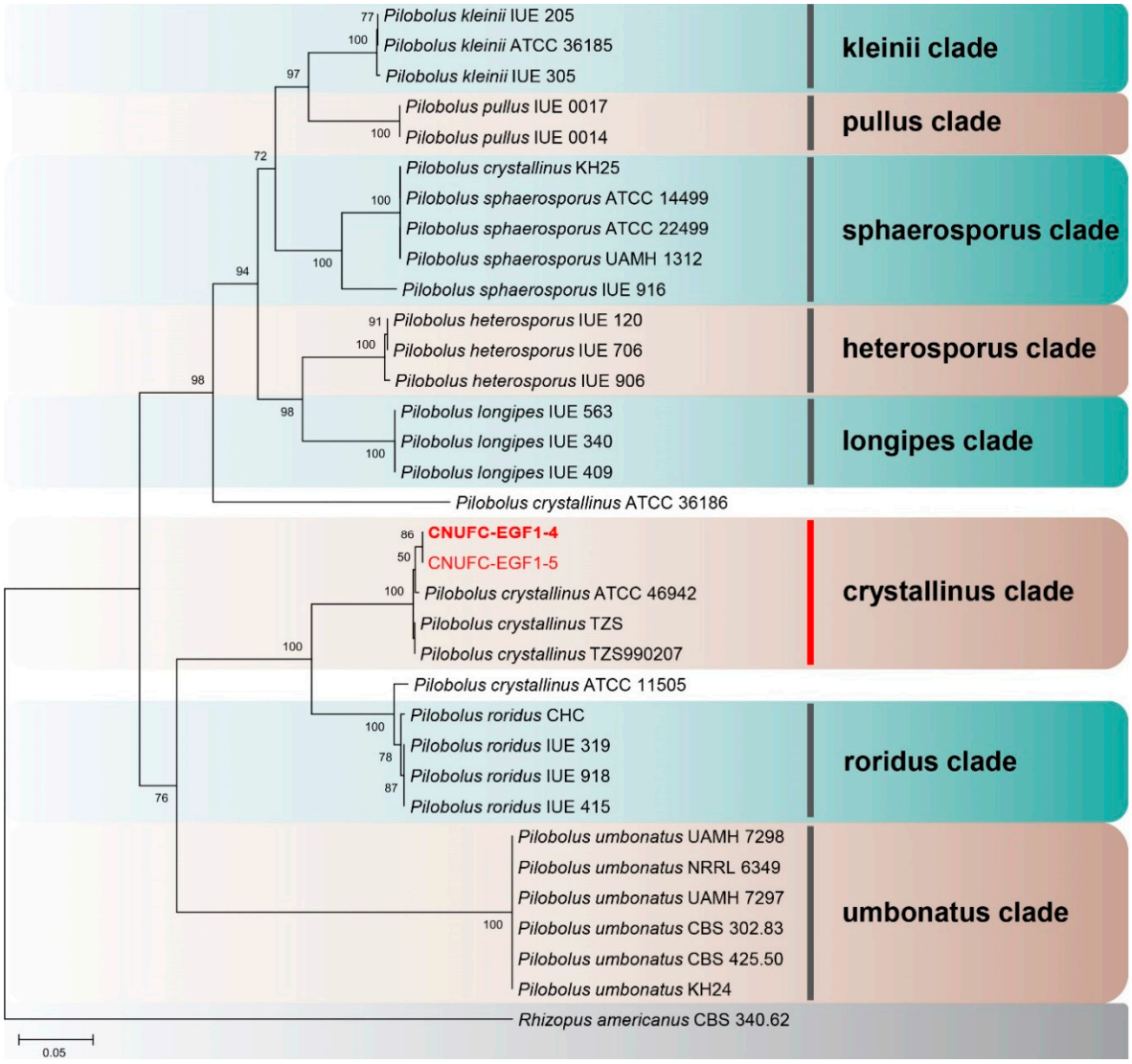

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Figure 2. Phylogenetic tree based on neighbor-joining analysis of internal transcribed spacer rDNA sequences for *Pilobolus crystallinus* CNUFC-EGF1-4 and *P. crystallinus* CNUFC-EGF1-5. *Rhizopus americanus* was used as an outgroup. Bootstrap support values of ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

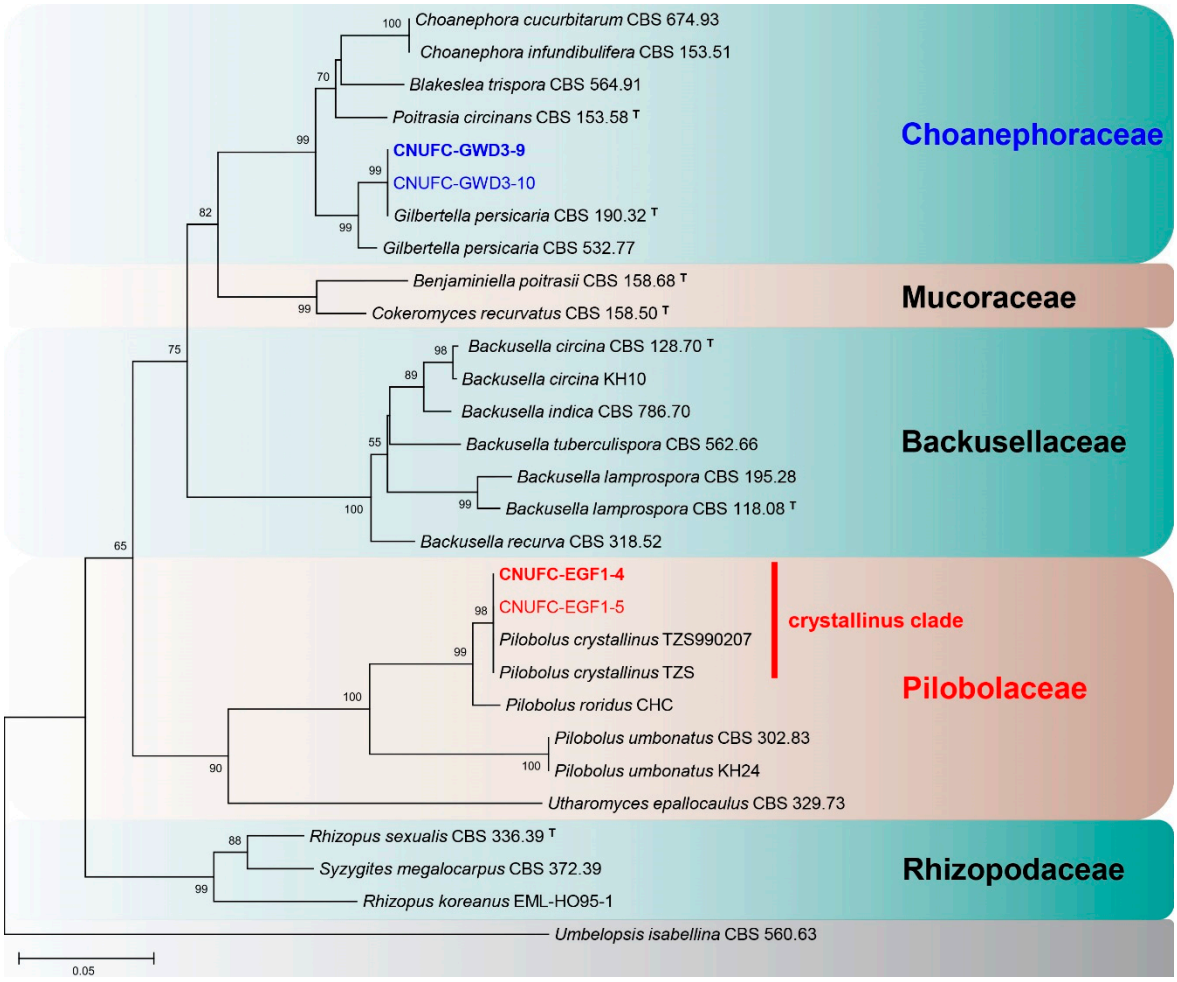

Figure 3. Phylogenetic tree based on neighbor-joining analysis of 28S rDNA sequences for *Gilbertella persicaria* CNUFC-GWD3-9, *G. persicaria* CNUFC-GWD3-10, *Pilobolus crystallinus* CNUFC-EGF1-4, and *P. crystallinus* CNUFC-EGF1-5. *Umbelopsis isabellina* was used as an outgroup. Bootstrap support values of ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

2.2. Morphological Characterization

Taxonomic descriptions of the morphological structures for the two species (*G. persicaria* CNUFC-GWD3-9 and *P. crystallinus* CNUFC-EGF1-4) are shown in details below.

2.2.1. CNUFC-GWD3-9 *Gilbertella persicaria*

Colonies grew rapidly at 25°C on SMA, filling the Petri dish after 2 days of incubation. The colony color was initially white and later grayish yellow. The colony reverse side was white and later pale yellow. Sporangiophores were 10.5–50.0 μm wide, variable in length, hyaline, light brown to grayish, sometimes branched, and uncommonly had a septum under the sporangia. The sporangia separated longitudinally into two halves, were globose to subglobose, many-spored, initially white-yellowish and then turning brown or black at maturity, and measured 36.5–250.5 × 37.2–253.5 μm. Columellae were variable in shape, ovoid to pyriform, subglobose, and measured 20.5–110.7 × 25.2–139.0 μm. Sporangiospores were irregular in shape, mainly ellipsoidal, and measured 5.9–15.5 × 4.5–8.9 μm. Chlamydospore formations were well defined on the medium. Zygospores were not observed. Subsidiarily, colonies grew slowly on SMA, PDA, and MEA at 5°C. Among these, the best mycelial

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growth and sporulation were on PDA at 5°C.

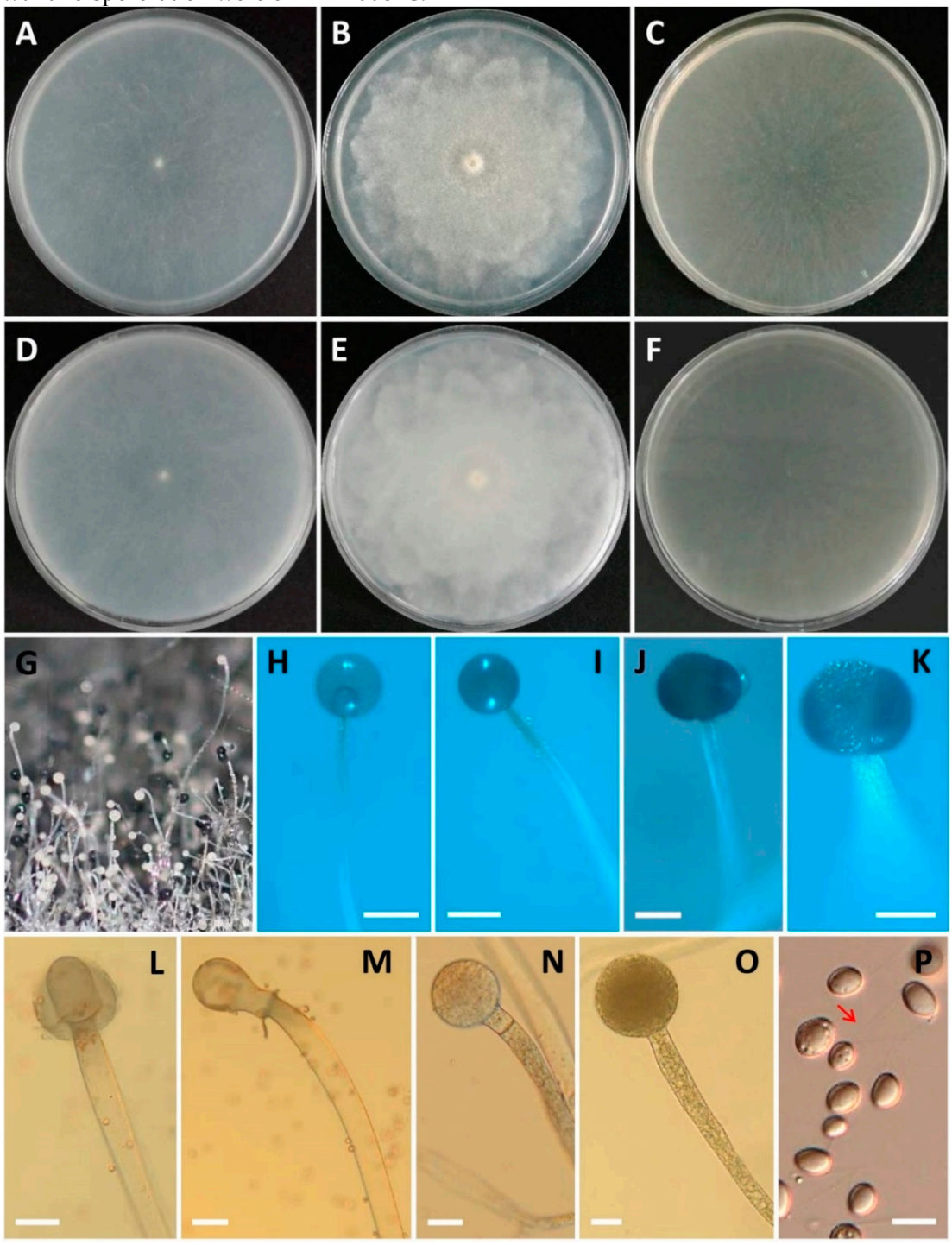

Figure 4. Morphology of *Gilbertella persicaria* CNUFC-GWD3-9. A, D: Colony on synthetic Mucor agar; B, E: Colony on potato dextrose agar; C, F: Colony on malt extract agar (A–C, top view; D–F, reverse view); G–I, N, O: Immature and mature sporangia and sporangiophores; J, K: Wall suturing in two equal halves; L, M: Columellae with collarette; P: Sporangiospores with appendages (red arrow) (scale bars H, I = 200 μm; J–M = 50 μm; N, O = 20 μm; P = 10 μm).

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2.2.2. Distinguishing Characters

The CNUFC-GWD3-9 isolate was similar to the description of *G. persicaria* as detailed by Hesseltine [8], in terms of the shape, size of the sporangiospores (5.9–15.5 × 4.5–8.9 μm), and maximum temperature. However, some morphological features differed. The size of columellae described by Hesseltine [8] was larger (40–119 × 20–170 μm) than that (20.5–110.7 × 25.2–139.0 μm) observed in our isolate. Our *G. persicaria* isolate presented sporangiophores that were sometimes branched, which was not described by Hesseltine [8]. Moreover, our *G. persicaria* isolate had a septum under the sporangia. In conclusion, comparing the morphology and culture characteristics of the isolate with previous descriptions [8], our present isolate was similar to *G. persicaria*, with some exceptions (Table 1).

Table 1. Morphological characteristics of CNUFC-GWD3-9 and the reference species *Gilbertella persicaria* grown on synthetic mucor agar medium at 25–26°C.

Characteristic	CNUFC-GWD3-9	*Gilbertella persicaria* [a]
Colony color	Rapid-growing, first white and then grayish yellow	Rapid-growing, first white and then grayish olive
Sporangiophores	10.5–50.0 μm in width, variable in length	Up to 40–50 μm in width, up to 3–4 mm in height
Sporangia	Many-spored, globose to subglobose, first white-yellowish and then brown or black when mature, 36.5–250.5 × 37.2–253.5 μm	Many-spored, globose to irregularly globose, first white and then yellow and then black and glistening when mature, 40–260 μm in diameter
Columellae	Variable in shape, ovoid to pyriform, subglobose, 20.5–110.7 × 25.2–139.0 μm	Variable in shape depending on size, 40–119 × 20–170 μm
Sporangiospores	Irregular in shape, mainly ellipsoidal, 5.9–15.5 × 4.5–8.9 μm	Short oval and rather irregular in shape, 5–13 × 4.5–11 μm, up to 8.6 × 17 μm
Chlamydospores	Present	Present
Zygospores	Not observed	Present

[a]From the description by Hesseltine [8].

2.2.3. CNUFC-EGF1-4 *Pilobolus crystallinus*

Trophocysts were subglobose to ellipsoidal, and measured 199.0–409.8 × 147.5–186.9 μm. Sporangiophores were 57.0–122.7 μm wide, variable in length, erect, nonseptate, and unbranched. Subsporangial vesicles were ovoid, with an orange ring at the base, and measured 298.0–677.9 × 175.0–548.7 μm. Sporangia were hemispherical, umbonate, yellow or brown when young and turning black at maturity, and measured 169.5–371.5 × 151.5–295.5 μm. Columellae were ellipsoidal to mammiform, and measured 110.3–186.7 × 122.1–230.5 μm. Sporangiospores were elliptical, hyaline, yellowish, and measured 6.0–8.5 × 4.0–5.5 μm.

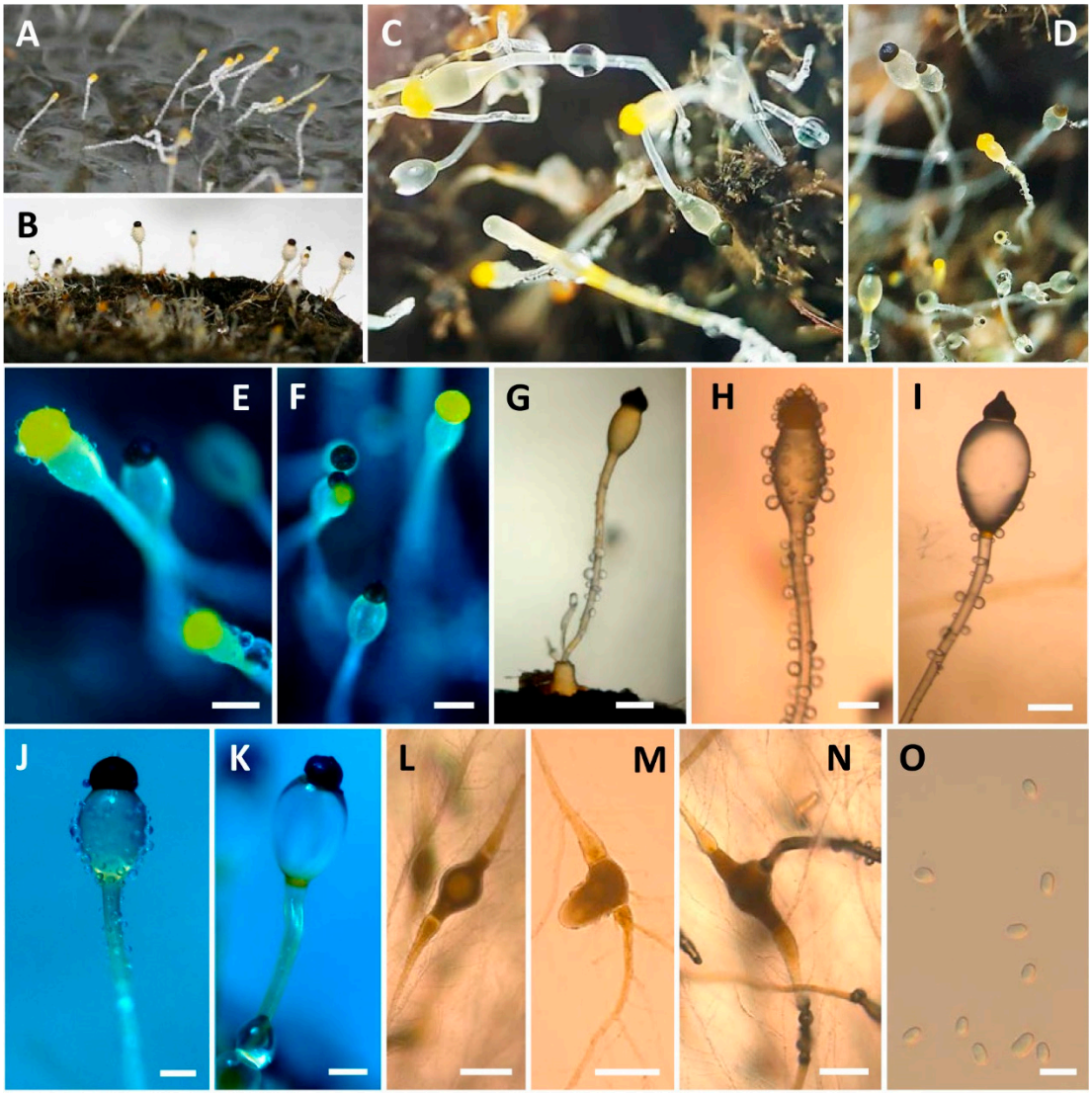

Figure 5. Morphology of *Pilobolus crystallinus* CNUFC-EGF1-4. A: Young sporangia and sporangiophores on dung agar medium; B–K: Yellow and black sporangia, subsporangial vesicles, and sporangiophores (B–G, J, K, on water deer dung); L–N: Substrate mycelia with trophocysts and rhizoidal extensions; O: Sporangiospores (scale bars E–N = 200 μm; O = 10 μm).

2.2.4. Distinguishing Characters

The sporangiospores of isolate CNUFC-EGF1-4 were morphologically similar to the description for those of *P. crystallinus* by Boedijn [22], some differences in other morphological characteristics were found (Table 2).

Table 2. Morphological characteristics of CNUFC-EGF1-4 and the reference species *Pilobolus crystallinus*.

Characteristic	CNUFC-EGF1-4	*Pilobolus crystallinus*[a]
Trophocysts	Subglobose to ellipsoidal, 199.0–409.8 × 147.5–186.9 μm	Oblong, 500–575 × 200–230 μm
Sporangiophores	Variable in length, 57.0–122.7 μm wide	5–15 mm long, 115–160 μm wide
Subsporangial vesicles	Ovoid, orange ring at the base, 298.0–677.9 × 175.0–548.7 μm	Oviform, colorless except for an orange ring at the base, 400–920 × 350–720 μm
Sporangia	Hemispherical, umbonate, first yellow or brown and then black when mature, 169.5–371.5 μm × 151.5–295.5 μm	Semiglobose, black, 237–529 μm wide near the base, 138–345 μm high
Columellae	Ellipsoidal to mammiform, 110.3–186.7 μm × 122.1–230.5 μm	Broadly conical, 92–287 μm high, 172–345 μm wide below
Sporangiospores	Elliptical, hyaline, yellowish, 6.0–8.5 × 4.0–5.5 μm	Elliptical, hyaline, dark yellow in mass, 7–10 × 4–6 μm
Zygospores	Not observed	Unknown

[a]From the description by Boedijn [22].

3. Discussion

Until now, the distribution and occurrence of Mucoralean species from dung and freshwater sources is poorly studied. Since there have been no reports related to *Gilbertella* and *Pilobolus* species in Korea, the purpose of this paper was to describe and illustrate two rare species: *Gilbertella* and *Pilobolus* from specific sources such as freshwater and water deer dung in Korea, respectively.

In our phylogenetic analyses, the isolates of CNUFC-GWD3-9 and CNUFC-GWD3-10 were grouped with strains of *G. persicaria* CBS 190.32 (type species) (Figure 1 and 3). Although, the morphological characteristics of isolate of *G. persicaria* in this study were similar to the ones previously described by Hesseltine [8], its sporangiospores were slightly narrower (up to 8.6–17 μm). Besides, the molecular data also proved that our isolate was identified as *G. persicaria.*

Species of *G. persicaria* have been reported to produce extracellular enzymes such as endoglucanase, β-glucosidase, lipase, and pectinase [23, 24, 25]. Interestingly, our strain, CNUFC-GWD3-9, showed pectinase activity. This finding suggests that the strain may be a source of novel enzyme.

G. persicaria were often isolated from peach, pear, tomato, and dragon fruit by other researchers [7, 11-15]. However, this is the first isolation of *G. persicaria* from freshwater source. Based on a recent literature, the Ascomycetes is diverse among freshwater fungi with approximately 622 species (170 genera), including more than 531 species of Hyphomycetes (55 genera), and species of Trichomycetes (3 orders, no longer regarded as fungi); whereas, the information about freshwater-derived fungi belonging to Basidiomycetes and Zygomycetes was rare [26, 27]. Hence, further understanding about the biodiversity of Zygomycets in freshwater is needed.

On the other hand, isolates CNUFC-EGF1-4 and CNUFC-EGF1-5 were clustered with *P. crystallinus* species in a well-supported clade (Figure 2 and 3). Although most of morphological features of our isolate were similar to those of *P. crystallinus* described by Boedijin [22], although there were several

differences in the diameter of subsporangial vesicles and sporangia. Subsporangial vesicles sizes reported in the literature range from 400-920 x 350-720 μm [22], which are larger than our maximum measurement. According to Foos et al. [28], although the size and shape of the sporangiospores have been detected to be stable within species [29], species descriptions typically give a large range of sporangiospore sizes. Moreover, the sizes of many structures used for species identification vary greatly depending on changes in the environmental conditions [28, 30]. In this study, rDNA ITS gene provided sufficient phylogenetic information for the separation of *Pilobolus* species (Figure 2). However, isolate KH25 named as *P. crystallinus* was clustered with the other *P. sphaerosporus* species. Besides, isolates ATCC 36186 and ATCC 11505 named as *P. crystallinus* were not clustered with the other *P. crystallinus* species. Our results revealed that the isolate *P. crystallinus* KH25 should be changed to *P. sphaerosporus*. In addition, based on the sequences of ITS rDNA, we showed that the group containing species *P. crystallinus* is polyphyletic.

Despite the wide intraspecific variation found among some taxa, the ITS and D1/D2 regions have been used as appropriate barcode markers for identifying mucoralean fungi at the species level [4, 5]. Currently, the traditional method of fungal identification is still mainly in use, as further studies are required to reconcile the molecular and morphological conceptions of families and genera. In the present study, we also used the molecular strategy for fungal identification at the level of species, specifically utilizing of ITS rDNA gene sequence and phylogenetic analysis. In 2011, Foos et al. [28] conducted sequence analysis of the ITS region of rRNA, small subunit of 18S rRNA, and LSU (23S) of mitochondrial rRNA, and showed that the genus *Pilobolus* is polyphyletic. The results revealed that molecular phylogenetic identification of *Pilobolus* species based on sequence analysis of pure culture isolates was more reliable than the traditional method of identification [28, 31]. Our phylogenetic trees also agree with those reported by Foos et al. [28]. Therefore, these results confirmed that the isolate CNUFC-EGF1-4 belongs to the species *P. crystallinus*. Although a large number of species of fungi have been reported from dung of different animal taxa, few have been reported from water deer dung. Thus, diversity of rare dung fungi or dung-derived fungi are to be investigated consistently.

4. Materials and Methods

4.1. Sampling and Isolation of Fungal Strain

Water deer dung samples were collected on Eulsukdo Island (35°6'17.92" N, 128°56'24.52" E; located in Busan, Korea) in June 2017. The samples were transferred to sterile 50-mL conical tubes (SPL Life Sciences Co., Pocheon, Korea), and stored at 4°C until examination. The fecal samples were placed onto sterile moist Whatman's filter paper in a Petri dish using sterile forceps, and incubated in a moist chamber at 25°C for 6–9 days.

Freshwater samples were collected from the Geum River (36°27'47.32" N, 127°6'3.24" E; located in Gongju, Korea) in August 2017. These samples were transported in sterile 50-mL conical tubes, and stored at 4°C until examination. Fungi were isolated by the direct plating method. In brief, plant debris in the freshwater samples was placed onto synthetic mucor agar (SMA; 40 g of dextrose, 2 g of asparagine, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of thiamine chloride, and 15 g of agar in 1 L of deionized water) using sterile forceps and incubated at 25°C for 1–3 days. To isolate pure cultures, individual colonies of varied morphologies were picked up, transferred to potato dextrose agar (39 g of PDA in 1 L of deionized water; Becton, Dickinson and Co., Sparks, MD, USA) plates, and subcultured until pure mycelia were obtained. All pure isolates, including those of *G. persicaria* and *P. crystallinus*, were stored in 20% glycerol at −80°C at the Environmental Microbiology Laboratory Fungarium (Chonnam National University, Gwangju, Korea), as CNUFC-GWD3-9 and CNUFC-EGF1-4, respectively. Strain CNUFC-EGF1-4 was also deposited at the Culture Collection of the National Institute of Biological Resources (NIBR, Incheon, Korea), whereas strain CNUFC-GWD3-9 was also deposited at the Culture Collection of the Nakdonggang National Institute of Biological Resources (NNIBR, Sangju, Korea).

4.2. DNA Extraction, PCR, and Sequencing

Genomic DNA was extracted directly from mycelia and spores of the fungal isolates, using the Solg Genomic DNA Prep Kit for fungi (SolGent Co. Ltd., Daejeon, Korea). The internal transcribed spacer (ITS) region and large subunit (LSU) of 28S rDNA were amplified with the primer pairs ITS1 and ITS4 [32], and LROR and LR5F [33, 34], respectively (Table 3). The PCR amplification mixture (total volume, 20 μL) contained fungal DNA template, 5 pmol/μL of each primer, and Accupower PCR Premix (*Taq* DNA polymerase, dNTPs, buffer, and a tracking dye; Bioneer Corp., Daejeon, Korea). The PCR products were purified using the Accuprep PCR Purification Kit (Bioneer Corp.) according to the manufacturer's instructions. DNA sequencing was performed on an ABI 3700 Automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA).

Table 3. Primers used in this study, with sequences and sources.

Gene	Product Name	Primer	Direction	Sequence (5′-3′)	Reference
ITS	Internal transcribed spacer	ITS1	Forward	TCCGTAGGTGAACCTGCG G	[32]
		ITS4	Reverse	TCCTCCGCTTATTGATAT GC	
LSU	Large subunit (28S)	LROR	Forward	ACCCGCTGAACTTAAGC	[33, 34]
		LR5F	Reverse	GCTATCCTGAGGGAAAC	

4.3. Phylogenetic Analysis

Fungal sequences (Table 1) were used for phylogenetic analysis through alignment with Clustal_X v.2.0 [35] and editing using Bioedit v.7.2.5 software [36]. Phylogenetic trees based on the ITS rDNA and D1/D2 sequences were constructed using the neighbor-joining method in MEGA 6 [37]. The reliability of the internal branches was assessed using the p-distance substitution model, with 1,000 bootstrap replications. The CNUFC-GWD3-9, CNUFC-GWD3-10, CNUFC-EGF1-4, and CNUFC-EGF1-5 sequences were deposited in the NCBI database under the accession numbers shown in Table 4.

Table 4. Taxa, collection numbers, sequences, and GenBank accession numbers used in this study

Taxon name	Collection No. (Isolate No.)	GenBank accession No.	
		ITS	LSU
Backusella circina	CBS 128.70 (T)	-	JN206529
B. circina	KH10	-	JX644493
B. indica	CBS 786.70	-	JN206526
B. lamprospora	CBS 195.28	-	JN206530
B. lamprospora	CBS 118.08 (T)	-	JN206531
B. recurva	CBS 318.52	-	JN206522
B. tuberculispora	CBS 562.66	-	JN206525
Benjaminiella poitrasii	CBS 158.68 (T)	-	JN206411
Blakeslea trispora	CBS 130.59	JN206227	-
Bl. trispora	EML-PUKI88	KY047144	-
Bl. trispora	CBS 564.91	JN206230	JN206515
Choanephora cucurbitarum	CBS 120.25	JN206231	-
C. cucurbitarum	CBS 674.93	JN206233	JN206514
C. infundibulifera	CBS 153.51	JN206236	JN206513
C. infundibulifera	CBS 155.51	JN206237	-
Cokeromyces recurvatus	CBS 158.50 (T)	-	HM849699

Gilbertella persicaria	CBS 785.97	JN206218	-
G. persicaria	CBS 190.32 (T)	HM999958	HM849691
G. persicaria	CBS 246.59	JN206222	-
G. persicaria	CBS 442.64	JN206219	-
G. persicaria	CBS 532.77	JN206224	JN206517
G. persicaria	CBS 565.91	JN206226	-
G. persicaria	**CNUFC-GWD3-9**	**MG906872**	**MG906876**
G. persicaria	**CNUFC-GWD3-10**	**MG906873**	**MG906877**
Hyphomucor assamensis	CBS 415.77	JN206211	-
Pilobolus crystallinus	ATCC 11505	FJ160947	-
P. crystallinus	ATCC 36186	FJ160949	-
P. crystallinus	ATCC 46942	FJ160958	-
P. crystallinus	KH25	JX644569	-
P. crystallinus	TZS	JN942691	JN982943
P. crystallinus	TZS990207	JN942689	JN982939
P. crystallinus	**CNUFC-EGF1-4**	**MG906874**	**MG906878**
P. crystallinus	**CNUFC-EGF1-5**	**MG906875**	**MG906879**
P. heterosporus	IUE 120	HM049566	-
P. heterosporus	IUE 706	HM049604	-
P. heterosporus	IUE 906	HM049615	-
P. kleinii	ATCC 36185	FJ160957	-
P. kleinii	IUE 205	HM049567	-
P. kleinii	IUE 305	HM049574	-
P. longipes	IUE 340	FJ160950	-
P. longipes	IUE 409	FJ160951	-
P. longipes	IUE 563	FJ160952	-
P. pullus	IUE 0014	HQ877876	-
P. pullus	IUE 0017	HQ877877	-
P. roridus	CHC	JN942692	JN982944
P. roridus	IUE 319	HM049579	-
P. roridus	IUE 415	FJ160948	-
P. roridus	IUE 918	HM049619	-
P. sphaerosporus	ATCC 14499	FJ160954	-
P. sphaerosporus	ATCC 22499	DQ059382	-
P. sphaerosporus	IUE 916	HM049616	-
P. sphaerosporus	UAMH 1312	FJ160953	-
P. umbonatus	CBS 302.83	JN206274	HM849665
P. umbonatus	CBS 425.50	JN206275	-
P. umbonatus	KH24	JX644571	JX644519
P. umbonatus	NRRL 6349	FJ160955	-
P. umbonatus	UAMH 7297	FJ160956	-
P. umbonatus	UAMH 7298	DQ058412	-
Poitrasia circinans	CBS 153.58 (T)	JN206239	JN206516
Pt. circinans	CBS 647.70	JN206240	-
Rhizopus americanus	CBS 340.62	HM999967	-
R. koreanus	EML-HO95-1	-	KU058196
R. sexualis	CBS 336.39 (T)	-	HM849673
Syzygites megalocarpus	CBS 372.39	-	JN206401
Umbelopsis isabellina	CBS 560.63	-	JN206573
Utharomyces epallocaulus	CBS 329.73	-	HM849660

Bold letters indicate the isolates and accession numbers determined in our study. ITS, internal transcribed spacer; ATCC, American Type Culture Collection (Manassas, VA, USA); CBS,

Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands); CNUFC, Chonnam National University Fungal Collection (Gwangju, South Korea); EML, Environmental Microbiology Laboratory (Fungarium, Chonnam National University, Gwangju, South Korea); NRRL (Agricultural Research Service Culture Collection, Peoria, IL, USA); T, ex-type strain.

4.4. Morphological Studies

For detailed morphological studies, strain CNUFC-GWD3-9 was cultured on SMA, PDA, and malt extract agar (33.6 g of MEA in 1 L of deionized water; Becton, Dickinson and Co.). The plates were incubated at 5°C, 15°C, 25°C, 35°C, and 40°C in the dark for 2–3 days. Samples were mounted in distilled water and observed using an Olympus BX51 microscope with differential interference contrast (DIC) optics (Olympus, Tokyo, Japan). CNUFC-EGF1-4 strain was cultured on dung agar medium (2 g of water deer dung and 2 g of agar in 100 mL of deionized water) and the plates were incubated at 20°C, 25°C, and 35°C in the dark for 7–14 days. Additionally, fungal spores of strain CNUFC-EGF1-4 were inoculated on surface-sterilized pieces of water deer dung by touching with a sterile needle, and the plates were then incubated at 25°C in the dark for 7–14 days. Samples were observed under an Olympus BX51 microscope with DIC optics.

4.5. Pectinase activity assay

To detect pectinase activity, we used the medium as described by Hankin and Anagnostakis (1975) [38]. The medium contained 1 g of yeast extract, 20 g of agar, 10 g of pectin (citrus), $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; K_2HPO_4, 1.0 g; $FeSO_4.7H_2O$, 0.01 g and 1000 ml of distilled water; pH 6.8 or 7.0. Strain CNUFC-GWD3-9 was cultured on potato dextrose agar (PDA, Becton, Dickinson and Co.) at 25°C for 7days. 7 agar pieces 6.5 mm of fungal mycelia were put in to 50 ml potato dextrose broth (PDB) in a 100 ml Erlenmeyer flask, previously sterilized at 121°C for 15 min. Strain CNUFC-GWD3-9 was incubated at 25°C for 5 days at 130 rpm in a horizontal shaker incubator. The broth culture was centrifuged at 13,000 ×*g* for 20 min at 4°C. A 50-µL aliquot of the supernatant was transferred to a paper disc (diameter, 8 mm), and then the disc was placed on the surface of a potato-dextrose-agar (PDA) plate (90 mm × 15 mm). After 3 days of incubation at 25°C, plates were flooded with a 1% aqueous solution of hexadecyltrimethylammonium bromide (Fisher Chemical Co., Fairlawn, N. J.). Clear zones around a colony indicated degradation of the pectin.

5. Conclusions

The analysis of two sequence datasets (ITS and LSU rDNA) and morphological characteristics represented that the two mucoralean strains CNUFC-GWD3-9 and CNUFC-EGF1-4 were consistent with *G. persicaria* and *P. crystallinus*, respectively, which have never been described in Korea.

G. persicaria showed high pectinase activity; accordingly, it may be a source of novel enzyme (data not shown).

Our findings contribute to the current knowledge of diversity of the order Mucorales in Korea. However, data regarding the diversity of the order Mucorales in Korea are still lacking, further studies on the classification of different orders and families within the Mucoromycotina are required to expand our knowledge of rare undiscovered taxa with specific habitats in Korea.

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Author Contributions: S.H.L. performed the experiments, and wrote the paper; T.T.T.N. reviewed and edited the paper; H.B.L. designed and edited the paper, and supervised the study.

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