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RESEARCH ARTICLE

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Molecular and Morphological Confirmation of Three Undescribed Species of *Mortierella* from Korea

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ABSTRACT

Three fungal isolates designated as CNUFC-YR329-1, CNUFC-PTS103-1, and CNUFC-PTS2-1 were discovered during a survey of fungal diversity of the order *Mortierellales* from freshwater and pine tree rhizosphere soil samples in Korea. The strains were analyzed morphologically and phylogenetically based on the internal transcribed spacer (ITS) and large subunit (LSU) of ribosomal DNA gene sequences. Based on their morphology and phylogeny, the three isolates were identified as *Mortierella elongata*, *M. horticola*, and *M. humilis*, respectively. To the best of our knowledge, *M. elongata*, *M. horticola*, and *M. humilis*, belonging to an undiscovered taxon, have not been previously described in Korea.

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1. Introduction

The genus *Mortierella* was described by Coemans (1863) with the type species *Mortierella polycephala* Coem [1]. Currently, *Mortierella* is a member of the family Mortierellaceae, order Mortierellales, subphylum Mortierellomycotina [2–4]. Until now, approximately 100 species of *Mortierella* have been described [5]. The species belonging to this genus are characterized by the production of a mainly coenocytic but irregularly septate mycelium. Sporangiophores are simple or variously branched, terminating with sporangia and occasionally with a swelling at the base. Sporangia are globose, with multiple or few spores, or a single spore. Species of *Mortierella* are frequently isolated from soil and dead or dying plant tissues, freshwater, or from animal fecal samples [6–11].

Many of the species are potential producers of γ-linolenic acid and arachidonic acid [12]. In addition, some members of the genus *Mortierella* assist crops and mycorrhizal fungi in phosphorus (P) acquisition [13] and also can synthesize and secrete oxalic acid [14]. They also have shown great capacity to decompose plant litter and degrade polyaromatic hydrocarbons [15].

In morphology-based taxonomy, the genus *Mortierella* is divided into nine sections: *Actinomortierella*, *Alpina*, *Haplosporangium*, *Hygrophila*, *Mortierella*, *Schmuckeri*, *Simplex*, *Spinosa*, and *Stylospora* [16]. However, recent molecular analyses do not support this classification system. Based on the sequences of the internal transcribed spacer (ITS) rDNA regions, Wagner et al. [5] reclassified this genus to seven groups: "*selenospora* and *parvispora*", "*verticillata-humillis*", "*lignicola*", "*mutabillis*, *globulifera* and *angusta*", "*strangulate* and *wolfii*", "*alpina* and *polycephala*", and "*gamsii*".

To date, six species of *Mortierella* have been reported in Korea: *M. alpina*, *M. ambigua*, *M. indohii*, *M. minutissima*, *M. oligospora*, and *M. zychae* [17–19]. *M. elongata* isolated from the root of *Pharagmites australis* in Korea was previously reported without a detailed description by Khalmuratova et al. [20]. A new *Mortierella* species, *M. fluviae*, was isolated from a freshwater sample collected at Yeongsan River located in Gwangju, Korea, in 2016 [10].

The objective of the present study was to perform morphological and molecular analyses to characterize three undescribed fungal species in Korea: *M. elongata*, *M. horticola*, and *M. humilis*.

2. Materials and methods

2.1. Isolation of fungal strains from freshwater and pine tree rhizosphere soil samples

Freshwater samples were collected from the Yeongsan River located in Gwangju (35° 10' 36.94" N 126° 55' 15.04" E), Korea. Soils were sampled from the rhizosphere of pine trees at Geumgol mountain located in Jindo (34° 28' 59.00" N 126° 15' 43.00" E), Korea. These samples were transferred

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in sterile 50 mL conical tubes and stored at 4 °C until examination. Fungi were isolated by a serial dilution plating method as described previously [21], and cultured on potato dextrose agar (PDA: 39 g PDA in 1 L of deionized water; Becton, Dickinson and Co., Sparks, MD, USA) supplemented with streptomycin sulfate 0.5 mg/mL, to suppress bacterial growth. After incubation at 25 °C for 3–7 days, individual hyphal tips of the developing fungal colonies were removed and placed onto PDA medium. Pure isolates were maintained in PDA slant tubes and stored in 20% glycerol at −80 °C at the Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, Korea, and were designated CNUFC-YR329-1, CNUFC-YR329-2, CNUFC-PTS103-1, CNUFC-PTS103-2, CNUFC-PTS2-1, and CNUFC-PTS2-2. Strain CNUFC-YR329-1 was also deposited at the Culture Collection of the Nakdonggang National Institute of Biological Resources (NNIBR, Sangju, Korea), and strains CNUFC-PTS103-1 and CNUFC-PTS2-1 were deposited at the Culture Collection of the National Institute of Biological Resources (NIBR, Incheon, Korea).

2.2. Morphological studies

For detailed morphological studies, CNUFC-YR329-1, CNUFC-PTS103-1, and CNUFC-PTS2-1 strains were cultured on PDA, malt extract agar (MEA: 20 g malt extract, and 20 g agar in 1 L of deionized water), oatmeal agar (OA; 30 g oatmeal extract, 15 g agar in 1 L of deionized water), and water agar (20 g agar in 1 L of deionized water). The plates were incubated at 10, 20, 25, 30, and 35 °C in the dark for 7 days. Fragments of mycelia were removed from cultures, placed on microscope slides with distilled water and lactophenol solution (Junsei Chemical Co. Ltd., Tokyo, Japan) and observed under a light microscope (Olympus, Tokyo, Japan).

2.3. DNA extraction, PCR, and sequencing

Genomic DNA was extracted directly from the mycelia of fungal isolates, using the Genomic DNA Prep Kit (Solgent Co. Ltd., Daejeon, South Korea). The ITS region and large subunit (LSU) ribosomal RNA were amplified with the primer pairs ITS4 and ITS5 [22], and LROR and LR5F [23], respectively. 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). 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 1. Taxa, collection numbers, sequences, and GenBank accession numbers used in this study.

Taxon name	Collection no. (Isolate no.)	GenBank accession no. ITS	28S
Mortierella alpina	CBS 219.35		KC018359
M. bisporalis	CBS 145.69	JX975857	KC018377
M. camargensis	CBS 221.58	JX975949	HQ667408
M. clonocystis	CBS 357.76	JX975899	HQ667395
M. elongata	FSU 823		KC018279
M. elongata	FSU 822	JX975978	
M. elongata	CBS 276.89	JX976111	KC018452
M. elongata	CBS 126.71	JX976101	
M. elongata	CBS 279.62	JX976089	KC018417
M. elongata	CBS 110517		KC018348
M. elongata	IHBF 2303	MF326586	
M. elongata	**CNUFC-YR329-1**	**MH737689**	**MH737683**
M. elongata	**CNUFC-YR329-2**	**MH737690**	**MH737684**
M. epicladia	CBS 246.75		KC018361
M. epicladia	CBS 355.76	JX976130	
M. epigama	CBS 161.76	JX976109	JX976158
M. epigama	CBS 489.70	JX976057	HQ667367
M. exigua	CBS 655.68	JX976047	
M. fluviae	EML-YR25716-1 (T)	KX227755	KX227753
M. globalpina	CBS 226.78	JX976006	JX976160
M. horticola	CBS 305.52 (T)	NR_111572	NG_042556
M. horticola	CBS 305.52	JX975874	JX976166
M. horticola	CBS 254.76		JX976166
M. horticola	CBS 869.68		JX976138
M. horticola	**CNUFC-PTS103-1**	**MH737691**	**MH737685**
M. horticola	**CNUFC-PTS103-2**	**MH737692**	**MH737686**
M. humilis	CBS 181.72		KC018405
M. humilis	CBS 222.35 (T)	NR_077209	JN940875
M. humilis	CBS 222.35	HQ630325	HQ667401
M. humilis	CBS 363.95		KC018443
M. humilis	W1	KT896653	
M. humilis	FSU 828	JN943015	
M. humilis	FSU 829	JN943013	
M. humilis	**CNUFC-PTS2-1**	**MH737693**	**MH737687**
M. humilis	**CNUFC-PTS2-2**	**MH737694**	**MH737688**
M. hypsicladia	CBS 116202 (T)	NR_111563	NG_042547
M. indohii	CBS 331.74	JX975860	
M. indohii	CBS 665.70		KC018357
M. minutissima	CBS 226.35	JX976092	
M. oligospora	CBS 191.79	JX975966	
M. oligospora	CBS 381.71		KC018368
M. polycephala	CBS 456.66	JX976034	
M. polygonia	CBS 685.71 (T)	NR_111562	NG_042546
M. sclerotiella	CBS 529.68 (T)	JX975988	HQ667387
M. verticilata	CBS 220.58	JX975905	JN940873
M. wolfii	CBS 651.93	JX975904	HQ667382
M. wolfii	CBS 612.70	HQ630304	HQ667381
M. zychae	CBS 316.52 (T)	NR_111576	NG_042559
Umbelopsis isabellina	NRRL 1757		JN940879

Bold letters indicate isolates and accession numbers determined in our study.
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; FSU: Friedrich Schiller University, Jena, Germany; ITS: internal transcribed spacer; NRRL (ARS Culture Collection, Peoria, Illinois); T: ex-type strain.

2.4. Phylogenetic analysis

Fungal sequences (Table 1) were initially aligned using Clustal_X v.2.1 [24] and Bioedit v. 7.2.6 software [25]. Phylogenetic analyses were performed using MEGA 6

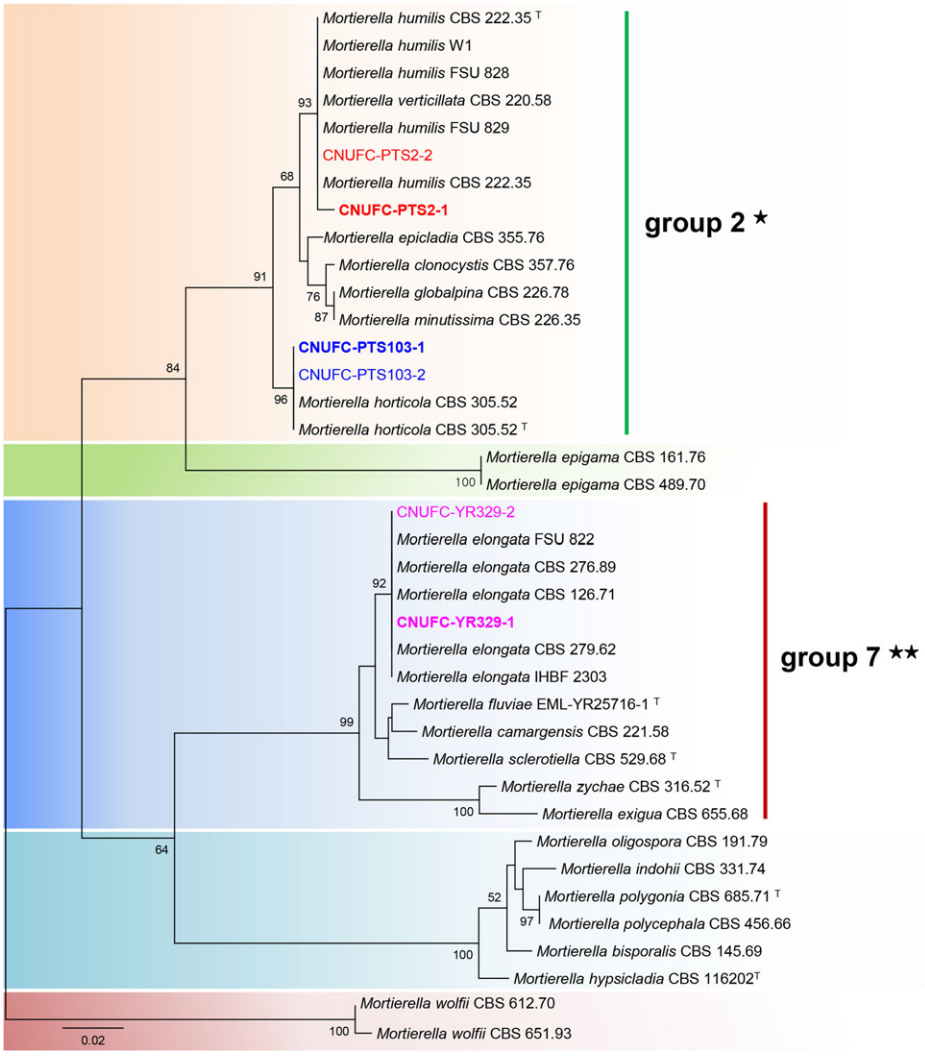

Figure 1. Phylogenetic tree based on maximum likelihood analysis of internal transcribed rDNA sequences for *Mortierella elongata* CNUFC-YR329-1, *M. elongata* CNUFC-YR329-2, *M. horticola* CNUFC-PTS103-1, *M. horticola* CNUFC-PTS103-2, *M. humilis* CNUFC-PTS2-1, and *M. humilis* CNUFC-PTS2-2. The sequences of *Mortierella wolfii* were used as the outgroups. Bootstrap support values ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position. The thick colored clade lines with the asterisk were constructed by a previous phylogenetic system described by Wagner et al. [5], whereas the clade lines marked by the asterisk were constructed by Wagner et al. [5] and Hyde et al. [10].

with the default settings [26]. Phylogenetic trees were constructed using the Maximum likelihood (ML) analysis with the ITS and LSU sequences. The sequences of *Mortierella wolfii* and *Umbelopsis isabellina* were used as the outgroups. The sequence identity was determined using the National Center for Biotechnology Information Basic Local Alignment Search Tool for nucleotides (BLASTn).

3. Results

3.1. Phylogenetic analysis

In the BLASTn analysis of the ITS sequences, CNUFC-YR329-1, CNUFC-PTS103-1, and CNUFC-PTS2-1 represented high identity values of 99.8% (607/608 bp) with *M. elongata* (GenBank accession no. MF326586), 99.8% (499/500 bp) with *M. horticola* (GenBank accession no. JX975874), and 100% (596/596 bp) with *M. humilis* (GenBank accession no. KT896653), respectively.

In the BLASTn analysis of the 28S sequence, CNUFC-YR329-1, CNUFC-PTS103-1, and CNUFC-PTS2-1 represented high identity values of 99.9% (973/974 bp) with *M. elongata* (GenBank accession no. KC018348), 100% (672/672 bp) with *M. horticola* (GenBank accession no. JX976138), and 100% (493/493 bp) with *M. humilis* (GenBank accession no. KC018443), respectively.

Based on the analysis of the ITS and 28S rDNA sequences, CNUFC-YR329-1, CNUFC-YR329-2, CNUFC-PTS103-1, CNUFC-PTS103-2, CNUFC-PTS2-1, and CNUFC-PTS2-2 strains were placed within the order Mortierellales (Figures 1 and 2).

3.2. Taxonomy

3.2.1. Taxonomy of CNUFC-YR329-1

Mortierella elongata Linnem., Pflanzenforschung 23: 43 (1941) [MB#301325] (Table 2, Figure 3).

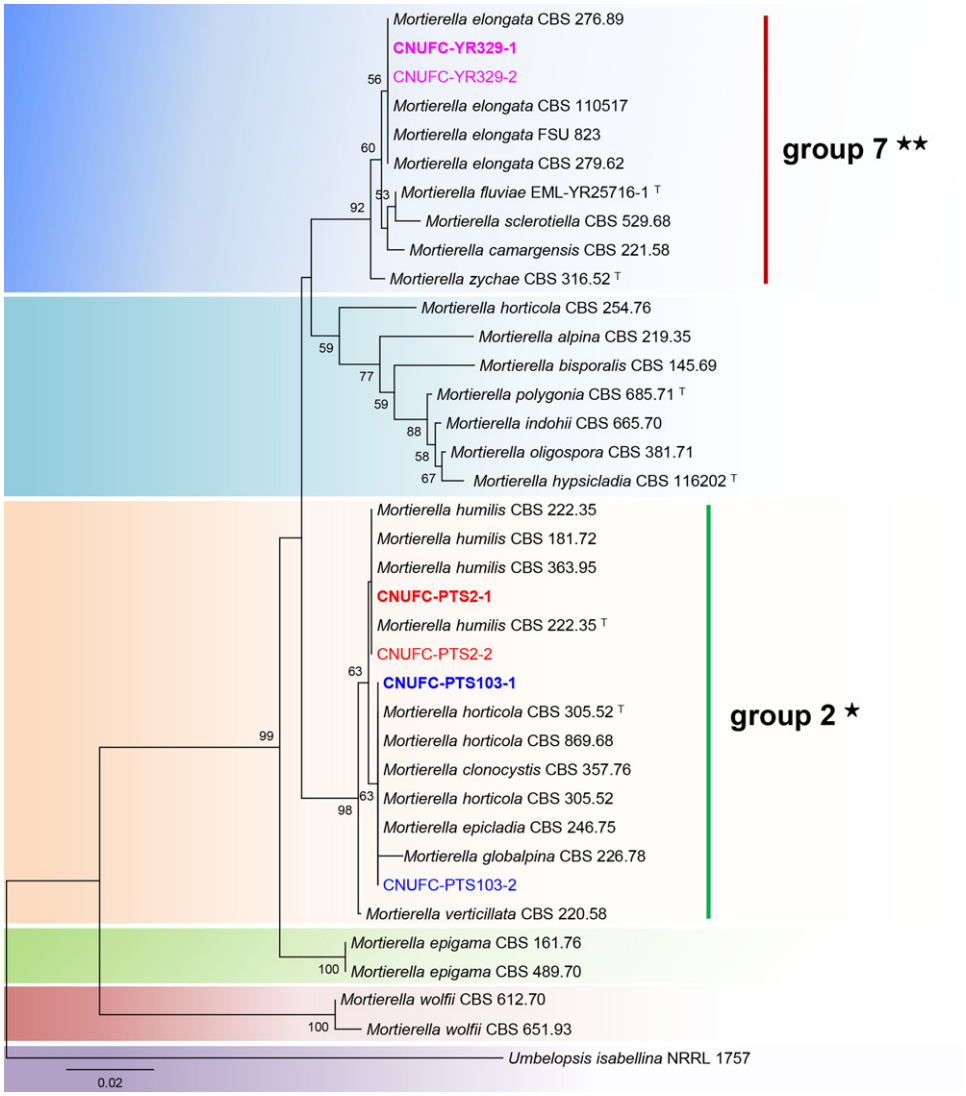

Figure 2. Phylogenetic tree based on maximum likelihood analysis of large subunit rDNA sequences for *Mortierella elongata* CNUFC-YR329-1, *M. elongata* CNUFC-YR329-2, *M. horticola* CNUFC-PTS103-1, *M. horticola* CNUFC-PTS103-2, *M. humilis* CNUFC-PTS2-1, and *M. humilis* CNUFC-PTS2-2. The sequence of *Umbelopsis isabellina* was used as the outgroup. Bootstrap support values ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position. The thick colored clade lines with the asterisk were constructed by a previous phylogenetic system described by Wagner et al. [5], whereas the clade lines marked by the asterisk were constructed by Wagner et al. [5] and Hyde et al. [10].

Table 2. Morphological characteristics of CNUFC-YR329-1 and the reference, *Mortierella elongata*.

Characteristics	CNUFC-YR329-1	*Mortierella elongata*[a]
Colony	Grew rapidly at 20 °C on PDA, white; reverse colony white and slightly zonate.	White, arachnoid to cottony
Sporangiophores	2.5–4.5 µm wide at the tip, variable in length, mostly branched	Up to 250 (–400) µm long, branched, 5–8(–12) µm wide at the base, 1.5–3.5 µm at the tip
Sporangia	Globose, multi-spores, 19.5–34.5 × 18.4–32.5 µm, with a small collarette	Globose, (15–)20–30 µm diameter, with a small collarette
Sporangiospores	Ellipsoidal to short cylindrical, reniform, 8.5–15.7 × 5.2–8.3 µm	Ellipsoidal to broadly ellipsoid or somewhat reniform, (5–)10–16(–10) × 5–8(–9.5) µm
Chlamydospores	Present	NA
Zygospores	Not observed	Globose to subglobose, (42–)54(–80) × (40–)52(–70) µm

[a]From the description by Kirk [6].
NA: not available.

Description: The strain grew rapidly at 20 °C on PDA, reaching 62–65 mm in diameter after 5 days incubation, and were white with abundant aerial hyphae. The reverse colony was white and slightly zonate. The colonies exhibited slower growth on PDA and WA than on OA. On OA, mycelial development was better than that on PDA and WA. However, a smaller number of sporangia were produced on OA. On WA, although the mycelial growth was sparse, the sporulation was excellent. Sporangiophores developed from aerial hyphae and were 2.5–4.5 μm at the tip and simple or branched. Sporangia were globose, with multi-spores and a collar after deliquescence. Sporangia were globose and measured 19.5–34.5 × 18.4–32.5 μm. Sporangiospores were ellipsoidal to short cylindrical, reniform, and measured 8.5–15.7 × 5.2–8.3 μm. Chlamydospore formation was

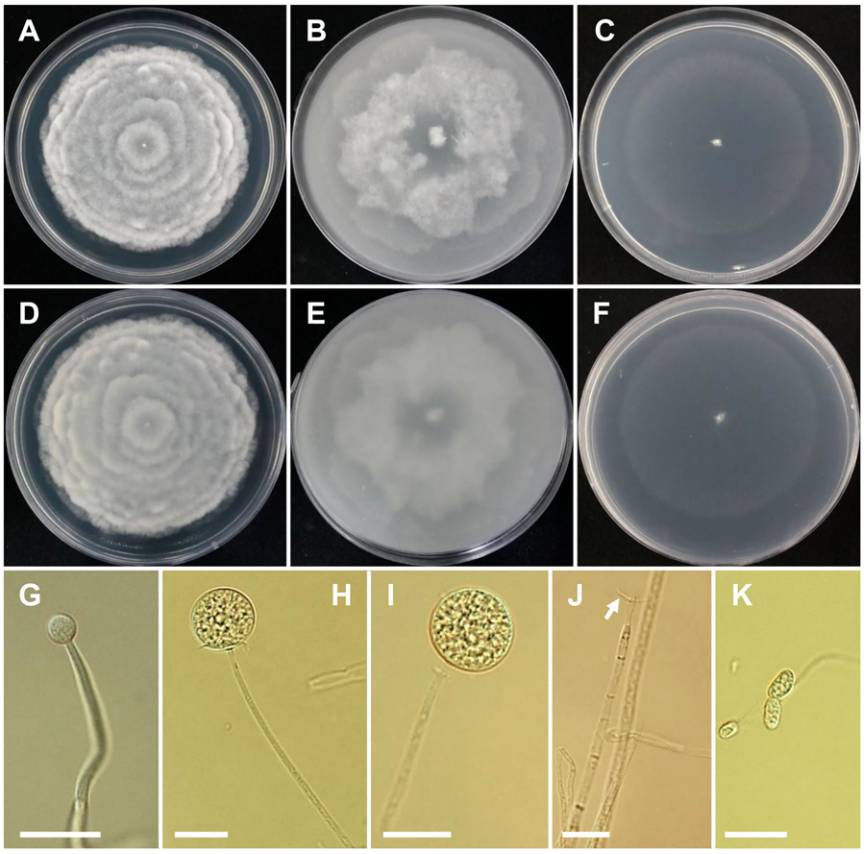

Figure 3. Morphology of *Mortierella elongata* CNUFC-YR329-1. A, D colony on potato dextrose agar; B, E colony on oatmeal agar; C, F colony on water agar; (A–C: observed view, D–F: reverse view). G–I young sporangia on sporangiophores; J the collar at the top of the sporangiophore (white arrow). K sporangiosphores (scale bars: G–K = 20 μm).

Table 3. Morphological characteristics of CNUFC-PTS103-1 and the reference, *Mortierella horticola*.

Characteristic	CNUFC-PTS103-1	*Mortierella horticola*[a]
Colony	Grew rapidly at 20 °C on PDA, cotton white, later turning to slightly beige, and white mixed with slightly yellow on the reverse colony	NA
Sporangiophores	Unbranched, 3.9–5.3 μm in width, variable in length	Always unbranched
Sporangia	Nearly globose, multi-spored, 12.0–20.0 × 12.0–19.0 μm	Always one-spored, globose, finely echinulate, minutely spinulose, 7–12 μm in diameter
Sporangiospores	Globose, smoothed, 4.9–5.4 × 4.9–5.8 μm	NA
Chlamydospores	Present	NA
Zygospores	Not observed	NA

[a]From the description by Linnem [7].
NA: not available.

abundant on the medium. Zygospores were not observed.

3.2.2 Taxonomy of CNUFC-PTS103-1

Mortierella horticola Linnem., Pflanzenforschung 23: 21 (1941) [MB#301329] (Table 3, Figure 4).

Description: The strain grew rapidly at 20 °C on PDA, attaining a diameter of 81 mm after 7 days of incubation. The colony color was initially cotton white and later became slightly beige with a similar flower shape. The reverse colony was also white mixed with slightly yellow and was irregularly zonate. Typical sporangia and sporangiospores were not observed although the PDA medium showed good mycelial growth. Sporangiophores were always unbranched. Sporangia were normally produced on OA after 7 days at 20 °C. Sporangia were nearly globose and measured 12.0–20.0 × 12.0–19.0 μm. Sporangiospores were globose, smooth, and measured 4.9–5.4 × 4.9–5.8 μm. Zygospores were not observed.

3.2.3 Taxonomy of CNUFC-PTS2-1

Mortierella humilis Linnem. ex W. Gams, Beitrag zu einer Flora der Mucorineae Marburgs, Diss. (1963) [MB#317898] (Table 4, Figure 5).

≡*Mortierella humilis* Linnem., Flora (Regensburg) 130: 209 (1936).

Description: The strain grew rapidly at 20 °C on PDA, reaching a diameter of 85 mm after 7 days of incubation and was cottony in the center with a white

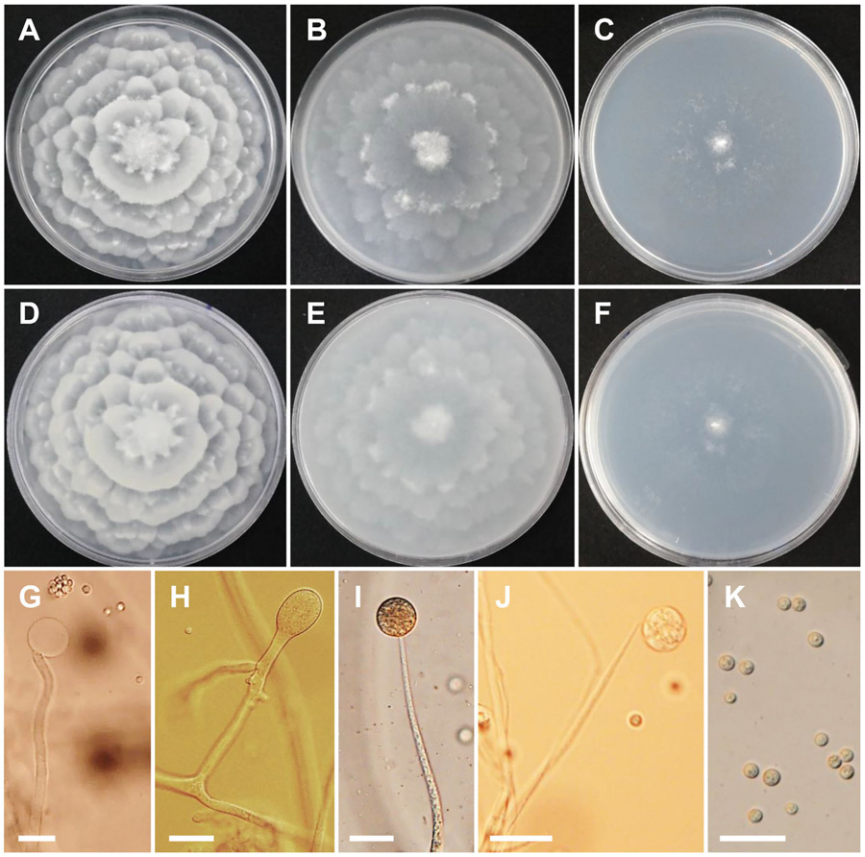

Figure 4. Morphology of *Mortierella horticola* CNUFC-PTS103-1. A, D colony on potato dextrose agar; B, E colony on oatmeal agar; C, F colony on water agar; (A–C: observed view, D–F: reverse view); G, H sporangiophores with different shapes of sporangia; I, J young and mature sporangia; K sporangiospores (scale bars: G–K = 20 μm).

Table 4. Morphological characteristics of CNUFC-PTS2-1 and the reference species, *Mortierella humilis*.

Characteristics	CNUFC-PTS2-1	*Mortierella humilis*[a]
Colony	Grew rapidly at 20 °C on PDA, cotton in the center with a white margin; reverse colony white and irregularly zonate	NA
Sporangiophores	Branched, 63.2 μm long	Branched, 50–200 μm long
Sporangia	Globose, finely spinulose, always one-spored, 8.2–13.1 × 7.9–12.1 μm	1-spored, 6–15 μm in diameter
Chlamydospores	Absent	Absent
Zygospores	Not observed	Globose to subglobose, (34–)46(–62) μm in diameter

[a]From the description by Chien et al. [11].
NA: not available.

margin. The reverse colony was white and irregularly zonate. Growth was rapid on MEA, producing a rosette colony and abundant aerial hyphae reaching 85 mm diameter after 7 days of incubation at 20 °C. For colonies grown on WA, aerial hyphae were dispersed on the agar surface reaching a diameter of 82 mm at 20 °C after 7 days of incubation. Sporangiophores were simple or branched, with a length of 63.2 μm. Sporangia were globose, finely spinulose, always single-spored, and measured 8.2–13.1 × 7.9–12.1 μm. Zygospores were not observed.

4. Discussion

Until now, few studies have reported new and undescribed species belonging to the order Mortierelles in Korea [17–19]. Finding of *M. elongata*, *M. horticola*, and *M. humilis* improves our knowledge regarding the occurrence and distribution of zygomycete species within the genus *Mortierella* known as an undiscovered taxon in Korea.

In maximum likelihood, phylogenetic tree using ITS and LSU regions, the two strains CNUFC-YR329-1 and CNUFC-YR329-2 were placed in group 7 "*gamsii*", along with some species from "*elongata*" and *M. fluviae*, *M. camargensis*, *M. sclerotiell*, *M. zychae*, and *M. exigua* as defined by Wagner et al. [5] and Hyde et al. [10]. CNUFC-YR329-1 was morphologically most similar to *M. elongata* described by Kirk [6]. However, the sporangiophores were slender (1.5–3.5 μm, according to

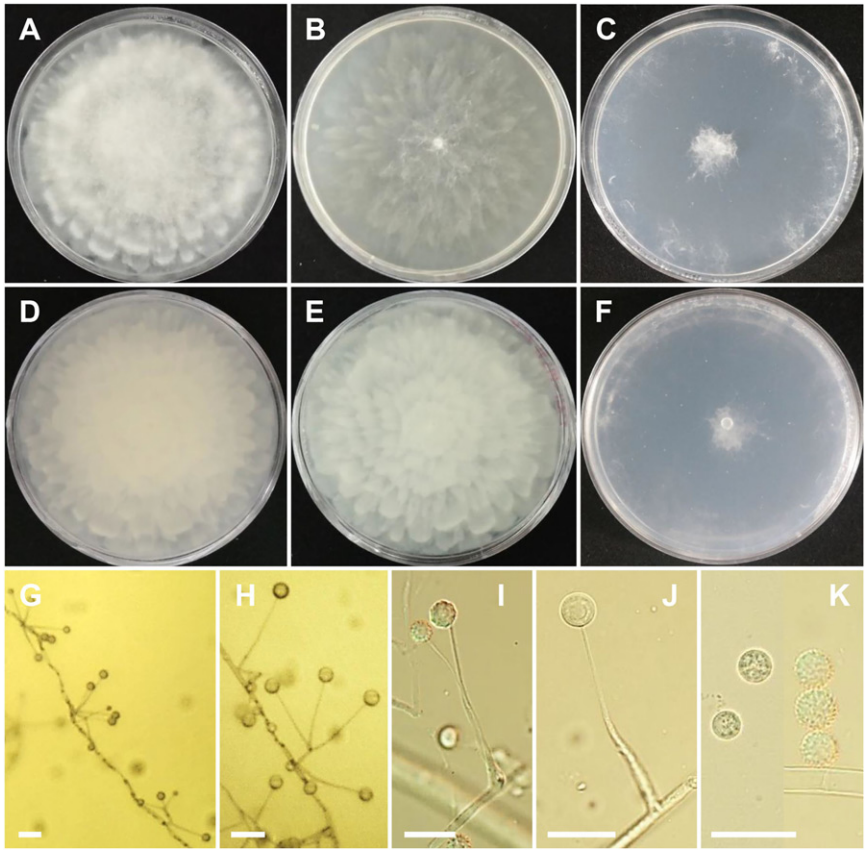

Figure 5. Morphology of *Mortierella humilis* CNUFC-PTS2-1. A, D colony on potato dextrose; B, E colony on malt extract agar; C, F colony on water agar; (A–C: observed view, D–F: reverse view); G, H sporangiophore showing different branches and sporangia (observed under stereo-microscope); I, J sporangiophores bearing single-spored sporangia (observed under light microscope). K detail of sporangia with a finely spinulose membrane (scale bars: G–K = 20 μm).

Kirk [6]) than those (2.5–4.5 μm) observed in our isolate.

M. elongata has been isolated from different niches including soil samples [5,27–29], a black fly from Quebec, Canada [5], an arsenic mine in Poland [30], *Spagnum fiscum* in Canada [31], as keratinophilic fungi from deer horn [32], and as a bacterial endosymbiont [33]. To the best of our knowledge, this is the first isolation of *M. elongata* from freshwater. *M. elongata* reportedly produces omega-6, omega-3, docosahexaenoic acid, arachidonic acid, palmitic acid, oleic acid, linoleic acid, and α-linolenic acid [34–37]. *M. elongata* also efficiently flocculates with the marine alga *Nannochloropsis oceanica*, which abundantly produces triacylglycerol, which can increase oil productivity [38]. Inoculation of *M. elongata* into soil significantly improves soil phosphatase and β-glucosidase activities and also increases the levels of plant indole acetic acid and plant biomass [29].

The phylogenetic analyses of ITS and LSU sequences showed that CNUFC-PTS103-1, CNUFC-PTS103-2, CNUFC-PTS2-1, and CNUFC-PTS2-2 belongs to group 2 "*verticillata-humillis*" as defined by Wagner et al. [5]. CNUFC-PTS103-1 displayed similar morphological characteristics with *M. horticola* described by Linnem [6], but is in contradiction to having multi-spored sporangia resembling *M. epicladia*. So, there is a possibility that *M. horticola* produces single-spored as well as multi-spored sporangia. *M. horticola* and *M. epicladia* were easily distinguishable in ITS tree, suggesting that the number of spores per sporangium is of no taxonomic relevance due to the lack of fixed spores in this group. *M. horticola* would have been expected to have evolved multi-spored sporangia as an adaptation to the environmental factors. Additional information regarding suitable morphological criteria and molecular markers is necessary for the correct validation of *M. horticola* [5].

M. horticola has been isolated from peatland soils [27], from *Spagnum fiscum* in Canada [31], from a sacred grove and disturbed forest in Northeast India [39], from washed root surfaces [40], as an endophyte [41], from the rhizosphere of *Meyna spinosa* Roxb [42], and from wheat field soil and agricultural soil from Germany and The Netherlands [5]. The higher amount of docosahexaenoic acid and production of omega-6 and omega-3 fatty acid was also detected in *M. horticola* [37].

The morphological characteristics of CNUFC-PTS2-1 agree well with the description of *M. humilis* by Chien et al. [11]. Molecular data revealed that

our isolates clustered within one clade with the type species, *M. humilis* CBS 222.35.

M. humilis has been isolated from *Sphagnum fuscum* [40], various soils and roots of herbaceous plants [43], forest soil from North Carolina, a *Pinus* forest in Mexico, stump bark from South Carolina, soil from The Netherlands, forest soil from China [5], Norway spruce stands on sod-podzolic soil [44], pea rhizosphere soil [45], and on heavily decayed wood [46]. *M. humilis* is reported to produce useful fatty acids, including arachidonic acid used in medicine, pharmacology, cosmetics, agriculture, and in food industry [36,47], eicosapentaenoic acid [48], and enzymes capable of degrading xylans, sugars (sucrose, galactose, fructose, mannose, maltose), paraffin, and chitin [31]. *M. humilis* can also degrade cellulose and lignin, which makes plants remnants readily available to other members of the ecological system, which enhances biological activity [49,50].

In comparison to *M. elongata*, *M. horticola,* and *M. humilis* have been a less-studied species. Further studies, including multi-gene analyses and observations of ultrastructure of uni- or multi-spores, are needed to unravel the phylogenetic relationship of the related *Mortierella* species. In addition, three species obtained from this study may potentially be highly valuable. Thus, the potential biological activities of *M. elongata*, *M. horticola*, and *M. humilis* should be further studied.

Disclosure statement

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