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

Thuong T. T. Nguyen, Se Won Park, Monmi Pangging and Hyang Burm Lee

Division of Food Technology, Biotechnology and Agrochemistry, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, Korea

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.

1. Introduction

The genus Mortierella was described by Coemans (1863) with the type species Mortierella polypephala Coem [1]. Currently, Mortierella is a member of the family Mortierellaceae, order Mortierellales, subphyllum 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-humilis”, “ligicola”, “mutabilis, globulifera and angusta”, “strangulate and wolfii”, “alpina and polypephala”, and “gamsit”.

To date, six species of Mortierella have been reported in Korea: M. alpina, M. ambiguca, M. indohii, M. minutissima, M. oligospora, and M. zychae [17–19]. M. elongata isolated from the root of Phragmites 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
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, 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).

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
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-PTS103-1, and 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).
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. Sporangioles developed from aerial hyphae and were 2.5–4.5 μm wide at the tip, variable in length, mostly branched. Sporangia were globose, 19.5–34.5 × 18.4–32.5 μm, with a small collarette. Sporangiospores were ellipsoidal to short cylindrical, reniform, and measured 8.5–15.7 × 5.2–8.3 μm. Chlamydospore formation was NA.

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

| Characteristics       | CNUFC-YR329-1                                      | Mortierella elongata* |
|-----------------------|----------------------------------------------------|-----------------------|
| Colony                | Grew rapidly at 20°C on PDA, white; reverse colony  | White, arachnoid to cottony |
|                       | white and slightly zonate.                         |                       |
| Sporangioles          | 2.5–4.5 μm wide at the tip, variable in length,   | Up to 250 (~400) μm long, branched, 5–8 (~12) μm wide       |
|                       | mostly branched                                    | at the base, 1.5–3.5 μm at the tip                           |
| Sporangia             | Globose, multi-spores, 19.5–34.5 × 18.4–32.5 μm,  | Globose, (15–)20–30 μm diameter, with a small collarette    |
|                       | with a small collarette                            |                       |
| Sporangiospores       | Ellipsoidal to short cylindrical, reniform,       | Ellipsoidal to broadly ellipsoid or somewhat reniform,      |
|                       | 8.5–15.7 × 5.2–8.3 μm                              | (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       |

*From the description by Kirk [6].

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. Sporangiohophores 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

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

| Characteristic | CNUFC-PTS103-1 | Mortierella horticola* |
|----------------|----------------|------------------------|
| 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 |
| Chlamydomspores | Present | NA |
| Zygospores     | Not observed | NA |

*From the description by Linnem [7].

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. sclerotii, 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

| Characteristics       | CNUFC-PTS2-1       | Mortierella humilis       |
|-----------------------|--------------------|---------------------------|
| 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 |

*From the description by Chien et al. [11].

NA: not available.
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 fuscum 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 fuscum 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**

No potential conflict of interest was reported by the authors.

**Funding**

This work was in part supported by the Graduate Program for the Undiscovered Taxa of Korea, and in part by the Project on Survey and Discovery of Indigenous Fungal Species of Korea funded by NIBR and Project on Discovery of Fungi from Freshwater and Collection of Fungarium funded by NNIBR of the Ministry of Environment (MOE), and in part carried out with the support of Cooperative Research Program for Agriculture Science and Technology Development [PJ013744], Rural Development Administration, Republic of Korea. This work was in part supported by the BK21 plus program through the National Research Foundation (NRF) funded by the Ministry of Education of Korea.

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