Members of the genus Mortierella of the order Mortierellales within the family Mortierellaceae are filamentous fungi commonly found in soil. The genus contains a large number (nearly 100) of validated species that can be found on almost any substrate and are often encountered in soils as saprophytes [1]. Mortierella polycephala was the first species of the genus described by Coemans [2]. Major members of the genus include Mortierella alliacea, Mortierella alpina, M. polycephala, Mortierella elongata, Mortierella spinosa, Mortierella gamsii, Mortierella isabellina, Mortierella humilis, and Mortierella reticulata.

Many filamentous fungi belonging to the genus Mortierella are promising candidates for use as producers of arachidonic acid and other polyunsaturated fatty acids (PUFAs) [3, 4]. PUFAs, particularly linoleic acid (18:2, n-6), α-linolenic acid (18:3, n-3), and arachidonic acid (20:4, n-6), are essential fatty acids vital for various mammalian biological functions [5].

Three new species belonging to Mortierella were isolated from soils collected in Gangwon-do, Chungcheongbuk-do, and Gyeonggi-do during a study of the fungal community in crop field soils. This study represents the first report of these species in Korea. The aim of this study was to compare the morphological and phylogenetic characteristics of the new species with previously described Mortierella spp. We also investigated the potential of the newly recorded species to produce arachidonic acid.

### MATERIALS AND METHODS

**Soil sampling and isolation of fungi.** Soil samples were collected in 2014 from agricultural fields at various locations in Gangwon-do (36°54′06.10″ N, 127°28′08.20″ E), Chungcheongbuk-do (36°46′04.61″ N, 127°30′00.52″ E), and Gyeonggi-do (37°25′45.60″ N, 127°08′14.15″ W), Korea. Each soil sample was collected from a depth of approximately 0–15 cm after removing the surface litter, sealed in a sterile soil sampling polythene bag, air dried, and stored in a plastic bag at 4°C until use. The fungi were isolated using the conventional dilution technique [6] and cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) supplemented with 100 μg/L chloramphenicol (a bacteriostatic agent) for 5–7 days at 28°C until fungal colony

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**Keywords**  
Arachidonic acid, Mortierella ambigua, Mortierella indohii, Mortierella zychae, Triphenyltetrazolium chloride
growth was observed. The pure cultures were maintained on the PDA slants at 4°C for future use.

Morphological characterization. Morphological characteristics of isolates KNU14-5, KNU14-14, and KNU14-17 were observed on PDA, malt extract agar (MEA; 30 g/L malt extract, 5 g/L mycological peptone, and 15 g/L agar), and MEA with 0.1% triphenyltetrazolium chloride (TTC) after inoculation in 9-cm petri dishes and incubation at 28°C for 5 days. Photomicrographs were taken with an HK 3.1 CMOS digital camera (KOPTIC Korea Optics, Seoul, Korea) attached to an Olympus BX50F-3 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and a 1450VP scanning electron microscope (Leo Electron Microscopy Ltd., Cambridge, UK).

Genomic DNA extraction, sequencing, and data analysis. Total genomic DNA was extracted from isolates KNU14-5, KNU14-14, and KNU14-17 using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The internal transcribed spacer regions (ITS1 and ITS2), including the 5.8S were amplified with the primers ITS1 and ITS4 [7]. Polymerase chain reaction (PCR) for amplifying the large subunit (LSU) 28S rDNA was done using the standard primers LR0R and LR5 [8]. The amplified PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. The PCR products were sequenced with the ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were compared with reference ITS and LSU rDNA sequences of Genbank at National Center for Biotechnology Information (NCBI) using the basic local alignment search tool [9]. The nucleotide sequences were deposited in GenBank and assigned accession numbers KP966613, KP966612, and KP055598 for isolates KNU14-5, KNU14-14, and KNU14-17, respectively. The sequences of closely related strains were aligned using the MultAlin program. Phylogenetic relationships were analyzed using molecular evolutionary genetic analysis (MEGA 6) software [10]. The neighbor-joining tree was constructed using the Kimura 2-parameter substitution model [11]. The phylogeny of the tree was inferred using the maximum-likelihood heuristic search option with the nearest-neighbor interchange. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

Qualitative analysis of arachidonic acid production. Arachidonic acid production of the isolates was assessed by TTC staining. Fresh mycelia were harvested by suction filtration and staining was performed according to the procedure described by Zhu et al. [12] with a slight modification; 1 mL of 0.6% TTC solution in 0.5 M phosphate buffer (pH 7.8) was added to 0.1 g of fresh mycelia in a cap tube and incubated in the dark for 1 hr at 28°C. Mycelia were then rinsed twice with sterile water and homogenized by grinding with a mortar pestle. The red triphenylformazan (TF) formed in mycelia was extracted three times with 2 mL of ethyl acetate at room temperature using fresh solvent for each extraction. The degree of staining was quantified by measuring the absorbance of TF in the ethyl acetate solvent at a wavelength of 485 nm.

RESULTS AND DISCUSSION

Molecular phylogeny of the isolate KNU14-5. KNU14-5 was isolated from crop field soil in Gangwon-do, Korea in July 2014. ITS rDNA and 28S rDNA sequences were compared to determine the phylogenetic relationship between the isolate KNU14-5 and previously described Mortierella species. The isolate was most closely related to M. zyhae CBS316.52 and formed a monophyletic group with bootstrap value of 96% (Fig. 1). The phylogenetic analysis showed that the isolate is M. zyhae. A synonym for this species is Mortierella brachyrhiza, E. Wolf (1954). This is a common species found especially in soil as saprophytes, but this is the first report of the isolation of M. zyhae in Korea.

Morphological characterization of the isolate KNU14-5. Photomicrographs of morphological structures of the isolate KNU14-5 are shown in Fig. 2. Colonies grew moderately fast on PDA, attaining a diameter of 76–79 mm in 5 days at 28°C. The front side of the colony was whitish and often dark whitish at the center. The reverse side of the colony was yellowish white and narrowly zonate. Extensive aerial mycelium production occurred at the center of the colony and numerous irregular bead-like hyphal swellings were formed. The colonies were also fast-growing on MEA, reaching a diameter of 77–80 mm in 5 days at 28°C. These colonies were a pale, whitish color and broadly zonate, producing a concentric pattern. Sporulation was abundant and the mycelium had a garlic-like odor. The sporangiophores were hyaline, erect, and simple. They were 190–1,000 μm long and tapered towards the apex. They were 8–12 μm wide at the base and 2–4 μm at the apex, bearing terminal sporangia. The sporangia were spherical, 15–35 μm in diameter, wrinkled, and non-columellate. The sporangiospores were hyaline, ellipsoidal, single-celled, and 6–10 μm × 4–6 μm in size. The chlamydospores were globose, catenate, clustered, and 15–19 μm in diameter.

Conclusions regarding the isolate KNU14-5. On the basis of the aforementioned taxonomical properties, the isolate was concluded to be a fungus belonging to the genus Mortierella [13-16] and the species zyhae [2, 15]. Typical swollen hyphae and oil droplets containing hyphae are characteristic features of M. zyhae and M. alpina, respectively. These fungi also contain ellipsoidal sporangiospores [16]. These characteristic structures were also observed in our study, clearly demonstrating that the KNU14-5 isolate is M. zyhae.
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Molecular phylogeny of the isolate KNU14-14. KNU14-14 was isolated from crop field soil in Chungcheongbuk-do, Korea in July 2014. ITS rDNA and 28S rDNA sequences were compared to determine the phylogenetic relationship between the isolate KNU14-14 and previously described Mortierella species. The isolate was most closely related to M. ambigua CBS 373.96 and formed a monophyletic group with bootstrap value of 100% (Fig. 1). This species is commonly found in soil as saprophytes, but this is the first report of the isolation of M. ambigua in Korea.

Morphological characterization of the isolate KNU14-14. Photomicrographs of the morphological structures of the isolate KNU14-14 are shown in Fig. 3. Colonies grew moderately fast on PDA, reaching a diameter of 64–68 mm in 5 days at 28°C. The front and reverse sides of the colony were milky white in color, cottony, and non-lobed. Aerial mycelium formed irregular bead-like hyphal swellings and sporulation was scanty. Colonies also grew fast on MEA, reaching a diameter of 65–73 mm in 5 days at 28°C. The colonies were a dirty whitish color, broadly zonate producing a concentric pattern, and lobed at the margin.
Fig. 2. A colony of Mortierella zychae KNU14-5 grown for 5 days on potato dextrose agar at 28°C (A) and on malt extract agar at 28°C (B). Mycelia (C), hypha containing oil droplets (D), hyphal swellings (E), mature chlamydospore (F), scanning electron microscope (SEM) of clustered chlamydomspores (G), sporangiophore with terminal sporangia under a compound microscope and SEM (H, I), sporangiospores (J) (scale bars: C–J = 10 µm).

Fig. 3. A colony of Mortierella ambigua KNU14-14 grown for 5 days on potato dextrose agar at 28°C (A) and on malt extract agar at 28°C (B). Hyphal swelling (C), chlamydomspore (D), sporangiophore with terminal sporangia under a compound microscope and scanning electron microscope (E, F), sporangiospores (G) (scale bars: C–G = 10 µm).
Sporulation was abundant. The sporangiophores were hyaline, erect, and simple. They were 25–450 μm long, tapered towards the apex. They were 7–25 μm wide at the base and 2–5 μm wide at the apex, bearing single terminal sporangia. The main sporangiophore frequently formed vesicles without sporangia. The sporangia were spherical, 3–35 μm in diameter, wrinkled, and non-columellate. The sporangiospores were globose to sub-globose, single-celled, and 2.5–5.5 μm in diameter. The chlamydospores were brown, globose, thick-walled, and 19–30 μm in diameter.

Conclusions regarding the isolate KNU14-14. The isolate KNU14-14 was assumed to belong to the genus Mortierella based on the morphologies of its sporangiophores and sporangia and its gross colony appearance. The isolate reasonably fits the description of M. ambiguа, which was originally isolated from garden soil [17]. The isolate KNU14-14 from crop field soil only slightly differs from the original description in its sizes of sporangiophores, sporangia, and sporangiospores. It has been reported that the asexual fruiting habit of Mortierella species may change and that one or more stages may not appear depending upon the culture conditions [18-20]. Thus, it can be concluded that no other described Mortierella species matches the studied isolate.

Molecular phylogeny of the isolate KNU14-17. KNU14-17 was isolated from crop field soil in Gyeonggi-do, Korea in July 2014. ITS rDNA and 28S rDNA sequences were compared to determine the phylogenetic relationship between the isolate KNU14-17 and previously described Mortierella species. The isolate was most closely related to the type strain CBS 720.71 of M. indohii and formed a monophyletic group with bootstrap value of 92% (Fig. 1). This is a common species found in soil, but this is the first report of its occurrence in Korea.

Morphological characterization of the isolate KNU14-17. Photomicrographs of morphological structures of the isolate KNU14-17 are shown in Fig. 4. Colonies grew fast on PDA, reaching a diameter of 70–73 mm in 5 days at 28°C. The colonies were snow-white in color and broadly zonate with scanty aerial mycelium. The colonies also grew rapidly on MEA, reaching a diameter of 75–79 mm in 5 days at 28°C. The reverse side of the colony was yellowish white. The front side of the colony was snow-white in color and narrowly zonate. The surface of the colony appeared mealy because of the large number of stylospores. Sporulation was abundant. Sporangiophores were absent. Stylospore-bearing stalks were erect, delicate, unbranched, and sometimes swelled at the tip. The stalks were 50–160 μm long and 1.5–2.5 μm wide. The stylospores were almost spherical,

Fig. 4. A colony of Mortierella indohii KNU14-17 grown for 5 days on potato dextrose agar at 28°C (A) and on malt extract agar at 28°C (B). Stalks bearing stylospores under a compound microscope and scanning electron microscope (C, D), a stalk with a stylospore (E, arrow indicates swollen tip of the stalk), stylospore (F) (scale bars: C, D = 10 μm, E = 2 μm, F = 20 μm).
12–18 μm in diameter, thick-walled, and covered with delicate spines.

Conclusions regarding the isolate KNU14-17. The isolate KNU14-17 is *M. indohii* based on its gross colony appearance and stylospores. The stylospores of the isolate KNU14-17 most closely resemble those of *M. polycephala*, which are commonly both aerial and submerged. The stylospores of this isolate differ from those of *M. polycephala* in that they are more perfectly globose and slightly larger. The isolate reasonably fits Chien's (1974) diagnosis [20] of *M. indohii*, which was originally isolated from the dung of ground hog in Memorial Park, Athens, Georgia. Stylospores are the major characteristics of *M. indohii*, a species with a close phylogenetic relationship to *Mortierella polygonia* and *Mortierella hypsicladia* [16]. Thus, our findings are in accordance with the findings of Wagner *et al.* [16].

Table 1. Triphenyltetrazolium chloride (TTC) staining values of fungal isolates

| Isolates                          | Staining value (A485 nm) |
|-----------------------------------|--------------------------|
| *Mortierella zychae* (KNU14-5)    | 2.012 ± 0.176            |
| *Mortierella ambigua* (KNU14-14) | 1.728 ± 0.285            |
| *Mortierella indohii* (KNU14-17) | 0.812 ± 0.236            |

Mean values are given of triplicate samples (± SE). Values with the different alphabetic superscripts are significantly different at $p \leq 0.05$ levels according to Duncan’s multiple range test.

Arachidonic acid production of isolates KNU14-5, KNU14-14, and KNU14-17. The isolates KNU14-5, KNU14-14, and KNU14-17 were stained with TTC to evaluate their arachidonic acid production potential. The results are presented in Table 1 and Fig. 5. The degree of staining was the highest in *M. zychae* (KNU14-5), with a
staining value of 2.012, followed by that in *M. ambigua* (KNU14-14) and *M. indohii* (KNU14-17) with staining values of 1.728 and 0.812, respectively. TTC reduction is commonly used as a biochemical marker for the viability of plant cells and tissues and as a measurement of bacterial respiratory activity [21, 22]. It is generally thought that TTC is reduced by dehydrogenases and absorbed by living cells [22] where it reacts with hydrogen atoms released by the dehydrogenase enzymes during cellular respiration [21]. There is a positive correlation ($r = 0.982$) between the degree of TTC staining and arachidonic acid content in mycelia lipids and fungi that did not produce arachidonic acid could not be stained [10, 24]. The results of TTC staining (Table 1) clearly revealed that the three newly recorded *Mortierella* species, especially *M. zychae* (KNU14-5) and *M. ambigua* (KNU14-14), could be potential producers of arachidonic acid. However, further studies on the yield of arachidonic acid by the present isolates under different culture conditions are needed.

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**REFERENCES**

1. Kirk PM, Cannon PF, Minter DW, Stalpers JA. Ainsworth and Bisby's dictionary of the fungi. 10th ed. Wallingford: CAB International; 2008.
2. Coemans E. Quelques hyphomycetes nouveaux. 1. Mortierella polyccephala et Mortiensella pectinata. Bull Acad R Sci Belg Cl Sci 2 1863;15:535-44.
3. Kendrick A, Ratledge C. Lipids of selected molds grown for production of n-3 and n-6 polyunsaturated fatty acids. Lipids 1992;27:15-20.
4. Botha A, Paul I, Roux C, Kock JL, Coetsee, DJ, Strauss T, Maree C. An isolation procedure for arachidonic acid producing *Mortierella* species. Antonie Van Leeuwenhoek 1999;75:253-6.
5. Merendino N, Costantini L, Manzi L, Molinari R, D’Eliseo D, Velotti F. Dietary ω-3 polyunsaturated fatty acid DHA: a potential adjuvant in the treatment of cancer. Biomed Res Int 2013;2013:310186.
6. Davet P, Rouxel F. Detection and isolation of soil fungi. Enfield: Science Publishers; 2000.
7. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. Academic press, Inc., New York. Academic Press; 1990. p. 315-22.
8. Vilgalys Lab, Duke University. Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA [Internet]. Durham: Duke University; 2002 [cited 2015 May 5]. Available from: http://wwwbiology.duke.edu/fungi/mycolab/primers.htm.
9. National Center for Biotechnology Information. Genbank overview [Internet]. Bethesda (MD): National Center for Biotechnology Information; 2015 [cited 2015 May 5]. Available from: http://www.ncbi.nlm.nih.gov/Blast.
10. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725-9.
11. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111-20.
12. Zhu M, Yu LJ, Liu Z, Xu HB. Isolating Mortierella alpina strains of high yield of arachidonic acid. Lett Appl Microbiol 2004;39:332-5.
13. Von Arx JA. The genera of fungi sporulating in pure culture. 3rd ed. Vaduz: J. Cramer; 1981.
14. Domsch KH, Gams W, Anderson TH. Compendium of soil fungi. 2nd ed. New York: Academic Press; 1980.
15. Zycha H, Siebmann R. Mucorales. Eine Beschreibung aller Gattungen und Arten dieser Pilzgruppen. Lehre: Verlag von J. Cramer; 1969.
16. Wagner L, Stielow B, Hoffmann K, Petkovits T, Papp T, Vágvölgyi C, De Hoog GS, Verkley G, Voigt K. A comprehensive molecular phylogeny of the Mortierellales (Mortierellomycotina) based on nuclear ribosomal DNA. Persoonia 2013;30:77-93.
17. Mehrotra BS, Baijal U, Mehrotra BR. Two new species of *Mortierella* from India. Mycologia 1963;55:289-96.
18. Van Tieghem P. Nouvelles recherches sur les Mucorinées. Ann Sci Nat Bot Ser 6 1875;1:5-175.
19. Van Tieghem P. Troisiéme mémoire sur les Mucorinées. Ann Sci Nat Bot Ser 6 1878;4:312-99.
20. Chien CY, Kuhlman EG, Gams W. Zygospores in two Mortierella species with "stylospores". Mycologia 1974;66:114-21.
21. Palta JP, Levitt J, Stadelmann EJ. Plant viability assay. Cryobiology 1978;15:249-55.
22. Ruklisha M, Paegle L. Metabolic fluxes and L-lysine synthesis by *Corynebacterium glutamicum* in relation to cellular total reducing activity. Process Biochem 2001;36:1233-40.
23. Musser DA, Oseroff AR. The use of tetrazolium salts to determine sites of damage to the mitochondrial electron transport chain in intact cells following *in vitro* photodynamic therapy with Photofrin II. Photochem Photobiol 1994;59:621-6.
24. Vadivelan G, Venkateswaran G. Production and enhancement of omega-3 fatty acid from *Mortierella alpina* CFR-GV15: its food and therapeutic application. Biomed Res Int 2014;2014:657414.