Phylogenetic Status of Two Undescribed Zygomycete Species from Korea: *Actinomucor elegans* and *Mucor minutus*

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Abstract During a survey of fungal diversity of the order Mucorales, three zygomycete isolates, CNUFC-YR113-1, CNUFC-KNU16-7, and CNUFC-BS1-1 were isolated from freshwater and soil samples in Korea. The strains were analyzed both morphologically and phylogenetically based on internal transcribed spacer and 28S rDNA gene sequences. Based on their morphology and phylogeny, the CNUFC-YR113-1 and CNUFC-KNU16-7 isolates were identified as *Actinomucor elegans*, and CNUFC-BS1-1 was identified as *Mucor minutus*. To the best of our knowledge, the species *A. elegans* and *M. minutus*, belonging to an undiscovered taxon, have not been previously described in Korea.

Keywords  *Actinomucor elegans*, *Mucor minutus*, Undiscovered taxa, Zygomycete fungi

*Actinomucor* and *Mucor* belong to the subphylum Mucoromycotina, order Mucorales, family Mucoraceae [1]. The genus *Actinomucor* was originally described in 1898 by Schostakowitsch [2]. Although the genus is closely related to *Mucor*, it differs in having branched stolons that give rise to rhizoids and sporangiophores. It is also distinct from the other two genera *Rhizopus* and *Absidia* in its arrangement of the columellae and sporangiophores. The genus originally contained two species, *A. elegans* (Eidam) C. R. Benj. & Hesselt., and *A. taiwanensis* S. C. Jong & G. F. Yuan [3, 4]. *A. taiwanensis* was differentiated from *A. elegans* by its larger sporangiospore size and by their differing maximum growth temperatures: 37°C for *A. taiwanensis* and 32°C for *A. elegans*. Later, Zheng and Liu [5] renamed *A. taiwanensis* to *A. elegans* var. *meitauzae* based on morphological characteristics and molecular analyses. Recently, Khan et al. [6] proposed the addition of a new variety, *A. elegans* var. *kuwaitensis*. In Index Fungorum (2017; http://www.indexfungorum.org), the genus *Actinomucor* contains only one species named *Actinomucor elegans*.

*Actinomucor* species are found in dung, soil, food, and human sources [5-7]. Some of them are commonly used for producing popular fermented soybean foods including Sufu and Chao [8]. In addition, *A. elegans* is also considered a good source of glycine aminopeptidase and glucosamine [9, 10]. *A. elegans* var. *elegans* has been reported as a potential biocontrol agent against the chafer beetle [11].

*Mucor* Fresen. (Mucoraceae, Mucorales) is characterized by the formation of non-apophysate sporangia, producing simple or branched sporangiophores without basal rhizoids. Zygospores have opposed, non-appendaged suspensors [12]. *Mucor* species have frequently been detected on substrates that support the growth of a fungal host, such as in soil, dung, fruit, and plants [13-15]. Several species are able to produce enzymes with biotechnological applications [16, 17], while some species are considered the causal agent of cutaneous zygomycosis in humans [18]. Although there are more than 300 named species described in the literature, only approximately 50 are known and described [15].
Mucor

(shape of sporangia as well as the mode of reproduction based on morphological characteristics such as size and regions of several mucoralean species, Walther transcribed spacer (ITS) and large subunit (LSU) rDNA observed that some Mucor species with curved sporangiophores were grouped with Backusella Hsett. & J. J. Ellis. Therefore, these Mucor species were transferred to Backusella.

In Korea, two new Mucor species have been currently reported by authors: Mucor koreanus from tangerine fruit [14] and Mucor stercorearius from rat feces [22]. Only seven species have been recorded: M. circinelloides, M. hiemalis, M. mucedo, M. piriformis, M. racemosus, M. fragilis, and M. irregularis [15, 23]. To our knowledge, there are no specific published literature records of these species in Korea.

The objective of the present study was to perform morphological and molecular analyses to characterize two unrecorded zygomycete species in Korea: Actinomucor elegans and Mucor minutus.

MATERIALS AND METHODS

Fungal strain isolation from freshwater and soil samples. Freshwater samples were collected from the Yeongsan River located in Gwangju, Korea. Soil samples were collected from the garden of the Chonnam National University located in Gwangju and a field in Gyeongnam, Korea. These samples were transported in sterile 50-mL Falcon tubes, and stored at 4°C. Freshwater samples were collected from the garden of the Chonnam National University located in Gwangju and a field in Gyeongnam, Korea. Soil samples were collected from the garden of the Chonnam National University located in Gwangju and a field in Gyeongnam, Korea.

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Two New Records of Zygomycete Species in Korea

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DNA extraction, PCR, and sequencing. Genomic DNA was extracted directly from the mycelia of fungal isolates, using the Solgent Genomic DNA prep Kit (Solgent Co. Ltd., Daejeon, Korea). The ITS region and large subunit of 28S rDNA were amplified with the primer pairs ITS4 and ITS5 [24], and LROR and LR5F [25], 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).

Phylogenetic analysis. The fungal sequences obtained from the GenBank database (Table 1) were aligned using Clustal_X v.1.83 [26] and edited with Bioedit v.5.0.9.1 [27].

Table 1. Taxa, collection numbers, sequences, and GenBank accession numbers used in this study

| Taxon name                  | Collection No. (isolate No.) | GenBank accession No. |
|-----------------------------|-----------------------------|-----------------------|
| Actinomucor elegans         | ATCC 46123                  | AM745430              |
| A. elegans                  | CBS 338.72                  | JN205824              |
| A. elegans                  | CBS111562                   | AB113009              |
| A. elegans                  | CBS 100.09                  | -                     |
| A. elegans                  | CBS154.86                   | -                     |
| A. elegans                  | CNUFC-YR113-1               | MG206066              |
| A. elegans                  | CNUFC-YR113-2               | MG206067              |
| A. elegans                  | CNUFC-KNU16-7               | MG206068              |
| A. elegans var. elegans     | ATCC22814’                  | MG206073              |

Two New Records of Zygomycete Species in Korea

Traditional taxonomy of Mucor species has been determined based on morphological characteristics such as size and shape of sporangia as well as the mode of reproduction (sexual or asexual).

Recently, molecular data have been used to evaluate mucoralean species [19, 20]. These studies indicated that Mucor is polyphyletic. Based on the phylogeny of internal transcribed spacer (ITS) and large subunit (LSU) rDNA regions of several mucoralean species, Walther et al. [21] observed that some Mucor species with curved sporangiophores were grouped with Backusella Hsett. & J. J. Ellis. Therefore, these Mucor species were transferred to Backusella.

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Table 1. Continued

| Taxon name                  | Collection No. (isolate No.) | GenBank accession No. |
|-----------------------------|-----------------------------|-----------------------|
|                            |                             | ITS 28S               |
| A. elegans var. kuwaitiensis| CBS117697                   | JN205823              |
| A. elegans var. meitauzae   | ATCC52370                   | AM745432              |
| A. elegans var. meitauzae   | CBS 111558                  | -                     |
| Backasella circina          | CBS 128.70                  | -                     |
| B. grandis                  | CBS 186.87                  | -                     |
| B. lamprosora               | CBS 118.08                  | -                     |
| Benjaminiella multispora    | CBS 421.70                  | -                     |
| Blakelea sinensis           | CBS 564.91                  | -                     |
| Choanephora infundibulifera | CBS 153.51                  | -                     |
| Cokeromyces recurvatus      | CBS 168.59                  | -                     |
| C. recurvatus               | CBS 158.50                  | -                     |
| Mucor aligarensis           | CBS 993.70                  | -                     |
| M. circinelloides           | B5-2                        | KT876701              |
| M. circinelloides           | CBS 108.16                  | JN205954              |
| M. fragilis                 | CBS 236.35                  | JN205979              |
| M. fragilis                 | EML-PUK106-1                | KY047147              |
| M. fragilis                 | EML-PUK106-2                | KY047150              |
| M. flavus                   | CBS 230.35                  | JN206061              |
| M. flavus                   | CBS 681.73                  | JN206070              |
| M. flavus                   | CBS 893.73                  | -                     |
| M. flavus                   | CBS 182.90                  | -                     |
| M. fuscus                   | CBS 132.22                  | JF723619              |
| M. fuscus                   | CBS 230.29                  | JN206204              |
| M. genevensis               | CBS 114.08                  | HM623318              |
| M. genevensis               | CBS 404.71                  | JN206042              |
| M. heterogamus              | CBS 338.74                  | JN206169              |
| M. heterogamus              | CBS 252.85                  | JN206490              |
| M. heterogamus              | CBS 405.58                  | JN206167              |
| M. hiemalis                 | CBS 242.35                  | JN206134              |
| M. hiemalis                 | CBS 115.18                  | JN206127              |
| M. irregularis              | CBS 977.68                  | JX976259              |
| M. irregularis              | EML-PUK112-1                | KY047151              |
| M. irregularis              | EML-PUK112-2                | KY047146              |
| M. koreanus                 | EML-QT1                     | KT936259              |
| M. koreanus                 | EML-QT2                     | KT936260              |
| M. luteus                   | CBS 243.35                  | JX976254              |
| M. minutus                  | CBS 586.67                  | JN206048              |
| M. minutus                  | CNUFC-BS1-1                 | MG206069              |
| M. minutus                  | CNUFC-BS1-2                 | MG206070              |
| M. mucedo                   | CBS 542.66                  | JN206086              |
| M. mucido                   | CBS 987.68                  | JN206089              |
| M. nidicola                 | EML-SBD1                    | KY047148              |
| M. nidicola                 | EML-SBD2                    | KY047149              |
| M. plasmaticus              | CBS 275.49                  | -                     |
| M. saturninus               | CBS 974.68                  | -                     |
| M. stercorea                | CNUFC-UK2-1                 | KX839689              |
| M. stercorea                | CNUFC-UK2-2                 | KX839680              |
| M. strictus                 | CBS 100.66                  | -                     |
| M. racemosus                | CBS 260.68                  | JF723556              |
| M. velutinosus              | UTHSC 04-1961               | JF299208              |
| M. velutinosus              | UTHSC 04-1981               | JF299212              |
| U. nana                     | NRRL 22420                  | KM017731              |

Bold letters indicate isolates and accession numbers determined in our study.

ITS, internal transcribed spacer; ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelmculures, Utrecht, The Netherlands; CNUFC, Chonnan National University Fungal Collection, Gwangju, South Korea; EML, Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, South Korea; NRRL (ARS Culture Collection, Peoria, Illinois); T, ex-type strain.
Phylogenetic analyses were performed using MEGA 6 software [28], and maximum likelihood was constructed by Kimura’s two-parameter correction method. The fungus *Umbelopsis nana* was used as an outgroup. The reliability of internal branches was assessed using the p-distance substitution model with 1,000 bootstrap replications.

**RESULTS**

**Phylogenetic analysis.** Phylogenetic analyses of the two sequence datasets (ITS and 28S rDNA) showed that the strains CNUFC-YR113-1, CNUFC-YR113-2, CNUFC-KNU16-7, CNUFC-BS1-1, and CNUFC-BS1-2 were placed within the same clade with species of *Actinomucor* and *Mucor* (Figs. 1 and 2).

In the BLASTn analysis of the ITS sequence, CNUFC-YR113-1 and CNUFC-BS1-1 represented 99.8% (535/536 bp) and 99.4% (613/617 bp) sequence identity values with *A. elegans* (GenBank accession No. JN205824) and *M. minutus* (GenBank accession No. JN206048), respectively.

In the BLASTn analysis of the 28S sequence, CNUFC-YR113-1 and CNUFC-BS1-1 strains showed 98.1% (634/636 bp) sequence identity values with *M. minutus* (GenBank accession No. JN206048), respectively.
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644 bp) and 100% (682/682 bp) identity values with *A. elegans* (GenBank accession No. JN205827) and *M. minutus* (GenBank accession No. JN206463), respectively.

**Taxonomy of CNUFC-YR113-1.**

*Actinomucor elegans* (Eidam) C. R. Benj. & Hesselt., Mycologia 49: 241 (1957) (Table 2, Fig. 3).

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**Table 2.** Morphological characteristics of CNUFC-YR113-1 and the reference *Actinomucor elegans* grown on synthetic mucor agar medium at 25°C.

| Character               | CNUFC-YR113-1                                      | *Actinomucor elegans* |
|------------------------|----------------------------------------------------|-----------------------|
| Colony color           | Rapid-growing, first white then deep olive-buff, reverse white | Rapid-growing, first white then deep olive-buff, reverse white to pale olive-buff |
| Sporangiohores         | 12.2–20.5 µm in width, variable in length          | Up to 30 µm in width, variable in length |
| Primary sporangia      | Globose to subglobose, multispored, 42.3–83.5 × 39.9–82.1 µm | Less than 80 µm, multispored |
| Secondary sporangia    | Globose to subglobose, multispored, 29.9–46.2 × 27.5–44.3 µm | Mostly 20–50 µm in diameter, multispored |
| Columellae inside      | Diverse in shape, oval, pyriform, oblong, 23.3–44.8 × 22.6–42.9 µm | Elongate-oval to pyriform, 50–60 × 30–40 µm |
| Primary sporangia      | Globose, 14.5–26.5 × 17.8–30.4 µm                   | Globose, 12–30 µm |
| Secondary sporangia    | Globose to subglobose, 6.1–8.5 × 5.8–8.1 µm         | Globose, mostly 6–8 µm in diameter |
| Chlamydospores         | Present                                            | Present               |
| Zygosporites           | Absent                                             | Unknown               |

*From the description by Benjamin and Hesseltine [3].*
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= Rhizopus elegans Eidam, Jahresber. Schles. Ges. Vaterl. Kultu. 61: 232 (1884).
= Mucor elegans (Eidam) J. Schröt., Kryptogamen-Flora von Schlesien 3-1: 207 (1886).
= Mucor corymbosus Harz, Bull. Soc. Imp. Nat. Moscou 44: 143 (1871).
= Actinomucor repens Schostak., Ber. Dtsch. Bot. Ges. 16: 155 (1898).
= Glomerula repens Bainier, Bull. Soc. Mycol. Fr. 19: 154 (1903).
= Mucor botryoides Lendn., Bull. Soc. Bot. Genève 2: 79 (1910).
= Mucor botryoides var. minor C.N. Jensen, Bull. Cornell Univ. Agric. Exp. Stn. 315: 457 (1912).
= Mucor cunninghamelloides Pispek, Acta Bot. Inst. Bot. Univ. Zagreb. 4: 91 (1929).
= Actinomucor corymbosus Naumov, Opredelitel Mukorovykh (Mucorales): 56 (1935).
= Actinomucor corymbosus f. palaestinus Rayss, Palestine J. Bot. 3: 162 (1945).

Description: Colonies grew rapidly at 25°C on SMA, filling the Petri dish after 5 days of incubation. The colony color was initially white, later deep olive-buff. Sporangiophores were 12.2–20.5 µm wide, erect, branched, irregular, and verticillate. Primary sporangia were globose to subglobose, and measured 42.3–83.5 × 39.9–82.1 µm. Secondary sporangia were formed with the same shape as the primary sporangia, and measured 29.9–46.2 × 27.5–44.3 µm. Columellae inside the primary sporangia were diverse in shape, oval, pyriform, oblong, and measured 23.3–44.8 × 22.6–42.9 µm. Columellae inside the secondary sporangia were globose, and measured 14.5–26.5 × 17.8–30.4 µm. Sporangiospores were globose to subglobose, and measured 6.1–8.5 × 5.8–8.1 µm. Chlamydospore formations were well-defined on the medium. Zygospores were not observed.

Taxonomy of CNUFC-BS1-1.
Mucor minutus (Baijal & B. S. Mehrotra) Schipper, Stud. Mycol. 10: 24 (1975) (Table 3, Fig. 4).
= Mucor griseo ochraceus var. minutus Baijal & B. S. Mehrotra, Sydowia 19: 206 (1966).
= Mucor saturninus var. minutus (Baijal & B. S. Mehrotra) Milko, Opredelitel mukoral’nykh gribov. 129 (1974).

Description: Colonies grew rapidly on SMA, attaining a diameter of 70–72 mm after 5 days at 25°C. The colony reverse was white. Sporangiophores were 12.2–20.5 µm wide, erect, branched, irregular, and verticillate. Primary sporangia were globose to subglobose, and measured 42.3–83.5 × 39.9–82.1 µm. Secondary sporangia were formed with the same shape as the primary sporangia, and measured 29.9–46.2 × 27.5–44.3 µm. Columellae inside the primary sporangia were diverse in shape, oval, pyriform, oblong, and measured 23.3–44.8 × 22.6–42.9 µm. Columellae inside the secondary sporangia were globose, and measured 14.5–26.5 × 17.8–30.4 µm. Sporangiospores were globose to subglobose, and measured 6.1–8.5 × 5.8–8.1 µm. Chlamydospore formations were well-defined on the medium. Zygospores were not observed.

Table 3. Morphological characteristics of CNUFC-BS1-1 and the reference species Mucor minutus grown on synthetic mucor agar medium at 25°C

| Character             | CNUFC-BS1-1                                      | Mucor minutus* |
|-----------------------|--------------------------------------------------|----------------|
| Colony color          | First white and later smoke gray                 | Smoke gray, up to 19 mm in height |
| Sporangiosphere       | 9–24.5 µm wide, variable in length               | Up to 20 µm, variable in length |
| Sporangia             | Globose, 37.1–109.8 µm × 36.4–103.4 µm            | Up to 175 µm |
| Columella             | Globose to ellipsoidal, 27.9–95.2 µm × 24.8–84.5 µm | Cylindrical to ellipsoidal, 110–135 µm in width |
| Sporangiospores       | Globose, 4.3–5.6 µm × 4.1–5.0 µm                  | Subspherical, 4–5 µm in diameter |
| Zygospore             | Absent                                           | Unknown |

*From the description by Schipper [29].
color was initially white, later turning to smoke gray. Sporangiophores were 9–24.5 µm wide, erect, mostly branched, and irregular. Sporangia were globose, and measured 37.1–109.8 µm × 36.5–103.4 µm. Columellae were globose to ellipsoidal, and measured 27.9–95.2 µm × 24.8–84.5 µm. Sporangiospores were globose, and measured 4.3–5.6 µm × 4.1–5.0 µm. Zygospores were not observed on artificial media.

**DISCUSSION**

Despite the wide intraspecific variation found among some taxa, the rDNA ITS and D1/D2 regions have been used as critical barcode markers for identifying mucoralean fungi at the species level, including taxa of *Actinomucor* and *Mucor* [21].

In the ITS and LSU phylogenetic trees, our strains CNUFC-BS1-1, CNUFC-BS1-2, and CNUFC-KNU16-7 were clustered within the elegans clade including *A. elegans*, *A. elegans* var. *meiatazae*, and var. *kuwaitiensis* in a well-supported clade. However, our strain CNUFC-YR113-1 differed from *A. elegans* var. *meiatazae* and *A. elegans* var. *kuwaitiensis* in sporangiospore size; CNUFC-YR113-1 strain exhibited smaller sporangiospores (6.1–8.5 × 5.8–8.1 µm) than *A. elegans* var. *meiatazae* (7–19.5 × 6–15 µm) and *A. elegans* var. *kuwaitiensis* (5–12 µm). The maximum growth temperature of our strain was 35°C, while *A. elegans* var. *meiatazae* and *A. elegans* var. *kuwaitiensis* were able to grow under higher temperatures up to 40°C.

Jong and Yuan [4] reported that growth temperature is a criterion for distinguishing between *A. elegans* and *A. taiwanensis*. These authors showed that *A. taiwanensis* has a maximum growth temperature of 37°C, while *A. elegans* does not grow at this temperature. Contrary to reports by Jong and Yuan [4], maximum growth temperature is less useful for distinguishing between the varieties [5, 6].

The morphological features of our isolates were in line with the description of *A. elegans* by Benjamin and Hesseltine [3], as the properties including shape, size of the sporangiospores (6–8 µm), and maximum temperature for growth were compared. Under these criteria, our isolate was identified as *A. elegans*.

In the tree based on D1/D2 sequence analyses, the strains CNUFC-BS1-1 and CNUFC-BS1-2 were placed into the minutus clade within the *M. flavus* group as presented by Walther et al. [21] including: *M. flavus*, *M. saturninus*, *M. aligarensis*, and *M. minutus* (Fig. 2), and formed a monophyletic group with *M. minutus* (type species). The CNUFC-BS1-1 isolate was morphologically most similar to *M. minutus* as described by Schipper [29], although there were differences in the shape and size of columellae. The size of columellae described by Schipper [29] was larger (110–135 µm) than those (27.9–95.2 × 24.8–84.5 µm) observed in our isolate. According to Schipper [29], the *M. minutus* species is similar in morphology and closely related to *M. flavus* because they produce columellae with the same size.

However, sporangiospores with different sizes and shapes have been observed. *M. minutus* has smaller sporangiospores (4–5 µm) than *M. flavus* (7–12 × 4–6.5 µm). Comparing the colony morphology and culture characteristics of the isolate with previous descriptions [29], the present isolate was similar to *M. minutus*, with some exceptions. Our *M. minutus* isolate presented one to three septa below the columella, which were not described by Schipper [29].

Recently, several studies have focused on the increased incidence of mucormycosis in both immunocompromised and immunocompetent patients [30]. Some species belonging to the order Mucorales (subphylum Mucoromycotina) are considered opportunistic pathogens. Particularly, four families, including Cunninghamellaceae, Lichtheimiaceae, Mucoraceae,
and Syncophalastraceae, have been described to be responsible for human infections [31].

More recently, A. elegans and A. elegans var. kuwaitiensis have been reported as the agent of mucormycosis in humans in several cases [6, 7, 32]. Morphological keys are available for identifying Actinomucor. However, it is still difficult to identify taxa to intraspecific rank in Actinomucor. Thus, taxonomic revision and phylogenetic analysis are needed in future studies.

Interestingly, A. elegans has been reported as protease enzyme for generation of small peptides with ACE-inhibitory activity from razor clam Sinonovacula constricta meat [33]. So this finding suggests that the strain CNUFC-YR113-1 may be a useful source for biotechnological applications.

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