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Research Article

Isolation and Characterization of Three Unrecorded Zygomycete Fungi in Korea: *Cunninghamella bertholletiae*, *Cunninghamella echinulata*, and *Cunninghamella elegans*

Thuong T. T. Nguyen[1], Young-Joon Choi[2] and Hyang Burm Lee[1,*]

[1]*Division of Food Technology, Biotechnology & Agrochemistry, College of Agriculture & Life Sciences, Chonnam National University, Gwangju 61186, Korea*
[2]*Department of Biology, College of Natural Sciences, Kunsan National University, Gunsan 54150, Korea*

Abstract In a survey of undiscovered taxa in Korea, three zygomycete fungal strains–EML-W31, EML-HGD1-1, and EML-RUS1-1–were isolated from freshwater, grasshopper fecal, and soil samples in Korea. On the basis of the morphological characteristics and phylogenetic analysis of internal transcribed spacer and 28S rDNA, the isolates of EML-W31, EML-HGD1-1, and EML-RUS1-1 were confirmed to be *Cunninghamella bertholletiae*, *Cunninghamella echinulata,* and *Cunninghamella elegans*, respectively. These species have not been previously described in Korea.

Keywords *Cunninghamella bertholletiae*, *Cunninghamella echinulata*, *Cunninghamella elegans*, Undiscovered taxa, Zygomycota

Mucorales is the largest order of fungi, classified into the subphylum Mucoromycotina [1], which consist of at least 57 genera within 15 families comprising 334 species [2]. Members of Mucorales can be easily isolated from soil, dung, water, stored grains, plants, and fungal masses [3, 4]. Many members in the order are characterized by rapid growth and the ability to colonize and sporulate on diverse, carbohydrate-rich, and terrestrial substrates.

The genus *Cunninghamella* (Cunninghamellaceae, Mucorales) was established in 1903 by Matruchot, and is comprised of species characterized by the formation of the pedicellate and unispored sporangia on the surface of the entire vesicle [5]. There are 14 accepted species in this genus [6-8]. *Cunninghamella* species are often found in soil, stored grains, and other organic substrates [3, 7-10]. Some are reported as human mucormycosis [11, 12], and some species are known to produce compounds that can destroy tumors [13]. The taxonomy of *Cunninghamella* has been determined based on morphological characteristics such as colony color, pattern of sporophore branching, vesicle shape and size, sporangiola shape, and the length of the spines in the sporangiola [5, 14]. In the study by Zheng and Chen [5], the genus *Cunninghamella* was monographed according to the morphological characteristics and maximum growth temperatures. Liu *et al.* [15], Walther *et al.* [16], and Yu *et al.* [6] sequenced the internal transcribed spacer (ITS), 28S, and elongation factor 1α gene regions of the accepted taxa and showed the phylogeny to be compatible with Zeng and Chen's taxonomical conclusions [5].

Knowledge of the taxonomy of some important species of mucoralean fungi in Korea has been limited. Only 60 taxa of zygomycetes, belonging to 21 genera and 9 families, have been found in Korea [17-19]. Within the Cunninghamellaceae, only 8 species have been roughly described [4, 17-19]. However, there is no published record of species belonging to this genus in Korea.

The objective of the present study was to perform morphological and molecular analyses to characterize three unrecorded zygomycete species–*Cunninghamella bertholletiae*, *Cunninghamella echinulata*, and *Cunninghamella elegans*–in Korea.

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*Corresponding author
E-mail: hblee@jnu.ac.kr

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MATERIALS AND METHODS

Isolation of fungal strains from grasshopper feces, freshwater, and soil samples. In July, grasshoppers were collected at the CNU Arboretum located in Chonnam National University, Gwangju, Korea and put into 50 mL conical tubes (SPL Life Sciences Co., Pocheon, Korea). Grasshoppers were transferred to the laboratory and after 6 hr, feces were obtained from the bottom of tube using sterile forceps, placed onto water agar (20 g/L agar in deionized water), and incubated at 25°C for 3–7 days. Under a stereomicroscope, hyphal tips were transferred to potato dextrose agar (PDA) plates, using tips of heat-stretched capillary tubes.

Soil samples were collected from the garden of Chonnam National University located in Gwangju. Freshwater samples were collected from a branch stream of the Nakdong River located in Gyeongsangbuk-do, Korea. These samples were transferred to sterile 50-mL conical tubes, and stored at 4°C until examination. Fungi were isolated by a serial dilution plating method. In this technique, 1 g of soil or 1 mL water was mixed with 9 mL of sterile distilled water and the solution was shaken for 15 min at room temperature, and serial dilutions were made ranging from 10^{-1} to 10^{-4}. A 0.1 mL aliquot from each dilution was transferred onto PDA and incubated at 25°C for 3–7 days.

To isolate pure cultures, individual colonies of varied morphologies were transferred to PDA plates. Pure isolates were maintained in PDA slant tubes and stored in 20% glycerol at −80°C at the Environmental Microbiology

Table 1. Sequences used in this study and GenBank accession numbers

Taxon name	Collection No. (isolate No.)	GenBank accession No.	
		ITS	28S
Cunninghamella bertholletiae	CBS 190.84	JN205878	HM849701
C. bertholletiae	CBS 693.68	JN205871	JN206600
C. bertholletiae	NRRL 1380^{NT}	AF254930	-
C. bertholletiae	CBS 779.68	JN205874	JN206599
C. bertholletiae	ATCC 42115	-	FJ345351
C. bertholletiae	**EML-W31**	**MF806022**	**MF806029**
C. binariae	CBS 158.28	JN205888	JN206602
C. binariae	CBS 481.66	JN205889	JN206603
C. blakesleeana	CBS 782.68	JN205869	JN206601
C. bigelovii	CGMCC 8094	KJ013403	KJ013405
C. clavata	CBS 100178^{T}	JN205890	JN206604
C. echinulata	CBS 656.85	JN205896	JN206598
C. echinulata	CBS 156.28	JN205895	HM849702
C. echinulata	CBS 545.75^{T}	JN205893	JN206597
C. echinulata	CBS 770.68	KJ183114	-
C. echinulata	**EML-HGD1-1**	**MF806020**	**MF806025**
C. echinulata	**EML-HGD1-2**	**MF806024**	**MF806026**
C. elegans	CBS 167.53	JN205882	HM849700
C. elegans	CBS 160.28^{NT}	AF254928	-
C. elegans	Cu-2	AF254927	-
C. elegans	CBS 773.68	JN205887	-
C. elegans	xsd08061	FJ792589	-
C. elegans	TUFC 20015	-	AB638466
C. elegans	TUFC 20040	-	AB638479
C. elegans	**EML-RUS1-1**	**MF806023**	**MF806027**
C. elegans	**EML-RUS1-2**	**MF806021**	**MF806028**
C. gigacellularis	URM 7400	KX238887	KX238889
C. gigacellularis	URM 7400	KX238886	KX238890
C. homothallica	CBS 168.53^{T}	JN205863	JN206605
C. intermedia	CBS 347.69^{T}	JN205892	JN206606
C. phaeospora	CBS 692.68	JN205864	HM849697
C. vesiculosa	CBS 989.96^{T}	JN205897	HM849693
Mortierella parvispora	CBS 311.52	EU484279	HM849689

Bold letters indicate isolates and accession numbers determined in our study.
ITS, internal transcribed spacer; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NRRL, ARS Culture Collection, Peoria, Illinois, USA; NT, ex-neotype strain; ATCC, American Type Culture Collection, Manassas, VA, USA; EML, Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, South Korea; T, ex-type strain; TUFC, Tottori University Fungal Culture Collection; URM, Universidade Federal de Pernambuco, Recife, Brazil.

Laboratory Fungarium, Chonnam National University, Gwangju, Korea.

Morphological studies. For detailed morphological studies, EML-W31, EML-HGD1-1, and EML-RUS1-1 strains were cultured on synthetic mucor agar (SMA; 40 g dextrose, 2 g asparagine, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.5 g thiamine chloride, and 15 g agar in 1 L of deionized water). The plates were incubated at 25°C in the dark for 5 days. Samples were mounted in a lactophenol solution (Junsei Chemical Co. Ltd., Tokyo, Japan) and observed under an Olympus BX51 microscope with DIC optics (Olympus, Tokyo, Japan). Fine fungal structures were observed by scanning electron microscopy (SEM) (Hitachi S4700; Hitachi, Tokyo, Japan). The isolates were fixed in 2.5% paraformaldehyde-glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 hr, and then washed with cacodylate buffer (Junsei Chemical Co. Ltd.). Cellular membranes were preserved by fixing the samples in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) diluted in cacodylate buffer for 1 hr. Samples were then washed again in cacodylate buffer, dehydrated in graded ethanol (Emsure, Darmstadt, Germany) and isoamyl acetate (Junsei Chemical Co. Ltd.), and dried in a fume hood. Finally, samples were sputter-coated with gold and observed under a Hitachi S4700 field emission scanning electron microscope at the Korea Basic Science Institute, Gwangju, Korea.

To investigate growth rates, EML-W31, EML-HGD1-1, and EML-RUS1-1 strains were incubated on PDA medium at 10°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C, and 42°C in the dark for 7 days.

DNA extraction, PCR, and sequencing. Genomic DNA was extracted directly from the mycelia of fungal isolates, using a genomic DNA prep kit (Solgent, Daejeon, Korea). The ITS region and large subunit of 28S rDNA were amplified with the primer pairs ITS4 and ITS5 [20]; LROR and LR5F [21, 22], respectively. The PCR amplification mixture (total volume, 20 µL) contained the fungal DNA template, 5 pmol/µL of each primer, and the Accupower PCR Premix (Bioneer, Daejeon, Korea). PCR products were purified using the Accuprep PCR Purification Kit

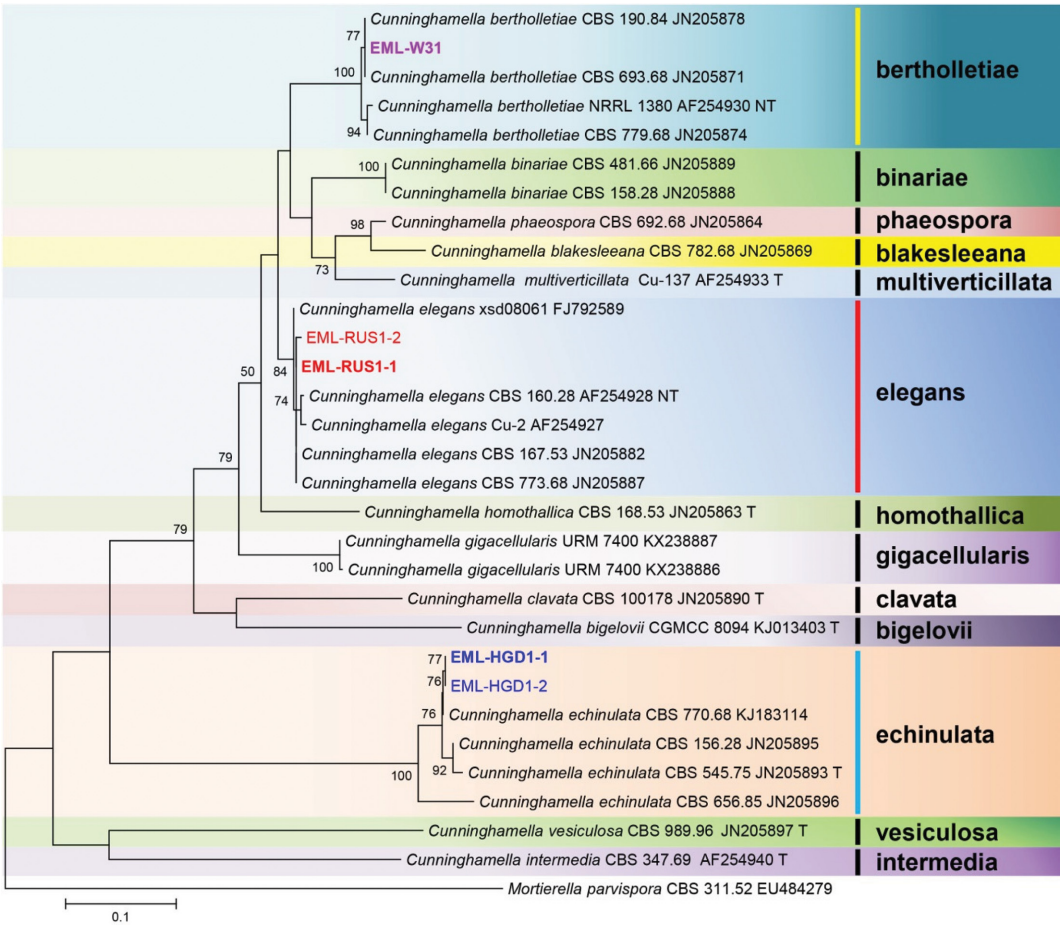

Fig. 1. Phylogenetic tree based on the maximum likelihood analysis of internal transcribed rDNA sequences for *Cunninghamella bertholletiae* EML-W31, *C. echinulata* EML-HGD1-1, *C. echinulata* EML-HGD1-2, *C. elegans* EML-RUS1-1, and *C. elegans* EML-RUS1-2. *Mortierella parvispora* was used as the outgroup. Bootstrap support values of ≥ 50% are indicated at the nodes. The bar indicates the number of substitutions per position.

(Bioneer) according to the manufacturer's instructions. DNA sequencing was performed on an ABI 3700 Automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). The EML-W31, EML-HGD1-1, EML-HGD1-2, EML-RUS1-1, and EML-RUS1-2 sequences were deposited in the NCBI database under accession numbers as shown in Table 1.

Phylogenetic analysis. Sequence data obtained from the GenBank database (Table 1) were aligned using Clustal_X v.1.83 [23] and Bioedit v. 5.0.9.1 software [24]. Phylogenetic analyses were performed using MEGA 6 software [25], and maximum likelihood was constructed by Kimura's two-parameter correction method. *Mortierella parvispora* was used as the outgroup. Reliability of the internal branches was assessed using the p-distance substitution model, with 1,000 bootstrap replications.

RESULTS

Phylogenetic analysis. BLASTN searches were performed against the ITS sequences of EML-W31, EML-HGD1-1, and EML-RUS1-1. Sequence similarity comparison revealed 100% (700/700 bp), 99.5% (657/660 bp), and 99.6% (674/677 bp) homologies with the three most closely related species, *Cunninghamella polymorpha* CBS 693.68 (current name: *C. bertholletiae*) (GenBank accession No. JN205871), *C. echinulata* CBS 770.68 (GenBank accession No. KJ183114), and *C. elegans* xsd08061 (GenBank accession No. FJ792589), respectively. On the basis of the 28S rDNA sequence analysis, EML-W31, EML-HGD1-1, and EML-RUS1-1 strains showed 99.8% (905/907 bp), 100% (628/628 bp), and 99.8% (659/660 bp) homologies with *C. bertholletiae* ATCC 42115 (GenBank accession FJ345351), *C. echinulata* CBS 156.28 (GenBank accession No. JN939199), and *C. elegans* CBS 167.53 (GenBank accession No. HM849700), respectively. On the basis of the ITS and 28S sequence analysis, the three isolates EML-W31, EML-HGD1-1, and EML-RUS1-1 were identical to *C. bertholletiae*, *C. echinulata*, and *C. elegans* (Figs. 1 and 2), respectively.

Taxonomy of EML-W31.

Cunninghamella bertholletiae Stadel, Über einen neuen Pilz, *Cunninghamella bertholletiae*: 1 (1911) (Table 2, Fig. 3).

Description: Colonies exhibited rapid growth on SMA, attaining a diameter of 76–78 mm after 4 days at 25°C. The colony color was initially white, later turning to gray. The colony reverse was pale gray and regularly zonate.

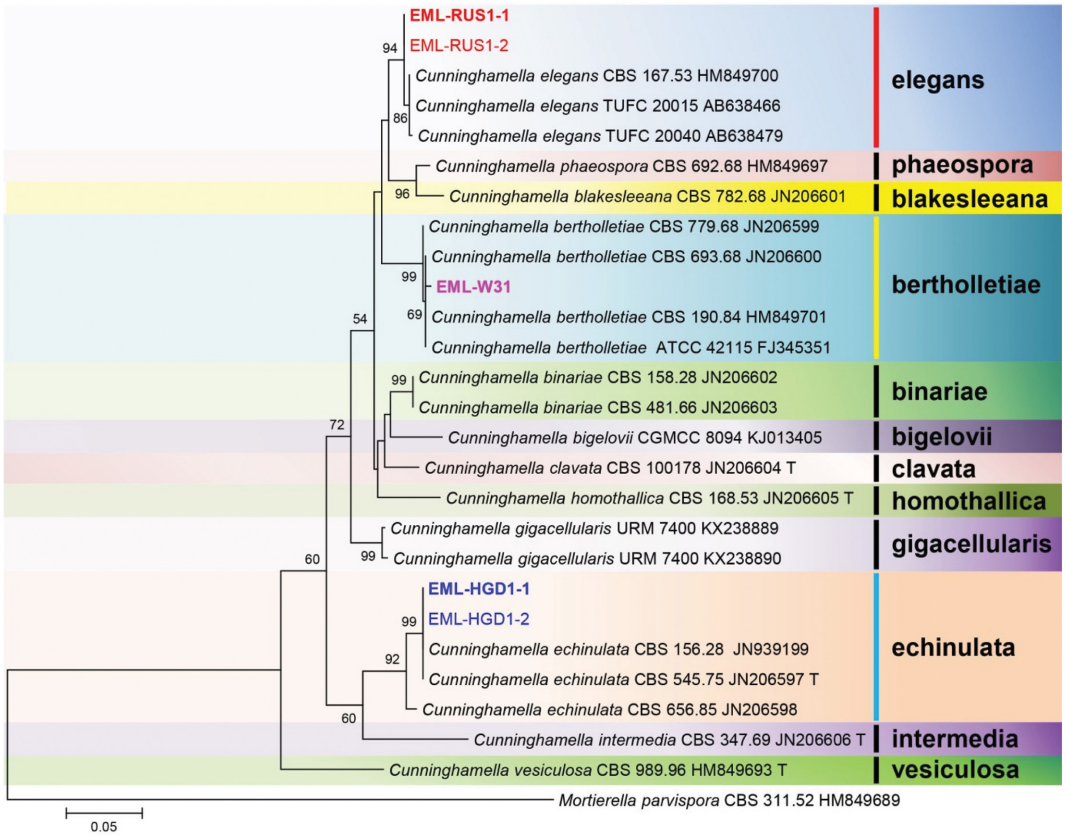

Fig. 2. Phylogenetic tree based on the maximum likelihood analysis of 28S rDNA sequences for *Cunninghamella bertholletiae* EML-W31, *C. echinulata* EML-HGD1-1, *C. echinulata* EML-HGD1-2, *C. elegans* EML-RUS1-1, and *C. elegans* EML-RUS1-2. *Mortierella parvispora* was used as the outgroup. Bootstrap support values of ≥ 50% are indicated at the nodes. The bar indicates the number of substitutions per position.

Table 2. Morphological characteristics of EML-W31 and the reference species *Cunninghamella bertholletiae* on synthetic mucor agar medium at 25°C

Character	EML-W31	*Cunninghamella bertholletiae*[a]
Colony color	Rapid growth, first white and later gray	Rapid growth, tannish-gray
Sporangiophore	6.3–12.5 µm in width, erect, diversely branched, irregular or verticillate	Erect, branched, irregular
Sporangiola	Globose, oval, 7.1–13.3 × 5.5–10.0 µm	Spherical, oval, 6–11 × 6–10 µm
Terminal vesicle	Globose to subglobose, 16.5–45.5 µm in diameter	Ovate, 15–52 µm in diameter
Lateral vesicle	Globose to subglobose, 11.6–30.5 µm in diameter	Ovate, 9–33 µm in diameter
Chladospore	Not observed	Unknown
Zygospore	Absent	Unknown

[a]From the description by Weitzman and Crist [14].

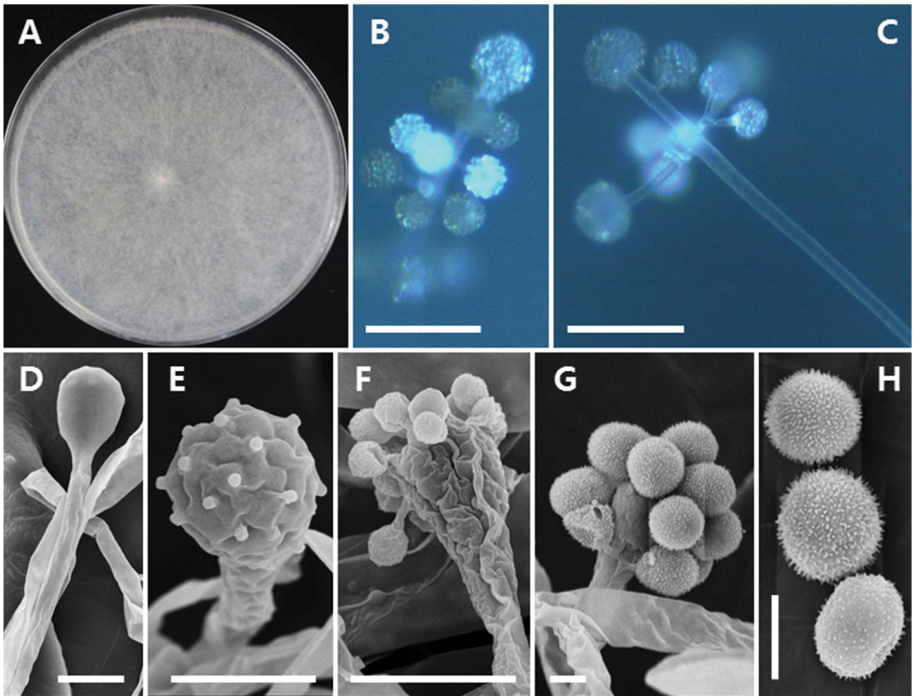

Fig. 3. Morphology of *Cunninghamella bertholletiae* EML-W31. A, Colonies on synthetic mucor agar; B, C, Sporangiola on branched sporangiophores (observed under light microscope); D, Sporangiophore with vesicle (observed under scanning electron microscopy [SEM]); E, Development of sporangiola on vesicles (observed under SEM); F, G, Vesicles bearing sporangiola (observed under SEM); H, Sporangiola with spines (observed under SEM) (scale bars: B, C = 100 µm, D–G = 10 µm, H = 5 µm).

Sporangiophores were 6.3–12.5 µm wide, erect, diversely branched, irregular, and verticillate. Sporangiola were subglobose to ovoidal, and measured 7.1–13.3 × 5.5–10.0 µm. Terminal vesicles were globose to subglobose, and measured 16.5–45.5 µm in diameter. Lateral vesicles were the same shape as terminal vesicles, and measured 11.6–30.5 µm in diameter. Chlamydospores and zygospores were not observed.

Taxonomy of EML-HGD1-1.

Cunninghamella echinulata (Thaxt.) Thaxt., Rhodora 5: 98 (1903) (Table 3, Fig. 4).

Description: Colonies exhibited rapid growth on SMA, attaining a diameter of 82–84 mm after 4 days at 25°C. The colony color was initially white, later becoming yellowish. Sporangiophores were 6.5–10.5 µm wide, erect, mostly branched, irregular, and verticillate. Sporangiola were oval, globose, and measured 11.2–15.5 × 10.4–15.2 µm. Terminal vesicles were globose to subglobose, and measured 20.7–37.8 µm in diameter. Lateral vesicles were a similar shape to the terminal vesicles, but smaller in size, and measured 10.5–26.3 µm in diameter. Chlamydospores were oval. Zygospores were not observed.

Taxonomy of EML-RUS1-1.

Cunninghamella elegans Lendn., Bull. Herb. Boissier 7: 250 (1907) (Table 4, Fig. 5).

Description: Colonies exhibited rapid growth on SMA, covering the petri dish after 4 days at 25°C. The colony color was initially white, later turning to a stormy gray.

Table 3. Morphological characteristics of EML-HGD1-1 and the reference species *Cunninghamella echinulata* on synthetic mucor agar medium at 25°C

Character	EML-HGD1-1	*Cunninghamella echinulata*[a]
Colony color	Rapid growth, first white and later yellowish	Rapid growth, first white and later pale ochraceous buff
Sporangiophore	6.5–10.5 μm in width, variable in length, mostly branched, irregular, verticillate	Up to 10 μm in width, 2 mm in length, mostly branched, irregular, opposite or verticillate
Sporangiola	Oval, globose, 11.2–15.5 × 10.4–15.2 μm in diameter	Mostly globose, 13.2–15.5 μm in diameter
Terminal vesicle	Globose to subglobose, 20.7–37.8 μm in diameter	Oval, globose, or pyriform, 21–41 μm in diameter
Lateral vesicle	Globose to subglobose to, 10.5–26.3 μm in diameter	Oval, globose, or pyriform, 13.5–32 × 9–24 μm
Chladospore	Present	Present
Zygospore	Absent	Unknown

[a]From the description by Baijal and Mehrotra [10].

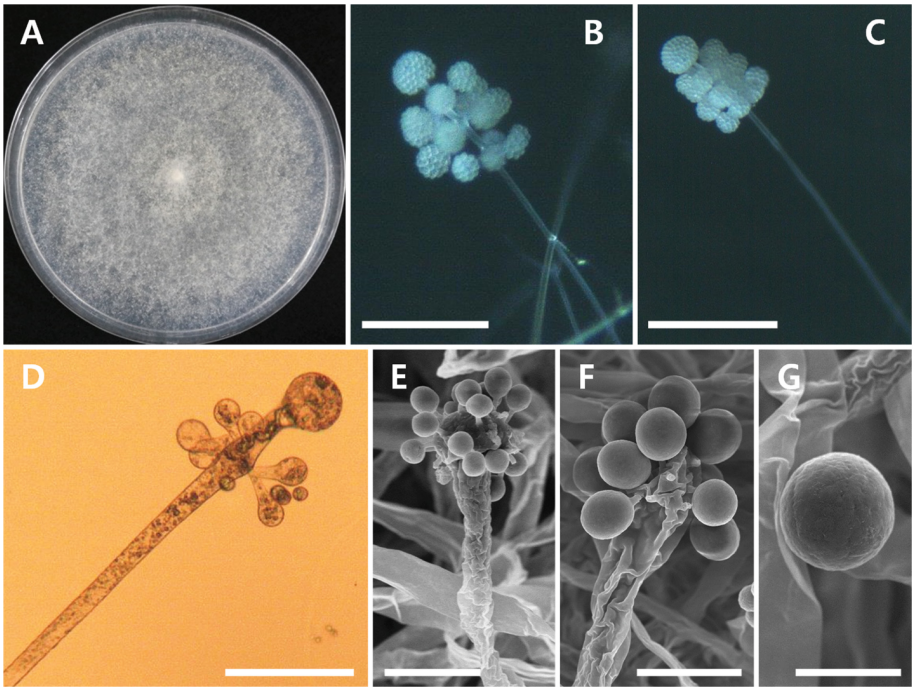

Fig. 4. Morphology of *Cunninghamella echinulata* EML-HGD1-1. A, Colonies on synthetic mucor agar; B, C, Sporangiophores with sporangiola (observed under light microscope); D, Sporangiophores with vesicles; E, F, Vesicles bearing sporangiola (observed under scanningelectron microscopy [SEM]); G, Smooth sporangiolum (observed under SEM) (scale bars: B, C = 100 μm, D = 50 μm, E, F = 20 μm, G = 10 μm).

Table 4. Morphological characteristics of EML-RUS1-1 and the reference species *Cunninghamella elegans* on synthetic mucor agar medium at 25°C

Character	EML-RUS1-1	*Cunninghamella elegans*[a]
Colony color	Rapid growth, first white and later storm gray	Rapid growth, first white and later gray
Sporangiophore	5.5–9.3 μm in width, variable in length, opposite, verticillate	Up to 12.5 μm in width, branched verticillate
Sporangiola	Subglobose to ellipsoidal, 7.3–11.6 × 6.1–10.5 μm in diameter	Globose, 6–10 μm in diameter
Terminal vesicle	Subglobose to oval, 18.3–35.5 μm in diameter	Subglobose to oval, 21.0–38.5 μm in diameter
Lateral vesicle	Oval to pyriform, 13.1–19.8 μm in diameter	Subglobose to oval, up to 20 μm in diameter
Chlamydospore	Not observed	Unknown
Zygospore	Absent	Unknown

[a]From the description by Baijal and Mehrotra [10].

Mycelial growth on SMA was sparse. Sporangiophores were 5.5–9.3 μm wide and erect and had solitary, opposite, or verticillate branches. Sporangiola were globose to ellipsoidal, and measured 7.3–11.6 × 6.1–10.5 μm. Terminal

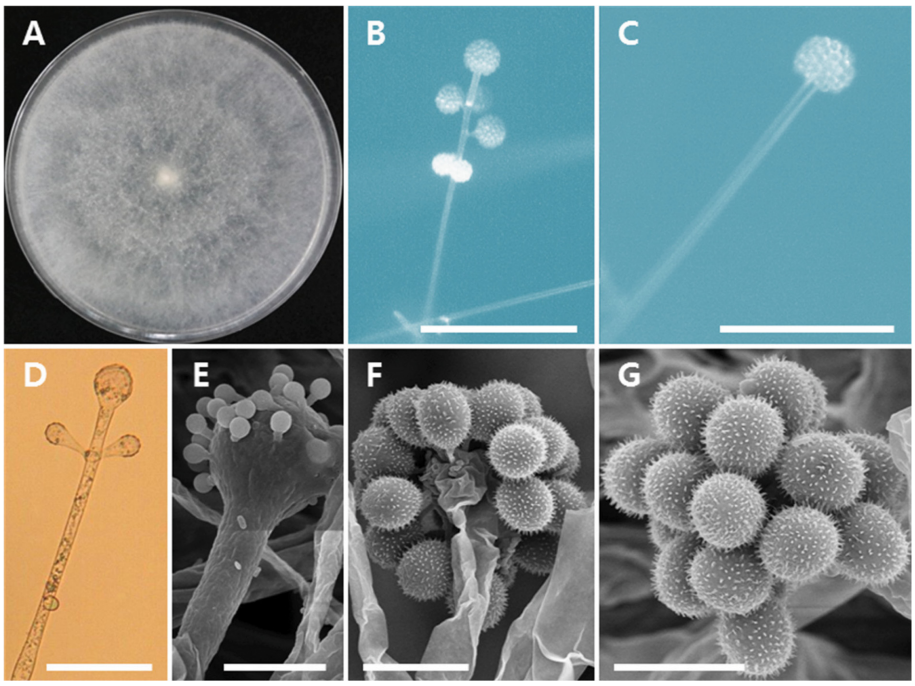

Fig. 5. Morphology of *Cunninghamella elegans* EML-RUS1-1. A, Colonies on synthetic mucor agar; B, C, Sporangiophores with sporangiola (observed under the light microscope); D, Sporangiophores with vesicles; E, Young sporangiola formed on a sporangiophore vesicle (observed under scanning electron microscopy [SEM]); F, G, Sporangiola with spines (observed under SEM) (scale bars: B = 200 μm, C = 100 μm, D = 50 μm, E–G = 10 μm).

vesicles were subglobose to oval and measured 18.3–35.5 μm in diameter. Lateral vesicles were oval to pyriform and measured 13.1–19.8 μm in diameter. Chlamydospores and zygospores were not observed.

Mycelial growth. The results regarding mycelial growth at different temperatures are shown in Fig. 6. The average growth rates of EML-W31, EML-HGD1-1, and EML-RUS1-1 on PDA were 19.5 mm, 21 mm, and 26.5 mm per day, respectively, at 25°C. The optimal temperature for growth was 30°C for EML-RUS1-1 and 35°C for EML-HGD1-1 and EML-RUS1-1. Slow growth was observed at 10°C and no growth was observed at 40°C for EML-RUS1-1 and 42°C for EML-W31 and EML-HGD1-1.

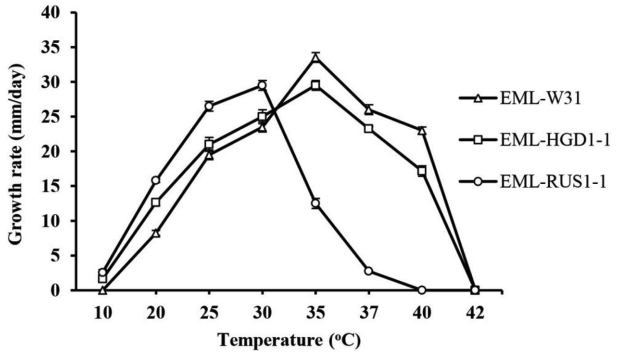

Fig. 6. Effect of temperature on mycelial growth of *Cunninghamella bertholletiae* EML-W31, *C. echinulata* EML-HGD1-1, and *C. elegans* EML-RUS1-1. Mycelia were grown on potato dextrose agar at different temperatures of 10°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C, and 42°C.

DISCUSSION

In this paper, the detailed morphologies of *Cunninghamella* species were described based on ultrastructures revealed by SEM and light microscopy. Observing the detailed morphologies of sporangiola and sporangiophores under SEM are valuable tools in classifying a diverse group within Cunninghamellaceae.

In the studies by Samson [26], Baijal and Mehrotra [10], and Lunn and Shipton [27], *C. bertholletiae* and *C. elegans* were considered to be the same species because there were no significant differences in the morphological characteristics. Generally, temperature plays an important role in fungal growth. In this study, the effect of temperature on the growth of different *Cunninghamella* species varied with the species. According to Weitzman and Crist [14], growth capacity at 40°C, or at temperatures above this was a key parameter used to distinguish the species of *C. elegans* and *C. bertholletiae*. These authors showed that the species of *C. elegans* has a maximum growth temperature of 37°C; whereas the maximum growth temperature of *C. bertholletiae* is between 40°C and 45°C.

Of the three species studied in this work, only *C. bertholletiae* and *C. echinulata* had a maximum growth temperature at 40°C, and the maximum growth temperature

for *C. elegans* was 37°C. The observed growth temperature of *C. bertholletiae* and *C. echinulata* suggested that these species of *Cunninghamella* have thermotolerance. Our results are in agreement with those reported by Weitzman and Crist [14] in that temperature tolerance is a criterion for distinguishing the difference between *C. bertholletiae* and *C. elegans* species.

Although *C. bertholletiae* and *C. echinulata* have a similar temperature tolerance, *C. bertholletiae* is morphologically different from *C. echinulata* because it forms long branches on the sporangiophore.

The morphological characteristics of the *Cunninghamella echinulata* isolate studied were similar to those previously described by Baijal and Mehrotra [10]. However, our *Cunninghamella echinulata* isolate had sporangiophores with swellings, which was not described by Baijal and Mehrotra [10].

Despite the wide intraspecific variation found among some taxa, the ITS region is the barcode marker for mucoralean species identification. The identification of the isolates was confirmed by DNA sequencing of ITS and 28S rDNA. In the phylogenetic trees based on the ITS and 28S sequences, EML-W31 strain was placed into the *bertholletiae* clade, EML-HGD1-1 and EML-HGD1-2 strains into the echinulata clade, and EML-RUS1-1 and EML-RUS1-2 strains into the *elegans* clade, along with representative species of *C. bertholletiae*, *C. echinulata*, and *C. elegans* (Figs. 1 and 2). The results of our molecular data analysis were consistent with the phylogeny presented by Walther *et al.* [16] and Yu *et al.* [6].

Pispek [28] described a new species, *C. polymorpha*, on the basis of the morphological characteristics of variously branched sporangiophores and globose or ovoid to ellipsoidal conidia. Weitzman and Crist [14, 29] later demonstrated that *C. polymorpha* was a synonym of *C. bertholletiae* on the basis of mating and morphology studies. Recently, Walther *et al.* [16] and Yu *et al.* [6], who performed phylogenetic analysis of sequences of *C. polymorpha*, indicated that this species was a synonym of *C. bertholletiae*.

Morphologically, the species of *C. bertholletiae* reported here had a close similarity with the description by Weitzman and Crist [14], although there were differences in the size of the terminal vesicles. The sizes of terminal vesicles were potentially larger (15–52 μm according to Weitzman and Crist [14]) than those (16.5–45.5 μm) observed in our isolate.

In recent studies on zygomycetous fungi belonging to undiscovered taxa in Korea, eight new species have been registered in Index Fungorum [4, 18, 30, 31].

Our results suggest that our knowledge on the diversity of zygomycetous fungi is small and more undescribed and novel taxa in zygomycota remain to be discovered.

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