MYCOBIOLOGY
2018, VOL. 46, NO. 4, 317–327
https://doi.org/10.1080/12298093.2018.1538071

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Isolation and Characterization of Three Zygomycetous Fungi in Korea: *Backusella circina*, *Circinella muscae*, and *Mucor ramosissimus*

Thuong T. T. Nguyen and Hyang Burm Lee

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

ABSTRACT

While surveying undiscovered fungal taxa in Korea, three rare zygomycetous fungal strains, CNUFC-PTF2-1, CNUFC-TF3-1, and CNUFC-ESAF3-1, were isolated from soil, leaf, and freshwater samples, respectively. The strains were analyzed morphologically as well as phylogenetically based on the internal transcribed spacer region and 28S rDNA sequences. Sequence analysis of the two loci revealed that the isolates, CNUFC-PTF2-1, CNUFC-TF3-1, and CNUFC-ESAF3-1, were identified as *Backusella circina*, *Circinella muscae*, and *Mucor ramosissimus*, respectively. These species have not yet been previously described in Korea.

ARTICLE HISTORY
Received 27 April 2018
Revised 23 August 2018
Accepted 24 August 2018

KEYWORDS
Backusella circina; *Circinella muscae*; *Mucor ramosissimus*; taxonomy; zygomycetous fungus

1. Introduction

The Mucorales, which is classified into the subphylum Mucoromycotina [1], is the largest order of fungi. Members of this group are ubiquitous saprophytes in nature. They are commonly found in soil and decaying vegetation, and can also be found in grains [2–4]. Mucorales members grow and invade quickly on easily digestible substrates, such as those containing starches, sugars, and hemicelluloses [2]. Currently, 14 families are placed in this order based on the analysis of a multigene (act-1, EF-1α, 18S, and 28S rRNA) data set [3].

The genus *Backusella*, which belongs to the subphylum Mucoromycotina, order Mucorales, and family Backusellaceae, was established by Ellis & Hesseltine (1969) with the type species *B. circina* [5]. Species belonging to this genus are characterized by the production of both sporangia and sporangiola, as well as by the formation of transitorily curved sporangiophores [4–6]. They are typically isolated from soil, leaf litter, and other plant debris, as well as from dung samples, such as those from human, agouti, and insects [7–10]. For a long time, the genus *Backusella* contained only three species: *B. circina*, *B. lamprospora*, and *B. ctenidia*. However, Walther et al. [4] recently revised the order Mucorales based on internal transcribed spacer (ITS) and 28S rDNA sequence data and transferred some species of *Mucor* to the genus *Backusella* because they had transitorily recurved sporangiophores, while *B. ctenidia* was transferred to the genus *Mucor*. Based on these criteria, this genus now comprises 13 species, among which one species is registered in Korea (Source: www.indexfungorum.org as of April 2018).

The genus *Circinella*, which belongs to the subphylum Mucoromycotina, order Mucorales, and family Lichtheimiaceae, was established in 1873 by van Tieghem and Le Monnier [11]. It is closely related to *Mucor*, but differs in that it has sporangiophores with circinate branches bearing sporangia [12]. Members of this genus are characterized by the production of sporangiophores bearing circinate branches terminated by globose multispored sporangia with persistent sporangial walls [12]. After Hesseltine and Fennell [12] monographed this genus, several additional species were included [13–16]. To date, 10 species belonging to this genus are known according to the Index Fungorum (www.indexfungorum.org). They are commonly isolated from soil, dung, sand beach, and hydrocarbon-polluted sand [2,17,18].

The genus *Mucor* belongs to the subphylum Mucoromycotina, order Mucorales, and family Mucoraceae, and was described by Fresenius in 1850, comprising the largest number of species within the Mucorales [19]. Specimens of this genus are characterized by the formation of non-apophysate sporangia and production of simple or branched sporangiophores without basal rhizoids. Zygospores have opposed, non-appendaged suspensors [20]. *Mucor* species are easily isolated from soil, fruits, vegetables, stored grains, insect, and dung [2,21–24]. Several species of this genus are of great interest to the

CONTACT Hyang Burm Lee hblee@jnu.ac.kr

biotechnology industry due to their ability to produce enzymes such as proteases, amylase, lipases, phytase, and polygalacturonase [25–27], while some species are considered as the causal agents of cutaneous zygomycosis in humans [28]. The traditional taxonomic classification of *Mucor* species was determined based on morphological characteristics such as size and shape of the sporangia and mode of reproduction (sexual or asexual). Recently, molecular studies have been performed to evaluate mucoralean species [3,4]. These studies have indicated that *Mucor* is polyphyletic. Based on the phylogenetic analysis of ITS and large subunit rDNA regions of several mucoralean species, Walther et al. [4] observed that some *Mucor* species with curved sporangiophores were grouped with *Backusella* Hesselt. & J. J. Ellis. Therefore, these *Mucor* species were transferred to *Backusella*. Nine species have been recorded, including three new species from freshwater, tangerine fruit, and rat feces samples in Korea [10,23].

During an inventory of fungal species from soil, leaf, and freshwater samples, three interesting fungal strains belonging to the order Mucorales were assigned to the genera *Backusella*, *Circinella*, and *Mucor*.

The objective of this study was to morphologically and molecularly characterize three unrecorded species in Korea: *B. circina*, *C. muscae*, and *M. ramosissimus*.

2. Materials and methods

2.1. Isolation of fungal strains from leaf, soil, and freshwater samples

Leaves of *Toxicodendron sylvestre* were collected from Daegak Mountain, Sinsi Island, Gunsan, Korea. Collected samples were stored in sterile polyethylene bags. Samples were cleaned under running tap water to remove debris before use. Leaf tissue pieces were cut into small fragments, surface-disinfested with 2% NaOCl solution and 70% ethanol for 1 min each, washed three times with sterile distilled water, plated on potato dextrose agar (PDA; BD Biosciences, Franklin Lakes, NJ) supplemented with the antibiotic streptomycin sulfate (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO), and incubated at 25 °C for 3–7 days.

Soil samples were collected from Geumgol Mountain, Jin Island (Jindo), Korea. Freshwater samples were collected from Eulsukdo, Busan, Korea. The samples were transported in sterile 50-mL Falcon tubes and stored at 4 °C until examination. Fungi were isolated using the serial dilution plating method. In this technique, 1 mL of water or 1 g of soil was mixed with 9 mL of sterile distilled water and shaken for 15 min at 25 °C; serial dilutions ranging from 10^{-1} to 10^{-4} were prepared. An aliquot of 0.1 mL from each dilution was transferred to PDA supplemented with the antibiotic streptomycin sulfate (0.5 mg/mL) and incubated at 25 °C for 3–7 days. To isolate pure cultures, individual colonies with various morphologies were picked, transferred to PDA, and subcultured until pure mycelia were obtained. All pure isolates, including those of *B. circina*, *C. muscae*, and *M. ramosissimus*, were stored in 20% glycerol at −80 °C at the Chonnam National University Fungal Collection (CNUFC), Gwangju, Korea. *B. circina*, *C. muscae*, and *M. ramosissimus* strains isolated in our study were designated CNUFC-PTF2-1 and CNUFC-PTF2-2, CNUFC-TF3-1 and CNUFC-TF3-2, CNUFC-ESAF3-1 and CNUFC-ESAF3-2, respectively. Strain CNUFC-PTF2-1 was also deposited at the Culture Collection of the National Institute of Biological Resources (NIBR, Incheon, Korea), strain CNUFC-TF3-1 was deposited at Korean Agricultural Culture Collection (Wanju, Korea), and strain CNUFC-ESAF3-1 was deposited at the Culture Collection of the Nakdonggang National Institute of Biological Resources (NNIBR, Sangju, Korea).

2.2. Morphological studies

Pure cultures of *B. circina*, *C. muscae*, and *M. ramosissimus* 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 10, 15, 20, 25, 30, 35, and 40 °C in the dark for 5 days. Fragments of mycelia were removed from the cultures, placed on microscope slides with lactophenol solution (Junsei Chemical Co. Ltd., Tokyo, Japan) and observed under a light microscope (Olympus, Tokyo, Japan). The fine structure of *C. muscae* was observed using scanning electron microscopy (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 h and then washed with 0.05 M cacodylate buffer (Junsei Chemical Co. Ltd.). Cellular membranes were preserved by fixing the samples in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) diluted in 0.05 M cacodylate buffer for 1 h, washing again in 0.05 M cacodylate buffer, dehydrating in graded ethanol (Emsure, Darmstadt, Germany) and isoamyl acetate (Junsei Chemical Co. Ltd.), and drying in a fume hood. Finally, the 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).

2.3. DNA extraction, PCR, and sequencing

Genomic DNA was extracted directly from the mycelia of fungal isolates using the Solgent

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
Backusella circina	CBS 128.70^{T}	JN206258	JN206529
B. circina	KH1	JX644544	JX644491
B. circina	CBS 323.69	JN206259	–
B. circina	KH9	–	JX644492
B. circina	**CNUFC-PTF2-1**	**MH262302**	**MH262312**
B. circina	**CNUFC-PTF2-2**	**MH262303**	**MH262313**
B. constricta	URM7322	KT937159	KT937156
B. indica	CBS 786.70	JN206255	JN206526
B. gigacellularis	CCIBt 3866	KF742415	–
B. grandis	CBS 186.87^{T}	JN206252	JN206527
B. lamprospora	CBS 118.08^{T}	JN206268	JN206531
B. lamprospora	CBS 195.28	JN206271	JN206530
B. locustae	CNUFC-SFB2^{T}	KY449291	KY449290
B. locustae	CNUFC-SFB4	KY449293	KY449292
B. oblongielliptica	CBS 568.70	JN206278	JN206533
B. oblongispora	CBS 569.70	JN206251	JN206407
B. recurva	KH6	–	JX644497
B. recurva	KH7	–	JX644498
B. recurva	CBS 318.52	JN206261	–
B. recurva	CBS 673.75	JN206264	–
B. tuberculispora	CBS 562.66	JN206267	JN206525
B. tuberculispora	CBS 570.70	JN206266	–
B. variabilis	CBS 564.66	JN206254	KC012658
B. variabilis	CBS 564.66	JN206253	–
Circinella angarensis	CBS 173.62	JN205849	JN206551
C. chinensis	CBS 140.28	JN205855	JN206549
C. lacrymispora	CBS 101757	JN206289	JN206608
C. minor	CBS 142.81	JN205861	JN206552
C. mucoroides	CYD1000719	KF805760	KF805746
C. muscae	CCD1000215	–	KF805745
C. muscae	CBS 141.28	JN205853	JN206548
C. muscae	CBS 107.13	JN205854	–
C. muscae	CYR003	–	KF805748
C. muscae	D00122901	KF805764	KF805750
C. muscae	**CNUFC-TF3-1**	**MH262304**	**MH262314**
C. muscae	**CNUFC-TF3-2**	**MH262305**	**MH262315**
C. simplex	CBS 428.80	JN206213	JN206445
C. umbellata	CBS 160.49	JN205858	HM849722
C. umbellata	CBS 101.16	JN205857	JN206553
Mucor amphibiorum	CBS 763.74^{T}	HM999957	–
M. amphibiorum	NRRL28633	–	AF113466
M. circinelloides	CBS 338.71	JN205998	–
M. circinelloides	CBS 635.65	JN205997	–
M. circinelloides	UTHSC 04-1961	–	FN650657
M. circinelloides	UTHSC 06-1667	–	FN650656
M. circinelloides f. *janssenii*	CBS 232.29	JN206007	–
M. circinelloides f. *janssenii*	CBS 206.68	JN206004	–
M. circinelloides f. *janssenii*	CBS 205.68^{NT}	HM999952	–
M. circinelloides f. *janssenii*	CBS 526.68	–	JN206426
M. circinelloides f. *circinelloides*	CBS 195.68	–	NG_055735
M. circinelloides f. *circinelloides*	Kw1378	–	FM246460
M. circinelloides f. *lusitanicus*	CBS 108.17	JN205980	FN650665
M. circinelloides f. *lusitanicus*	CBS 851.71	JN205982	–
M. circinelloides f. *lusitanicus*	CBS 111228	JN205989	–
M. circinelloides f. *lusitanicus*	CBS 242.33	JN205987	–
M. circinelloides f. *lusitanicus*	CBS 968.68	HM999953	–
M. circinelloides f. *lusitanicus*	UTHSC 03-1823	–	FN650662
M. circinelloides f. *lusitanicus*	NRRL 3631	–	AF113467
M. circinelloides f. *lusitanicus*	CBS 236.35	JN205979	–
M. fragilis	CTSP F1	EU862184	EU862173
M. fragilis	FSU 6164	EU484238	–
M. plumbeus	CBS 634.74	HM999955	–
M. plumbeus	CBS 226.32	JN205916	–
M. indicus	CBS 226.29	HM999956	HM849690
M. sinensis	CBS 204.74	JN205899	–
M. racemosus	UWFP 788	–	AY213712
M. racemosus f. *chibinensis*	CBS 636.67	JN205904	–
M. racemosus f. *racemosus*	CBS 260.68^{T}	–	NG_055727
M. ramosissimus	CBS 135.65^{NT}	NR_103627	FN650666
M. ramosissimus	ATCC 28933	–	AY213715
M. ramosissimus	**CNUFC-ESAF3-1**	**MH262306**	**MH262316**
M. ramosissimus	**CNUFC-ESAF3-2**	**MH262307**	**MH262317**
Phascolomyces articulosus	CBS 113.76	JX665039	JN206547
Rhizomucor pusillus	CBS 354.68	AF461764	HM849716
Zychaea mexicana	CBS 441.76	JN205845	JN206545

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

ATCC: American Type Culture Collection, Manassas, VA, USA; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CNUFC: Chonnam National University Fungal Collection, Gwangju, South Korea; ITS: internal transcribed spacer; NRRL (ARS Culture Collection, Peoria, Illinois); UTHSC, Fungal Testing Laboratory, Department of Pathology at the University of Texas Health Science Center, San Antonio, Texas, USA; ^{T} and ^{NT}: ex-type and ex-neotype strains.

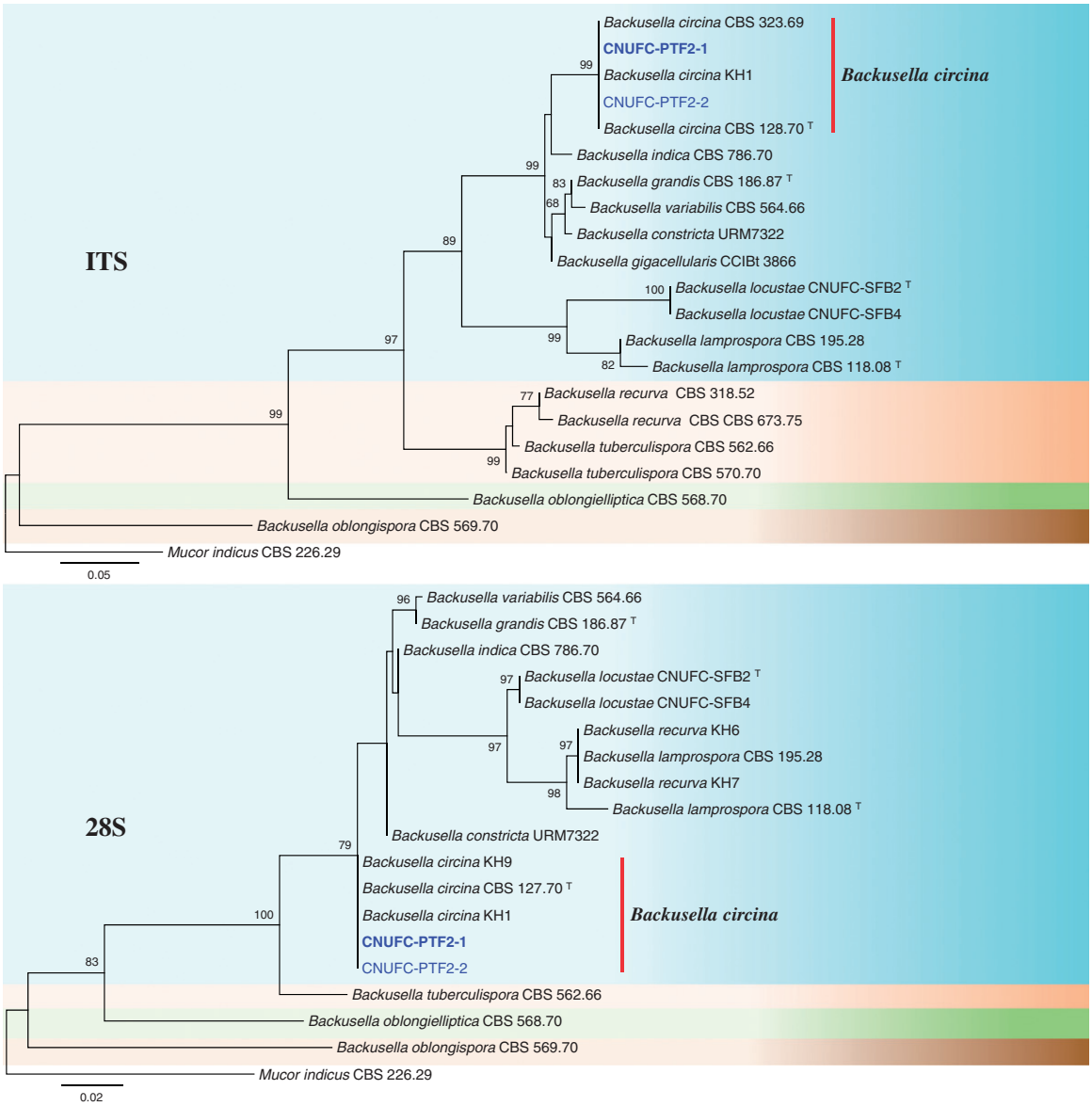

Figure 1. Phylogenetic tree based on maximum likelihood analysis of internal transcribed spacers (ITS) and 28S rDNA sequences for *Backusella circina* CNUFC-PTF2-1 and *B. circina* CNUFC-PTF2-2. *Mucor indicus* was used as the outgroup. Bootstrap support values ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

Genomic DNA prep Kit (Solgent Co. Ltd., Daejeon, South Korea). The ITS region and large subunit of 28S rDNA were amplified with the primer pairs ITS4 and ITS5 [29] and LROR and LR5F [30], respectively. The PCR 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 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).

2.4. Phylogenetic analysis

The fungal sequences obtained from the GenBank database (Table 1) were aligned using Clustal_X v.1.83 [31] and edited with Bioedit v.5.0.9.1 [32]. Phylogenetic analyses were performed using MEGA 6 software [33] and maximum likelihood (ML) was constructed by Kimura's two-parameter correction method. *M. amphibiorum*, *M. indicus*, and *Rhizomucor pusillus* were used as outgroups. The reliability of internal branches was assessed using the p-distance substitution model with 1000 bootstrap replicates. Sequence data were compared with similar sequences available in the GenBank databases using nucleotide Basic Local Alignment Search Tool (BLASTn).

3. Results

3.1. Phylogenetic analysis

A BLAST search of ITS sequences via the NCBI database indicated that the isolates CNUFC-PTF2-1, CNUFC-TF3-1, and CNUFC-ESAF3-1 most closely

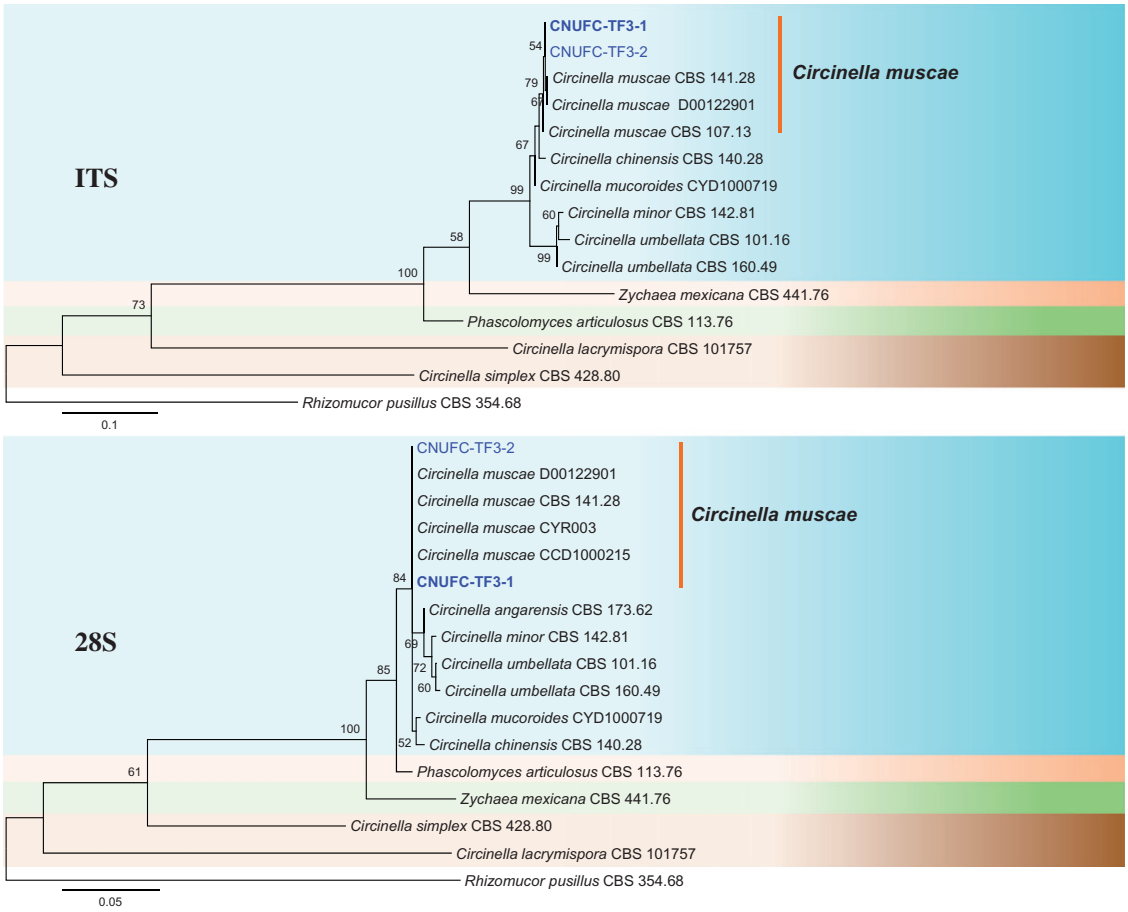

Figure 2. Phylogenetic tree based on maximum likelihood analysis of internal transcribed spacers (ITS) and 28S rDNA sequences for *Circinella muscae* CNUFC-TF3-1 and *C. muscae* CNUFC-TF3-2. *Rhizomucor pusillus* was used as the outgroup. Bootstrap support values ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

resembled *B. circina* (GenBank accession no. JX644544), *C. muscae* (GenBank accession no. KF805764), and *M. ramosissimus* (GenBank accession no. NR_103627) with 100% (637/637 bp), 99.8% (601/602 bp), and 99.8% (566/567 bp) homology, respectively. The 28S rDNA sequences of *B. circina* (GenBank accession no. JN206529), *C. muscae* (GenBank accession no. KF805750), and *M. ramosissimus* (GenBank accession no. FN650666) showed 100% (674/674 bp), 98.9% (550/556 bp), and 100% (694/694 bp) homology with the 28S rDNA sequences of the isolates CNUFC-PTF2-1, CNUFC-TF3-1, and CNUFC-ESAF3-1, respectively. Based on the ITS and 28S rDNA trees, the isolates CNUFC-PTF2-1, CNUFC-TF3-1, and CNUFC-ESAF3-1 were identical to *B. circina*, *C. muscae*, and *M. ramosissimus*, respectively (Figures 1–3).

Description: Colonies grew rapidly at 25 °C on SMA, filling the Petri plate (diameter, 90 mm) after 5 days of incubation. The colonies were initially white, but later turned light gray. The colony reverse was light gray. Sporangiophores were 6–20 μm wide, erect, branched, and irregular. Sporangia were globose to subglobose, multispored, and measured 27.5–66.5 μm × 27.0–63.5 μm. Columellae were subglobose to oblong, and measured 18.1–30.4 μm × 20.3–34.6 μm. Unispored sporangiola were abundant, globose to subglobose, wall spinulose, and measured 10.3–19.5 μm × 10.0–19.1 μm. Sporangiospores were subglobose to ovoid, and measured 6.5–11.0 μm × 6.0–10.2 μm. Zygospores were not observed on this medium. Optimal growth was observed at 25 °C, slow growth was observed at 10 and 35 °C, and no growth was observed at 37 °C.

3.2. Taxonomy

3.2.1. Taxonomy of CNUFC-PTF2-1

Backusella circina J.J. Ellis & Hesselt., Mycologia 61: 865 (1969) (Table 2, Figure 4).

=*Mucor pseudolamprosporus* H. Nagan. & Hirahara, Hiroshima Jogakuin College Bull.: 167 (1968)

3.2.2. Taxonomy of CNUFC-TF3-1

Circinella muscae (Sorokin) Berl. & De Toni, Sylloge Fungorum 7: 216 (1888) (Table 3, Figure 5).

≡*Helicostylum muscae* Sorokin, Bull. Soc. Imp. Nat. Moscou: 256 (1870)

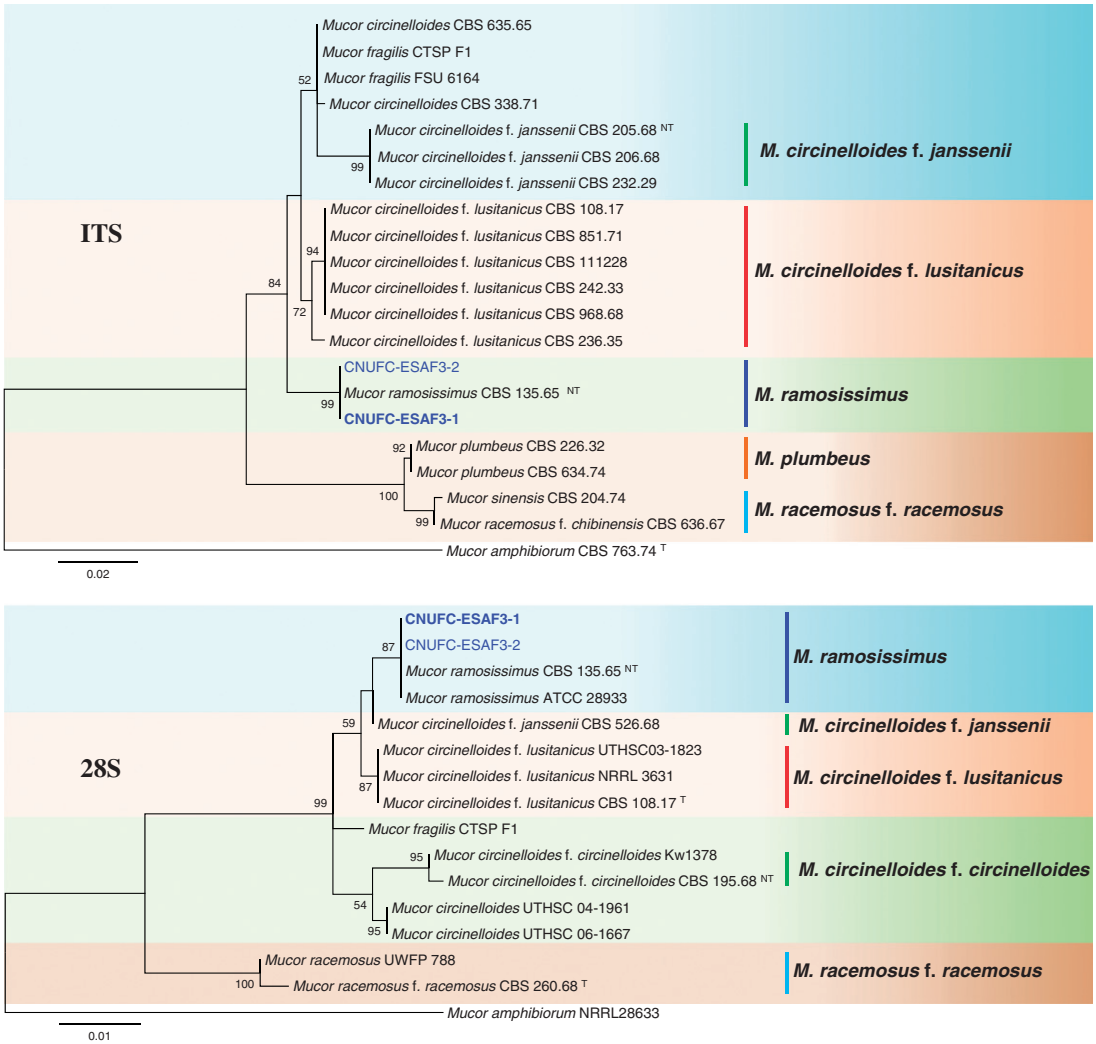

Figure 3. Phylogenetic tree based on maximum likelihood analysis of internal transcribed spacers (ITS) and 28S rDNA sequences for *Mucor ramosissimus* CNUFC-ESAF3-1 and *M. ramosissimus* CNUFC-ESAF3-2. *Mucor amphibiorum* was used as the outgroup. Bootstrap support values ≥50% are indicated at the nodes. The bar indicates the number of substitutions per position.

Table 2. Morphological characteristics of CNUFC-PTF2-1 compared to *Backusella circina* reference strain.

Character	CNUFC-PTF2-1	*Backusella circina*[a]
Colony color	Rapid-growing, first white then light gray, reverse light gray	Rapid-growing, first white then light olive-gray
Sporangiophores	6.0–20.0 µm in width, variable in length	Up to 9–16 µm in width, variable in length
Sporangia	Globose to subglobose, multispored, 27.5–66.5 × 27.0–63.5 µm	Globose and subglobose, 35–100 µm
Columellae	Subglobose to oblong, 18.1–30.4 × 20.3–34.6 µm	Subglobose to oblong, 11–35 × 11–30 µm
Unispored sporangiola	Globose to subglobose 10.3–19.5 × 10.0–19.1 µm	Globose to subglobose, (4.5–)6–16(–26) µm in diam., wall spinulose
Sporangiospores	Subglobose to ovoid, 6.5–11.0 × 6.0–10.2 µm	Subglobose to ovoid, (6.4–)7.2–10 (–12.8) × (5.6–)6.4–9.2 (–10) µm
Zygospores	Absent	Globose to subglobose, (35–)40–70(–80) µm in diam.

[a]From the description by Ellis and Hesseltine [5].

=*Circinella spinosa* Tiegh. & G. Le Monn., Annales des Sciences Naturelles Botanique 17: 305 (1873)

=*Circinella sydowii* Lendn., Bulletin de la Société Botanique de Genève 5: 29 (1913)

Description: Colonies grew rapidly at 25 °C on SMA, filling the Petri plate (diameter, 90 mm) after 7 days of incubation. The colonies were initially white, but later turned brown. The colony reverse was brown and irregularly zonate. Sporangiophores were 6.3–10.8 μm in width, variable in length, and often circinate below the sporangium. Sporangia were globose, yellow to dark gray, multispored, and measured 31.9–70.2 μm × 31.5–69.2 μm. Collumellae were diverse in shape, pyriform, subglobose, oval, conical, and measured 17.5–43.3 μm × 15.5–36.5 μm. Sporangiospores were globose and measured 4.1–9.5 μm. Zygospores were not observed on this medium. Optimal growth was observed at 25 °C, slow growth was

Figure 4. Morphology of *Backusella circina* CNUFC-PTF2-1. (A) Colony in synthetic mucor agar after 5 days at 25 °C; (B) Forming unispored sporangiola on curved short sporangiophores; (C) Sporangium on curved simple sporangiophores; (D–F) Columellae; (G) Sporangiospores (scale bars: B–G = 20 μm).

Table 3. Morphological characteristics of CNUFC-TF3-1 compared to *Circinella muscae* reference strain.

Character	CNUFC-TF3-1	*Circinella muscae*[a]
Colony color	Rapid-growing, first white then brown; reverse brown, irregularly zonate	Rapid-growing, first white then Saccardo's Olive
Sporangia	Globose, multispored, yellow to dark gray with age, 31.9–70.2 × 31.5–69.2 μm	Globose, multispored, 30–62 μm, some up to 100 μm
Columellae	Pyriform, subglobose, oval, conical, 17.5–43.3 × 15.5–36.5 μm	Pyriform, oblong, or conical 15–20 × 20–36 μm
Sporangiospores	Globose, 4.1–9.5 μm	Globose, sometimes short oval, 3–7 μm, mostly 5–6 μm
Zygospores	Absent	Globose, golden brown to reddish brown, 30–65 μm

[a]From the description by Hesseltine and Fennell [12].

observed at 10 and 35 °C, and no growth was observed at 40 °C.

3.2.3. Taxonomy of CNUFC-ESAF3-1

Mucor ramosissimus Samouts., Mater. Mikol. Fitopat. Ross.: 210 (1927) (Table 4, Figure 6)

Description: Colonies grew rapidly at 25 °C on SMA, filling the Petri plate (diameter, 90 mm) after 5 days of incubation. The color of the colony was gray. Sporangia were globose to subglobose and measured 15.5–60.1 μm × 14.8–56.3 μm. Columellae were globose, cylindrical–ellipsoidal, and measured 12.7–41.8 μm × 11.8–36.8 μm. Sporangiospores were subglobose, ellipsoidal, and measured 4.3–8.5 μm × 3.9–6.8 μm. Zygospores were not observed on this medium. Optimal growth was observed at 25 °C, slow growth was observed at 10 °C, and no growth was observed at 35 °C.

4. Discussion

To date, few studies have reported new and undescribed zygomycetous fungi in Korea [10,23,34,35]. Particularly, species of *Backusella* and *Circinella* are rarely found in Korea. Thus, our finding of *B. circina*, *C. muscae*, and *M. ramosissimus* species not only establishes new records, but also provides knowledge regarding the occurrence and distribution of rare species within these genera.

Variability in nucleotide sequences in the ITS region has been reported by several authors as a critical barcode marker for identifying fungi at the species level, including in the order Mucorales [36,37]. In previous studies, we successfully identified species of Mucorales using this marker [10,22,23,38].

In the ITS and 28S phylogenetic trees, CNUFC-PTF2-1 and CNUFC-PTF2-2 isolated from soil samples clustered in the clade containing *B. circina* CBS

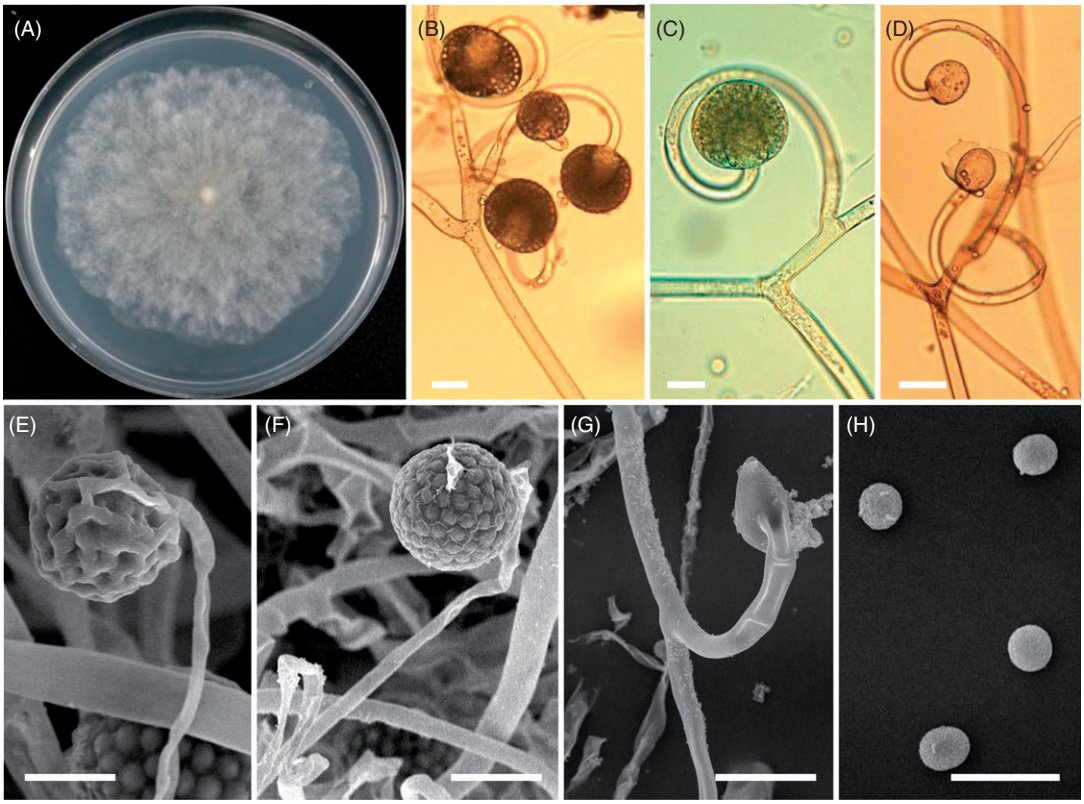

Figure 5. Morphology of *Circinella muscae* CNUFC-TF3-1. (A) Colony in synthetic mucor agar after 5 days at 25 °C; (B–D, G) Sporangia and columellae borne on circinate sporangiophores; (E, F) Young and mature multispored sporangia; (H) Sporangiospores. (B–D: observed under light microscope; E–H: observed by scanning electron microscopy) (scale bars: B–F, G = 20 μm, H = 10 μm).

Table 4. Morphological characteristics of CNUFC-ESAF3-1 compared to *Mucor ramosissimus* reference strain.

Character	CNUFC-ESAF3-1	*Mucor ramosissimus*[a]
Colony color	Rapid-growing, gray	Deep olive gray to mouse gray
Sporangia	Globose to subglobose, 15.5–60.1 × 14.8–56.3 μm	Globose, up to 70 (80) μm
Columellae	Globose, cylindrical–ellipsoidal, 12.7–41.8 × 11.8–36.8 μm	Applanate, up to 40 × 50 μm
Sporangiospores	Subglobose, ellipsoidal, 4.3–8.5 × 3.9–6.8 μm	Subglobose to broadly ellipsoidal, 4–7(8) μm in diam. or 5–8 × 4.5–6 μm
Zygospores	Absent	Absent

[a]From the description by Schipper [21].

128.70 (type species) (Figure 1). Although the morphological features of our isolate were similar to those of *B. circina* described by Ellis and Hesseltine [5], there were differences in the diameter of sporangia and unispored sporangiola. Sporangia sizes reported in literature range from 35 to 100 μm [5], which are larger than our maximum measurements. The unispored sporangiola (4.5–)6–16(–26) μm [5] was larger than that of our isolate. The *B. circina* strain may exhibit morphological similarities to *B. lamprospora*, such as the production of subglobose sporangiospores [7]. However, *B. circina* differs from *B. lamprospora* in its production of large numbers of spiny-walled and unispored sporangiola. Furthermore, in the phylogenetic tree, the strain formed a separate branch from that of *B. lamprospora*. Molecular data confirmed the morphological identification of CNUFC-PTF2-1 as *B. circina*.

Isolates CNUFC-TF3-1 and CNUFC-TF3-2 formed a group with strains of *C. muscae* (Figure 2). The results of our analysis of molecular data were consistent with the phylogeny presented by Walther et al. [4]. Comparing the colony morphology and culture characteristics of the isolate on SMA medium with previous descriptions by Hesseltine and Fennell [12], the present isolate was generally similar to isolates of *C. muscae*. Gonzalez et al. [17] and Zheng et al. [18] isolated this species from sand beach and hydrocarbon-polluted sand, respectively. Accordingly, this is the first reported isolation of *C. muscae* from a leaf of *T. sylvestre*. *C. muscae* has been shown to transform androst-4-ene-3,17-dione and produce extracellular enzymes such as proteases [39,40]. This finding suggests that strain CNUFC-TF3-1 may be useful in biotechnological applications and requires further investigation.

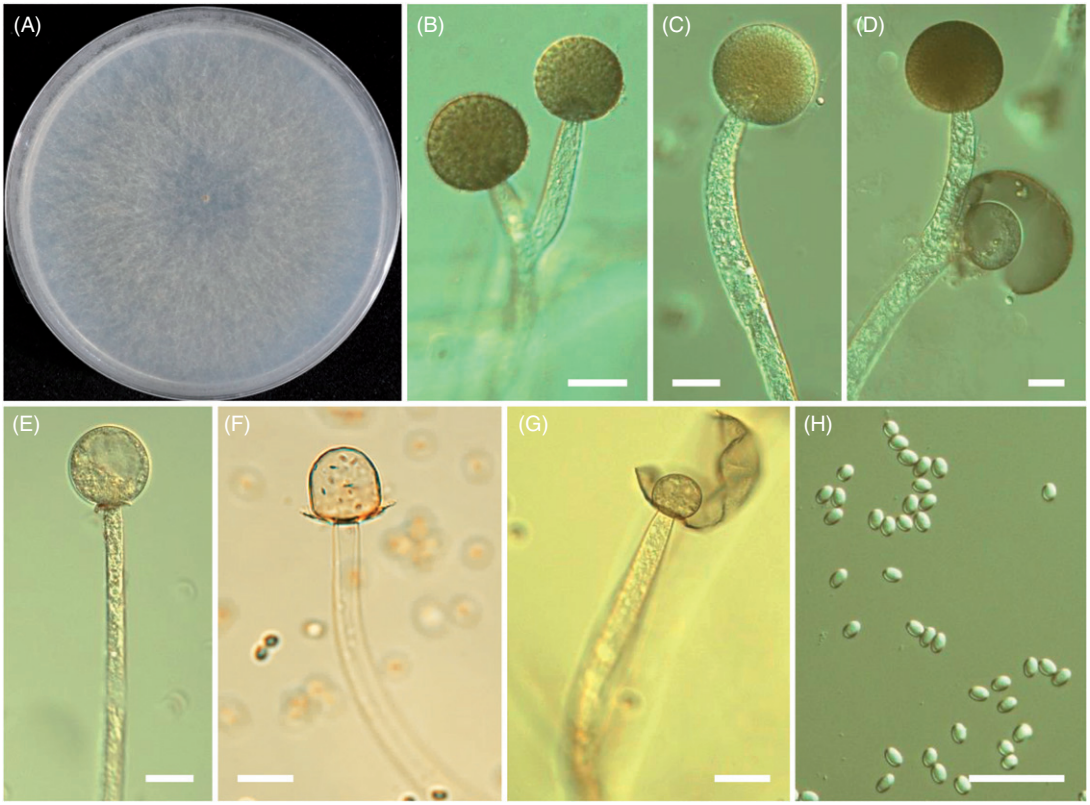

Figure 6. Morphology of *Mucor ramosissimus* CNUFC-ESAF3-1. (A) Colonies in synthetic mucor agar after 5 days at 25 °C; (B–D) Branched sporangiophores and sporangia; (E–G) Columella with clear collar present; (H) Sporangiospores (scale bars: B–H = 20 μm).

Isolate CNUFC-ESAF3-1 was grouped with strains of *M. ramosissimus* CBS 135.65 (neo-type species) based on phylogenetic analyses (Figure 3). The morphological characteristics of the *M. ramosissimus* isolate in this study were similar to those previously described by Schipper [21]. However, sporangia were smaller than that of *M. ramosissimus* described by Schipper (up to 70–80 μm). The *M. ramosissimus* strain may be confused with *M. circinelloides* due to its production of subglobose sporangiospores and sympodially branched sporangiophores. However, there are clear genetic differences between these two species. The results of our analysis of molecular data of this species were consistent with the phylogeny presented by Álvarez et al. [41] and Walther et al. [4]. Species of *M. ramosissimus* produce extracellular enzymes, such as endopolygalacturonase and lipase, and secondary metabolites, such as phytoalexin elicitor [42–44]. Recently, several studies have focused on applying Mucorales members to produce ethanol and biomass by-product [45]. Particularly, *M. ramosissimus* has been reported as a potential ethanol-producing mold [46].

This is the first report of *B. circina*, *C. muscae*, and *M. ramosissimus* in Korea. Future studies should investigate their ability to produce extracellular enzymes and potential applications in biotechnology.

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.

ORCID

Hyang Burm Lee http://orcid.org/0000-0001-5353-2586

References

[1] Hibbett DS, Binder M, Bischoff JF, et al. A higher-level phylogenetic classification of the fungi. Mycol Res. 2007;111:509–547.

[2] Benny GL. The methods used by Dr. R. K. Benjamin, and other mycologists, to isolate Zygomycetes. Aliso. 2008;26:37–61.
[3] Hoffmann K, Pawłowska J, Walther G, et al. The family structure of the Mucorales: a synoptic revision based on comprehensive multigene-genealogies. Persoonia. 2013;30:57–76.
[4] Walther G, Pawłowska J, Alastruey-Izquierdo A, et al. DNA barcoding in Mucorales: an inventory of biodiversity. Persoonia. 2013;30:11–47.
[5] Ellis JJ, Hesseltine CW. Two new members of the Mucorales. Mycologia. 1969;61:863–872.
[6] Kirk PM. Nomenclatural novelties. Index Fungorum. 2012;11:1–1.
[7] Benny GL, Benjamin RK. Observations on *Thamnidiaceae* (Mucorales). New taxa, new combinations, and notes on selected species. Aliso. 1975;8:301–351.
[8] de Souza JI, Marano AV, Pires-Zottarelli CLA, et al. A new species of *Backusella* (Mucorales) from a Cerrado reserve in Southeast Brazil. Mycol Prog. 2014;13:975–980.
[9] Lima DX, Voigt K, de Souza CAF, et al. Description of *Backusella constricta* sp. nov. (Mucorales, ex Zygomycota) from the Brazilian Atlantic Rainforest, including a key to species of *Backusella*. Phytotaxa. 2016;289:59–68.
[10] Wanasinghe DN, Phukhamsakda C, Hyde KD, et al. Fungal diversity notes 709–839: taxonomic and phylogenetic contributions to fungal taxa with an emphasis on fungi on *Rosaceae*. Fungal Divers. 2018;89:1–236.
[11] van Tieghem P, Le Monnier G. Recherches sur les Mucorinées. Ann Sci Nat. 1873;17:261–399.
[12] Hesseltine CW, Fennell DI. The genus *Circinella*. Mycologia. 1955;47:193–212.
[13] Hesseltine CW, Ellis JJ. Notes on Mucorales, especially *Absidia*. Mycologia. 1961;53:406–426.
[14] Faurel L, Schotter G. Notes mycologiques VI. Sur quelques champignons coprophiles d'Afrique Equatoriale. Cah La Maboké. 1965;3:123–133.
[15] Patil SD, Kale JC. A new species of *Circinella* van Tiegh. and Le Monn. Curr Sci. 1981;50:544–544.
[16] Arambarri AM, Cabello MN. *Circinella lacrymispora* sp. nov., a new mucoral isolated from Argentine soils. Mycotaxon. 1996;57:145–149.
[17] González MC, Murueta-Figueroa N, Medina-Ortiz C, et al. New record of *Circinella muscae* from a hydrocarbon polluted sand beach of Tabasco, Mexico. Mycotaxon. 2010;113:111–117.
[18] Zheng RY, Liu XY, Wang YN. *Circinella* (Mucorales, Mucoromycotina) from China. Mycotaxon. 2017;132:43–62.
[19] Spatafora JW, Benny GL, Lazarus K, et al. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia. 2016;108:1028–1046.
[20] Benny GL, Humber RA, Voigt K. Zygomycetous fungi: Phylum Entomophthoromycota and subphyla Kickxellomycotina, Mortierellomycotina, Mucoromycotina, and Zoopagomycotina. In: McLaughlin DJ, Spatafora JW, editors. Mycota VII, Part A, Systematics and evolution. New York (NY): Springer-Verlag; 2014. p. 209–250.
[21] Schipper MAA. On *Mucor circinelloides*, *Mucor racemosus* and related species. Stud Mycol. 1976; 12:1–40.
[22] Nguyen TT, Duong TT, Lee HB. Characterization of two new records of Mucoralean species isolated from gut of soldier fly larva in Korea. Mycobiology. 2016;44:310–313.
[23] Nguyen TT, Jung HY, Lee YS, et al. Phylogenetic status of two undescribed zygomycete species from Korea: *Actinomucor elegans* and *Mucor minutus*. Mycobiology. 2017;45:344–352.
[24] Souza CAFD, Lima DX, Gurgel LMS, et al. Coprophilous Mucorales (ex Zygomycota) from three areas in the semi-arid of Pernambuco, Brazil. Braz J Microbiol. 2017;48:79–86.
[25] Thompson DP, Eribo BE. Extracellular enzyme production by *Rhizopus* and *Mucor* species on solid media. Can J Microbiol. 1984;30:126–128.
[26] Alves MH, Campos-Takaki GM, Porto ALF, et al. Screening of *Mucor* spp. for the production of amylase, lipase, polygalacturonase and protease. Braz J Microbiol. 2002;33:325–330.
[27] de Souza PM, Bittencourt MLA, Caprara CC, et al. A biotechnology perspective of fungal proteases. Braz J Microbiol. 2015;46:337–346.
[28] Ribes JA, Vanover-Sams CL, Baker DJ. Zygomycetes in human disease. Clin Microbiol Rev. 2000;13:236–301.
[29] White TJ, Bruns T, Lee S, et al. 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*. San Diego, CA, USA: Academic Press; 1990. p. 315–322.
[30] Lee HB. Molecular phylogenetic status of Korean strain of *Podosphaera xanthii*, a causal pathogen of powdery mildew on Japanese thistle (*Cirsium japonicum*) in Korea. J Microbiol. 2012;50:1075–1080.
[31] Thompson JD, Gibson TJ, Plewniak F, et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–4882.
[32] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–98.
[33] Tamura K, Stecher G, Peterson D, et al. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–2729.
[34] Lee YS, Jung HY, Lee HB, et al. National list of species of Korea Ascomycota, Glomeromycota, Zygomycota, Myxomycota, Oomycota.Korea: National Institute of Biological Resources Ministry of Environment; 2015.
[35] Nguyen TT, Choi YJ, Lee HB. Isolation and characterization of three unrecorded Zygomycete fungi in Korea: *Cunninghamella bertholletiae*, *Cunninghamella echinulata*, and *Cunninghamella elegans*. Mycobiology. 2017;45:318–326.
[36] Schwarz P, Bretagne S, Gantier JC, et al. Molecular identification of Zygomycetes from culture and experimentally infected tissues. J Clin Microbiol. 2006;44:340–349.
[37] White MM, James TY, O'Donnell K, et al. Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. Mycologia. 2006;98:872–884.
[38] Li GJ, Hyde HD, Zhao RL, et al. Fungal diversity notes 253-366: taxonomic and phylogenetic contributions to fungal taxa. Fungal Divers. 2016;78: 1–237.

[39] de Azevedo Santiago ALCM, de Souza-Motta CM. Isolation of Mucorales from processed maize (*Zea mays* L.) and screening for protease activity. Braz J Microbiol. 2008;39:698–700.

[40] Heidary M, Habibi Z. Microbial transformation of androst-4-ene-3,17-dione by three fungal species *Absidia griseolla* var. *igachii*, *Circinella muscae* and *Trichoderma virens*. J Mol Catalysis B Enzymatic. 2016;126:32–36.

[41] Álvarez E, Cano J, Stchigel AM, et al. Two new species of *Mucor* from clinical samples. Med Mycol. 2011;49:62–72.

[42] Simões K, Dietrich SMC, Hahn MG, et al. Purification and characterization of a phytoalexin elicitor from spores of the saprobe *Mucor ramosissimus*. Rev Bras Bot. 2005;28:735–744.

[43] Marques MR, Buckeridge MS, Braga MR, et al. Characterization of an extracellular endopolygalacturonase from the saprobe *Mucor ramosissimus* Samutsevitsch and its action as trigger of defensive response in tropical plants. Mycopathologia. 2006; 162:337–346.

[44] Ko HS, Taguchi H, Takizawa K, et al. The enzymatic approach of zygomycosis – causing Mucorales. Kor J Med Mycol. 2007;12:9–17.

[45] Satari B, Karimi K. Mucoralean fungi for sustainable production of bioethanol and biologically active molecules. Appl Microbiol Biotechnol. 2018; 102:1097–1117.

[46] Schwarz P, Lortholary O, Dromer F, et al. Carbon assimilation profiles as a tool for identification of zygomycetes. J Clin Microbiol. 2007;45:1433–1439.