RESEARCH ARTICLE

First Report of *Chloridium chlamydosporum* Isolated in Korea

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ABSTRACT

A fungal strain designated KNUF-21-010 was isolated from soil obtained in Yangsan-si, Gyeongsangnam-do, Korea. Cultural characteristics, morphological features, and multilocus sequence analysis based on the internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (*LSU*), and translation elongation factor 1-alpha (*tef1-α*) genes were employed to identify the strain. The strain was cultivated on potato carrot agar (PCA) and oatmeal agar (OA), and after 4 weeks of incubation at 25°C, colony diameters ranged from 58.4–64.2 mm on PCA and 68.0–72.4 mm on OA. Brown, unbranched conidiophores; hyaline to pale brown hyphae; obovate to ellipsoidal conidia; and globose to subglobose chlamydospores were observed. These features closely matched those of *Chloridium chlamydosporum*. BLAST analysis of the ITS, *LSU*, and *tef1-α* sequences reveals that strain KNUF-21-010 shared > 99% sequence similarity with *Chl. chlamydosporum*. Phylogenetic analysis further confirmed that the strain formed a highly supported clade with *Chl. chlamydosporum*. Based on cultural, morphological, and molecular phylogenetic analyses, strain KNUF-21-010 was identified as *Chl. chlamydosporum*. This study represents the first report of *Chl. chlamydosporum* in Korea.

Keywords: *Chloridium chlamydosporum*, Saprophytic fungi, Soil fungi

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INTRODUCTION

The genus *Chloridium* was established by Link in 1809, with *Chloridium virescens* designated as the type species [1]. *Chloridium* comprises saprophytic fungi and is classified in the family Chaetosphaeriaceae, order Chaetosphaeriales, and class Sordariomycetes. Chaetosphaeriaceae, one of the largest families within Sordariomycetes, is commonly found on various decaying plant substrates, including leaves, fruits, branches, bark, and wood [2]. In this family, *Chloridium* also thrives on decaying plant materials in terrestrial and freshwater habitats [3]. Traditionally, species in *Chloridium* are regarded as saprophytic fungus that primarily grows on wood or other plant debris [4]. However, recent studies show that some species are frequently isolated from soil, indicating that members of *Chloridium* can also be classified as soilborne fungi [3,5].

The genus *Chloridium* was originally proposed for a single species, *Chl. viride* [1], which is

morphologically characterized based on pigmented, unbranched, erect, caespitose conidiophores and hyaline, globose conidia that adhere in green masses at the tip [3]. Subsequent taxonomic studies show that *Chl. viride* is conspecific with *Dematium virescens* (1794) [3]. According to the principle of nomenclatural priority, the epithet "virescens" takes precedence over *Chl. viride*, and the species name is accordingly revised to *Chl. virescens* [4].

Chl. chlamydosporum is initially described under the genus *Bisporomyces* based on its distinct morphological characteristics, including conidiophores that produce pairs of conidia at the tip of the phialide [6,7]. In subsequent years, the taxonomic relationships among *Bisporomyces chlamydosporum*, *Chl. virescens*, and *Cirrhomyces caudiger* was debated owing to their morphological similarities. Hughes later synonymizes the genera *Cirrhomyces* and *Bisporomyces* with *Chloridium*, recognizing five species within the *Chloridium*, including *Chl. chlamydosporum* [8].

The application of molecular phylogenetic methods in recent fungal taxonomy studies enables a more accurate understanding of taxonomic and phylogenetic relationships [2,3]. Traditionally circumscribed based on morphological characteristics, the genus *Chloridium* undergoes continuous taxonomic revisions at the genus and species levels with the advent of molecular phylogenetics. This approach addresses the limitations of morphology-based classification, and subsequent studies reveal that *Chloridium* is a polyphyletic group [2,3,9,10]. Since the genus *Chloridium* is revealed to be polyphyletic, researchers collect genetic data from *Chloridium* species and conduct phylogenetic analyses to revise its classification [2,3]. These studies highlight the importance of accurate classification through molecular approaches in fungal taxonomy.

This study aims to isolate the strain KNUF-21-010, presumed to be *Chloridium chlamydosporum,* from soil collected in Yangsan-si, Gyeongsangnam-do, which has not previously been revealed in Korea. To achieve accurate identification of this fungus, cultural, morphological, and molecular phylogenetic analyses were conducted. Multilocus sequence analysis (MLSA) was performed based on the internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (*LSU*), and translation elongation factor 1-alpha (*tef1-α*).

MATERIALS AND METHODS

Sample collection and fungal isolation

The strain KNUF-21-010 was collected from the soil in Yangsan-si, Gyeongsangnam-do, Korea (35°30'53.1"N 129°03'08.1"E). The standard serial dilution method was performed to isolate fungal strains from the soil [11]. One gram of collected soil sample was added to 10 mL of sterile distilled water and mixed thoroughly by vortexing. A 100 μL of the suspension was spread onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates, which were incubated in the dark at 25°C for approximately 3 days. Single colonies were transferred to a fresh PDA plate and incubated at 25°C. This step was repeated to obtain pure cultures, and several fungal strains were isolated. DNA was extracted, and polymerase chain reaction (PCR) amplification of the ITS regions was conducted. Among these strains, the ITS sequence of KNUF-

21-010 was selected for further taxonomic study because it indicated a potentially unreported fungal species in Korea. Subsequently, cultural, morphological, and molecular phylogenetic analyses were conducted to identify the strain. The isolate KNUF-21-010 was deposited at the National Institute of Biological Resources as a metabolically inactive culture (NIBRFGC000509190).

Morphological and cultural characterization

The cultural characteristics of strain KNUF-21-010 were examined on potato carrot agar (PCA; HiMedia, Mumbai, India) and oatmeal agar (OA; Difco, Detroit, MI, USA) plates. Colonies on both media were incubated at 25°C for 4 weeks in the dark. The features of the colonies on each medium, including color, shape, and size, were compared after 4 weeks of incubation. Colonies were imaged after 3 weeks using a Canon EOS 5D Mark III digital camera (Canon, Tokyo, Japan). To observe morphological characteristics, slide cultures were prepared from colonies grown on PCA. The morphological features, including hyphae, conidiophore, chlamydospores, conidia, and conidiogenous cells, were observed after 1~2 weeks of incubation at 25°C in the dark, under a light microscope (BX50, Olympus, Tokyo, Japan).

DNA extraction, PCR amplification, and sequencing

The strain KNUF-21-010 was incubated on PDA at 25°C for 2 weeks. DNA was extracted from the hyphae of this colony using a HiGene Genomic DNA Prep Kit (Biofact, Daejeon, South Korea), following the protocol of the manufacturer. The extracted DNA was used to amplify the ITS regions, *LSU,* and *tef1-α*. The ITS regions were amplified with the primer pair V9G/LR8 [12,13], and the *tef1-α* was amplified with the primer pair EF1-983F/EF1-2218R [14]. PCR amplicons were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The purified amplicons were sequenced using Macrogen (Sejong, South Korea). Sequencing of the *LSU* was performed using primer JS1, JS7, JS8, and LR7 [15]. The ITS, *LSU*, and *tef1-α* sequences of strain KNUF-21-010 were deposited in GenBank under the accession numbers LC886056 (ITS), LC886057 (*LSU*), and LC886058 (*tef1-α*).

Molecular phylogenetic analyses

Sequences of each region were compared with those of other species using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Table 1 presents the assembled DNA data registered in NCBI, used to determine the phylogenetic position of strain KNUF-21-010 relative to other species. The collected gene data from each region were aligned using Clustal X (version 2.0). Phylogenetic trees were constructed using the maximum likelihood method with the Kimura 2-parameter model and 1,000 bootstrap replicates [16]. The trees were generated using MEGA software (version 12.0) [17].

Table 1. List of species used in phylogenetic analysis along with their GenBank accession numbers

Species	Strain	GenBank accession numbers		
		ITS	*LSU*	*tef1-α*
Chloridium bellum	CBS 709.73A[T]	OP455360	OP455466	OP464934
Chloridium biforme	ICMP 23429[T]	OP455363	OP455470	OP464937
Chloridium caesium	CBS 145633	OP455367	OP455474	OP464941
Chloridium caudigerum	CBS 138691	OP455374	OP455481	OP464947
Chloridium chlamydosporum	CBS 114.41[T]	OP455385	OP455492	OP464958
Chloridium chlamydosporum	CBS 149052	OP455389	OP455496	OP464962
Chloridium chlamydosporum	**KNUF-21-010**	**LC886056**	**LC886057**	**LC886058**
Chloridium chloridioides	CBS 239.75A	OP455394	OP455501	OP464967
Chloridium detriticola	CBS 345.67[T]	MH858992	MH870689	OP464974
Chloridium elongatum	CBS 147816[T]	OP455403	OP455510	OP464978
Chloridium gamisii	CBS 667.75[T]	OP455415	OP455522	OP464990
Chloridium guttiferum	CBS 126073[T]	MH864068	MH875524	OP464991
Chloridium humicola	CBS 420.73[T]	OP455417	OP455524	OP464993
Chloridium novae-zelandiae	ICMP 22736[T]	OP455423	OP455530	OP464998
Chloridium peruense	CBS 126074[T]	OP455424	OP455531	OP464999
Chloridium setosum	CBS 263.76A	OP455427	OP455534	OP465002
Chloridium subglobosum	CBS 134152	OP455425	OP455532	OP465000
Chloridium virescens	CBS 145481	OP455439	OP455547	OP465014
Chloridium volubile	CBS 144661[T]	OP455446	OP455554	OP465018
Lomaantha folliculata	CBS 147152	OL654105	OL654162	OL654033

ITS: internal transcribed spacer regions; *LSU*: large subunit ribosomal RNA gene; *tef1-α*: translation elongation factor-1 alpha. [T]Type strain. The strain isolated in this study is indicated in bold.

RESULTS

Cultural and morphology characteristics

Colonies were grown on PCA and OA to compare the cultural characteristics of strain KNUF-21-010 with those of *Chl. chlamydosporum* and *Chl. peruense*. On PCA, colonies of strain KNUF-21-010 were whitish beige at the center, cinnamon to brown towards the periphery, and dark olivaceous grey towards the margin, reaching 58.4–64.2 mm in diameter after 4 weeks of incubation. They were circular, flat, margin fimbriate, velvety to cobwebby in texture, with a brown reverse (Fig. 1A, 1B). On OA, colonies were olivaceous dark grey, sometimes producing brown pigment. They were circular, flat, velvety to cobwebby, with an entire margin and a brown reverse (Fig. 1C, 1D). The colonies peaked at 68.0–72.4 mm in diameter after 4 weeks of incubation. Following microscopic observation of KNUF-21-010, the features of its conidiophores, conidia, chlamydospores, and vegetative hyphae were examined and compared. The conidiophores of KNUF-21-010 were brown, becoming paler toward the apex, macronematous, straight, unbranched, septate, and cylindrical, measuring 34–152(–245) × 2.7–3.5(–4.07) μm (Fig. 2A, 2B). Phialides that form conidia were present at the apices of the conidiophores (Fig. 2C). KNUF-21-010 produced chlamydospores that were lateral, sessile, intercalary, terminal, solitary, or arranged in short chains; globose to subglobose, thick-walled, smooth, and brown (Fig. 2D–F). The chlamydospores peaked at 4.75–6.21 × 4.16–5.41 μm. The strain also produced hyaline conidia that turned pale brown with age (Fig.

2G). The conidia were obovate to ellipsoidal, smooth, aseptate, with dimensions of 4.32–5.00 × 2.81–3.80 μm. The vegetative hyphae were hyaline to pale brown, septate, smooth, and 2.16–3.86 μm wide. Most microscopic features observed in KNUF-21-010 corresponded to those of *Chl. chlamydosporum*, the reference species (Table 2). *Chl. peruense* exhibited greater growth on PCA and OA media and produced smaller conidia than KNUF-21-010 and *Chl. chlamydosporum*.

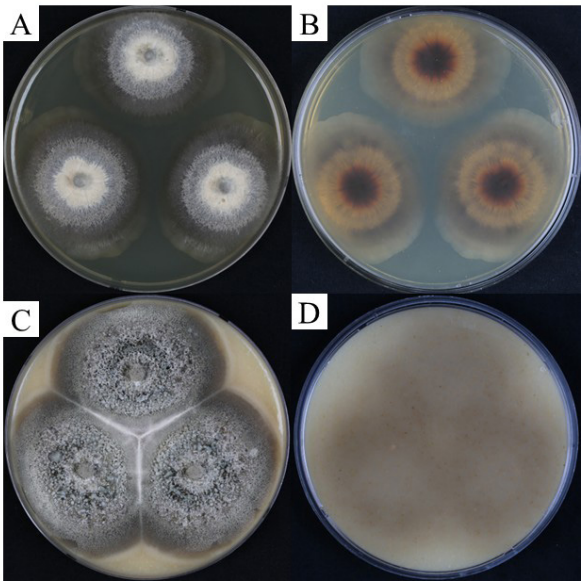

Fig. 1. Colony of KNUF-21-010 (*Chloridium chlamydosporum*) on oatmeal agar (OA) and potato carrot agar (PCA) after 28 days at 25°C. A, B: Colony on PCA; C, D: Colony on OA.

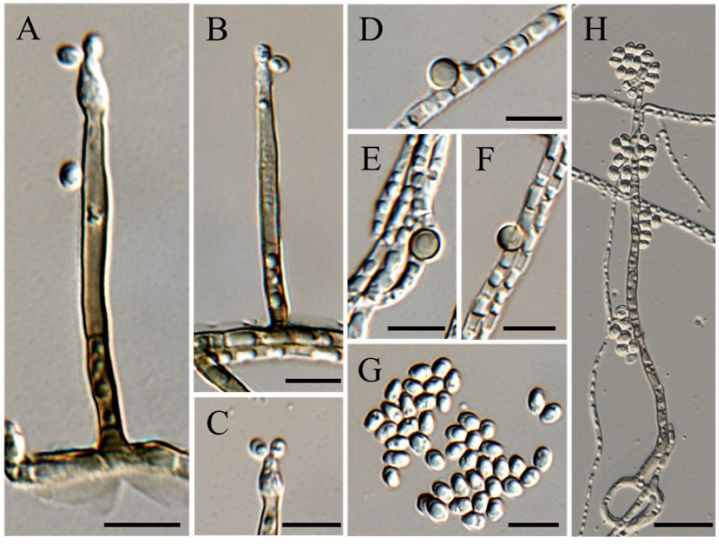

Fig. 2. Morphological characteristics of *Chloridium chlamydosporum*. A–B: Conidiophores; C: Tip of the phialide; D–F: Chlamydospores; G: Conidia; H: Conidiophores with multiple conidia. Scale bars: A–G, 10 μm; H, 20 μm.

Table 2. Comparison of morphological characteristics of KNUF–21–010 with *Chloridium chlamydosporum* and *Chloridium peruense*

Characteristic		*Chloridium chlamydosporum* KNUF- 21-010	*Chloridium chlamydosporum* CBS 114.41^{T}	*Chloridium peruense* CBS 126074^{T}
Colony	Color	Whitish beige in the center and dark olivaceous grey towards the margin on PCA; Olivaceous dark grey on OA	Beige in the center and dark olivaceous grey towards the margin on PCA; Olivaceous dark grey on OA	Beige-pink to dark brown on PCA; Beige grey, olivaceous brown at the margin, on OA
	Shape	Circular, flat, margin fim briate,velvety to cobwebby on PCA; Circular, flat, velvety to cobwebby, margin entire on OA	Circular, flat, margin fimbriate, velvety, cobwebby at the margin on PCA; Circular, flat, margin entire, velvety to cobwebby on OA;	Circular, flat, margin fimbriate, sparsely lanose on PCA; Circular, flat, margin fimbriate, velvety, farinose on OA
	Size(mm)	58.4–64.2 on PCA; 68.0–72.4 on OA	64–67 on PCA;69–70 on OA	86–88 on PCA;78–79 on OA
conidiophores	Color	Brown and paler towards the apex	Brown, paler towards the apex	Brown and paler towards the apex
	Shape	Macronematous, straight, unbranched, septate, cylindrical	macronematous, solitary, straight, unbranched, septate, cylindrical	solitary, straight or slightly flexuous, unbranched, septate, cylindrical
	Size(μm)	34–152 (–245) × 2. 7–3.5(–4.07)	43–164(–245) × 2.5–3.5(–4.5) μm	74–175 × 2.5–3
Conidia	Color	Hyaline	hyaline, pale brown upon ageing	Hyaline
	Shape	Obovate to ellipsoidal, smooth, aseptate	obovate to ellip soidal-obovate, smooth, aseptate	Obovate to ellip soidal-obovate, aseptate, smooth
	Size(μm)	4.32–5.00 × 2.81–3.80	3.5–5.0 × 2.0–3.0	3.0–4.0 × 2.0–2.5
Chlamydospores	Color	Brown	Brown	Brown
	Shape	Lateral, sessile, intercalary, terminal, solitary or in short chains, globose to subglobose, thick-walled, smooth	Lateral, sessile, sometimes terminal, intercalary, solitary or in short chains, globose, subglobose, thick-walled, smooth	Lateral, sessile, sometimes terminal or intercalary, solitary, globose, subglobose or pyriform, thick-walled, smooth
	Size(μm)	4.75–6.21 × 4.16–5.41	4–6(–7) × 4–5	4.5–6 × 4–5.5
Vegetative hyphae	Color	Hyaline to pale brown	hyaline to pale brown	N/A
	Shape	Septate, smooth	Branched, septate,smooth.	
	Size	2.16–3.86	1.5–3	

PCA: potato carrot agar; OA: oatmeal agar.

Molecular phylogeny analysis

Sequencing of the ITS regions, *LSU,* and *tef1-α* yielded sequences of 581 bp, 1,765 bp, and 966 bp, respectively. Sequences from KNUF-21-010 were analyzed using the NCBI BLAST tools and showed a high level of similarity within the three regions. The ITS regions of KNUF-21-010 showed 99.79% identity with *Chl. chlamydosporum* (CBS 114.41; OP455385), 99.60% with *Chl. humicola* (WZ-918; OP163808), and 99.16% with *Chl. virescens* (KUNCC 24-17978; PQ22234). The *LSU* of KNUF-21-010 exhibited 99.89% identity with *Chl. chlamydosporum* (CBS 114.41; OP455492), 99.66% with *Chl. detriticola* (M.R. 3774; OP455508), and 99.49% with *Chl. peruense* (CBS 126074; OP455531). The *tef1-α* of the strain exhibited 99.88% identity with *Chl. chlamydosporum* (CBS 149052; OP464962), 98.45% with *Chl. peruense* (CBS 126074; OP464999), and 98.24% with *Chl. guttiferum* (CBS 126073; OP464991). BLAST results for all sequence regions of the strain showed > 99% identity with *Chl. chlamydosporum*. These results reveal that the strain KNUF-21-010 is *Chl. chlamydosporum*. To clarify this identification, MLSA was conducted using the ITS regions, *LSU,* and *tef1-α*. The phylogenetic tree (Fig. 3), constructed from the combined three loci (ITS, *LSU*, and *tef1-α*), placed KNUF-21-010 in a clade with *Chl. chlamydosporum, Chl. peruense,* and *Chl. detriticola* (90% bootstrap). Among the species analyzed, KNUF-21-010 was

clearly distinct from *Chl. peruense* and *Chl. detriticola*. Within this clade, the strain formed a small clade with *Chl. chlamydospore* (CBS 149052) and *Chl. chlamydosporum* (CBS 114.41^{T}) with 95% bootstrap. KNUF-21-010 and *Chl. chlamydosporum* (CBS 149052) clustered with 100% bootstrap, indicating that KNUF-21-010 represents a new strain of *Chl. chlamydosporum*.

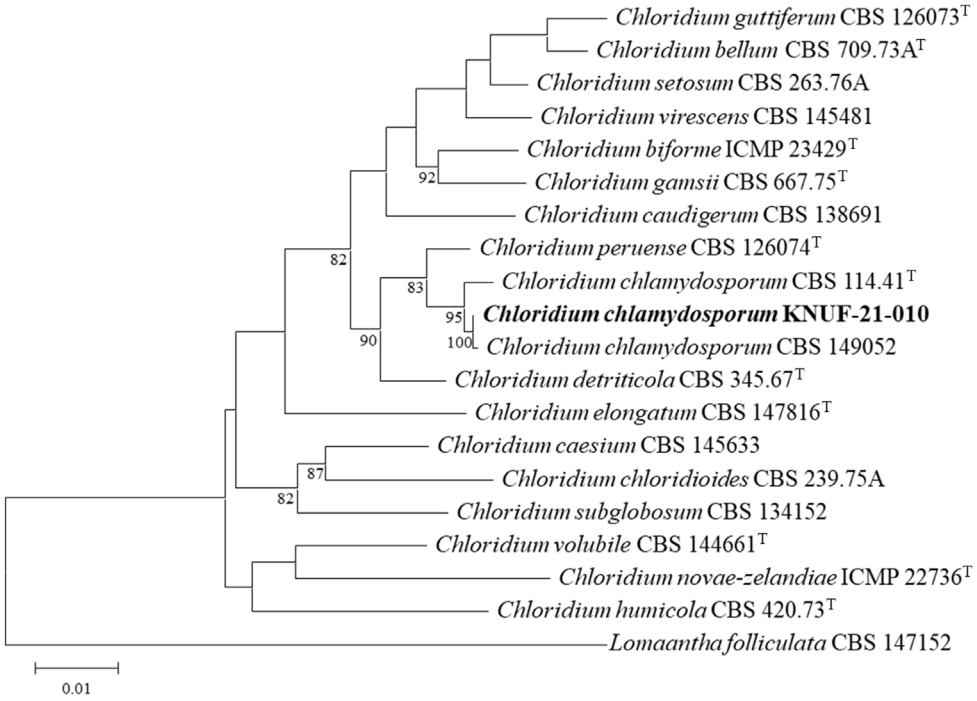

Fig. 3. The maximum likelihood phylogenetic tree based on the internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (*LSU*), and translation elongation factor 1-alpha (*tef1-α*) sequences. The tree shows the position of the KNUF-21-010 within the genus *Chloridium*. The fungal isolate newly determined in this study is highlighted in bold. Bootstrap values (> 80%) based on 1000 replicates were shown at nodes. Bar, 0.01 substitutions per nucleotide position.

DISCUSSION

Chloridium is a saprophytic fungal genus, with *Chl. virescens* which is designated as the type species [3]. In this study, *Chl. chlamydosporum*, a species not previously observed in Korea, was isolated from soil samples collected in Yangsan-si, Gyeongsangnam-do. For identification, its cultural and morphological characteristics, and genetic data were examined. Based on MLSA of the ITS regions, *LSU,* and *tef1-α*, the phylogenetic tree shows that our strain clustered in a distinct clade with the two reference strains of *Chl. chlamydosporum* (CBS 114.41^{T} and CBS 149052), thereby supporting its identification as *Chl. chlamydosporum*. Fungi of the family Chaetosphaeriaceae, which is one of the largest families within the class Sordariomycetes, are widely distributed and typically inhabit decaying leaves, fruits, and woody substrates in terrestrial and aquatic ecosystems [2,3].

The family Chaetosphaeriaceae was first reveal by Locquin in 1984 [18]. However, because it lacked a description compliant with the International Code of Botanical Nomenclature, it was considered invalidly published and was subsequently validated by Réblová et al. (1999) [19]. The introduction of molecular

phylogenetic approaches into fungal classification has significantly enhanced taxonomic and phylogenetic understanding, resulting in continuous reclassification within this family [2]. Owing to the presence of closely related polyphyletic genera within the Chaetosphaeriaceae family, accurate classification of genera, including *Chaetosphaeria, Chloridium, Codinaea, Cryptophiale, Dictyochaeta* and *Kionochaeta* remains necessary [2].

After Link established the genus *Chloridium*, von Höhnel (1903) identified a new genus, *Cirrhomyces*, with *C. caudiger* designated as the type species [3]. *Cirrhomyces* was distinguished from *Chloridium* based on the formation of multiple conidia. *C. caudiger* is characterized based on dematiaceous, fasciculate conidiophores and hyaline, oblong conidia adhering in white cirrhi. In 1940, van Beyma described *Bisporomyces chlamydosporum*, a fungus distinguished by its unique structure of paired conidia at the tip of the phialide [7]. Owing to the morphological similarities among *B. chlamydosporum, Chl. virescens,* and *C. caudiger*, considerable debate arose regarding their taxonomic placement. Hughes (1958) synonymized *Cirrhomyces* and *Bisporomyces* with *Chloridium*, recognizing five species within the genus: *Chl. botryoideum, Chl. caudigerum, Chl. chlamydosporum, Chl. minutum,* and *Chl. virescens* [8]. Gams and Holubová-Jechová (1976) subsequently classify *Chloridium* into three sections—*Chloridium, Gongromeriza*, and *Psilobotrys*—based on morphological characteristics such as the conidiogenous locus and the presence of chlamydospores [4].

In *Chloridium*, molecular data for some species have only been studied in the 21st century [3]. Traditionally, *Chloridium* was classified based on morphological traits; the recent application of molecular phylogenetics has prompted ongoing revisions at the genus and species levels. Based on molecular analyses of the *LSU* sequence, the hypothesis dividing the genus into three sections was rejected [20], and several studies have demonstrated the polyphyletic nature of *Chloridium* [2,3,9,10]. Wu and Diao (2022) attempt to redefine *Chloridium* based on ITS and *LSU* data, considering it paraphyletic, including parts of Adautomilanezia and Sporoschisma [2]. However, Réblová et al. (2022) demonstrate that *Chloridium* forms a separate monophyletic clade distinct from the Adautomilanezia and Sporoschisma group, using ITS and *LSU* data refined with Gblocks [3].

Chloridium has traditionally been regarded as a saprophytic genus primarily occurring on wood or other plant debris [4], but some species have also been reported to be isolated from soil [3]. In particular, *Chloridium* may contribute to increasing nitrogen-use efficiency and agricultural productivity in acidic soil ecosystems through heterotrophic nitrification [21]. Therefore, given its isolation from Korean soils, *Chl. chlamydosporum* warrants further investigation to clarify its potential roles in organic matter decomposition and contribution to the nitrification process within these soil ecosystems.

This study provides the first evidence that *Chl. chlamydosporum* occurs in Korean soils, contributing to the ecological understanding and biogeographical expansion of the genus *Chloridium*. *Chloridium* is a globally distributed fungal genus; however, only a few species have been revealed in Korea, indicating that research on this group remains in its early stages [22]. Therefore, additional research is required to identify previously unrecorded or novel *Chloridium* species and to better understand their distribution and diversity in Korean ecosystems.

CONFLICTS OF INTERESTS

The authors declare no potential conflicts of interest.

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