RESEARCH ARTICLE

New Records of Two Unreported Fungal Species: *Cladophialophora floridana* and *Chloridium setosum* Isolated from Soil in Korea

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ABSTRACT

During an exploration of fungal diversity in Korean soil, this study isolated two fungal strains, designated KNUF-23-321L and KNUF-23-314, and identified them as previously unrecorded species. Morphological and cultural features were examined for initial classification. The characteristics of strains KNUF-23-321L and KNUF-23-314 matched those of *Cladophialophora floridana* SR3028^{T}, and *Chloridium setosum* CGMCC 3.20741^{T}, respectively. To further pinpoint their identities and evolutionary relationships, molecular phylogenetic analyses were conducted using the internal transcribed spacer (ITS) regions and 28S rDNA large subunit (LSU) on strain KNUF-23-321L which showed high similarity of 98.6 and 100%, respectively, with strain *C. floridana* SR3028^{T}. For the strain KNUF-23-314, it showed a high similarity of 100% with strain *Ch. setosum* CBS 263.76A with both ITS regions and LSU genes. These combined morphological and phylogenetical analyses revealed that KNUF-23-321L was identified as *C. floridana*, and KNUF-23-314 was identified as *Ch. setosum.* To the best of our knowledge, these are the first reports of *C. floridana* and *Ch. setosum* in Korea.

Keywords: *Chloridium setosum, Cladophialophora floridana*, phylogeny, soil fungi, unreported species

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INTRODUCTION

Soil, as the primary habitat within natural ecosystems, harbors a diverse array of microorganisms [1]. Among the diverse soil microorganisms, fungi can have positive effects on plants and soils as mycorrhizal fungi, decomposers, and biosorbents [2]. It is estimated that more than 1.5 million species of fungi exist worldwide, with over 150,000 described species reported to date [3].

The fungal genus *Cladophialophora*, of the phylum Ascomycota, class Eurotiomycetes, order Chaetothyriales, and family Herpotrichiellaceae, is morphologically characterized by conidia that germinate through blastic, acropetal conidiogenesis, forming interconnected chains of conidia [4,5]. This genus is

widely distributed in plant residues, soil, skin lesions, and lower extremities and has been reported to be pathogenic in plants and mammals, including humans, with three species affecting plants and 11 humans [5,6]. It was first described in the 1980s, and currently, 68 species are registered worldwide [5]. Three species, *C. hostae*, *C. pucciniophila*, and *C. lanosa*, have been reported in Korea, which were isolated from rust fungi conidia, daylily (*Hemerocallis* sp.), and soil, respectively [7-9].

The genus *Chloridium* belongs to the phylum Ascomycota, class Sordariomycetes, order Chaetosphaeriales, and family Chaetosphaeriaceae [10]. *Chloridium* species have been isolated from a variety of sources, including soil, trees, and mosses, and are known to be endophytic or saprophytic in association with a variety of plants, including grasses and trees [11]. The first reported *Chloridium* species was *Ch. virescens*, and a total of 38 species have been identified to date [10]. However, only two, *Ch. virescens* and *Ch. apiculatum*, have been isolated and reported in Korea, and domestic studies of this genus are relatively scarce compared to overseas studies.

To expand the known fungal species diversity in Korea and discover new species, fungi were isolated from a soil specimen obtained in Korea. In this study, two fungal strains, designated as KNUF-23-321L and KNUF-23-314, were subjected to a comprehensive analysis assessing their cultural, molecular, and morphological attributes. The findings are documented and reported below.

MATERIALS AND METHODS

Sample collection and isolation of fungal strains

The fungal strains used in this study were isolated from the soil samples collected in Gachang-myeon, Daegu (35°47'14.1"N 128°33'19.7"E) and Geochang-gun, Gyeongsangnam-do (35°50'01.8"N, 127°47'44.0"E) in Korea. Using an autoclaved spatula, soil samples were taken from the field to a depth of 15-30 cm. The samples were then air-dried and kept at 4°C in a plastic bag. For isolation, 1 g from each soil sample was suspended in 10 mL of sterile distilled water, gently vortexed, and serially diluted. A 100 μL volume was then spread onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates, which were then cultured for 2-3 days at 25°C [12]. Germinating single colonies were then transferred to new PDA plates at 25°C, and based on specific characteristics, pure cultures of isolates KNUF-23-314 and KNUF-23-321L were selected for further molecular analyses and cultural and morphological characterization. The specimens have been stored at the National Institute of Biological Resources (NIBR) and are identified by the accession numbers NIBRFGC000510718 and NIBRFGC000510719, respectively.

Cultural and morphological characterization

Following previous studies, the cultural and morphological characteristics of the two fungal strains were assessed using cultural media based on their genus. The strain KNUF-23-321L was cultured on PDA for 20 days at 25°C, while KNUF-23-314 was cultured on PDA for 28 days at 25°C [6]. Additionally, KNUF-

23-321L was grown for 28 days at 25°C on malt extract agar (MEA; Difco, Detroit, MI, USA) [11]. The growth of the fungi was measured, and characteristics of the colonies, such as their color, form, and dimensions, were documented. Morphological features were examined using a light microscope (BX-50; Olympus, Tokyo, Japan).

Genomic DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

For the molecular identification of strains KNUF-23-314 and KNUF-23-321L, total genomic DNA was extracted using the HiGene™ Genomic DNA Prep Kit for fungi (Biofact, Daejeon, Korea). The internal transcribed spacer (ITS) region of the total genomic DNA extracted from the samples was amplified using the ITS1F/ITS4 primer pair, and the large subunit (LSU) gene of the 28S rDNA was amplified using the LROR/LR5 primer pair [13-15]. Amplification was confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide. The resulting amplification products were purified utilizing ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and then subjected to the sequencing services provided by Solgent (Daejeon, Korea).

Phylogenetic analyses

The sequences of KNUF-23-314 and KNUF-23-321L were analyzed for similarity against those available in the National Center for Biotechnology Information (NCBI) database utilizing the Basic Local Alignment Search Tool (BLAST), and a number of related sequences were downloaded from the database for phylogenetic analyses (Table 1). Phylogenetic trees were constructed based on concatenated ITS regions and LSU gene sequences using the neighbor-joining (NJ) method in MEGA version X [16,17]. The evolutionary distance matrices for the NJ analysis were produced using Kimura's two-parameter model, and bootstrap values derived from 1,000 resamples were produced to provide branch support [18].

Table 1. GenBank strain and accession numbers of the strains utilized for the phylogenetic analyses in this study

Species Name	Strain Number	GenBank accession number	
		ITS	LSU
Chloridium bellum var. *bellum*	CBS 709.73A	NR_185446	OP455466
Chloridium bellum var. *bellum*	CBS 709.73B^{T}	OP455361	OP455467
Chloridium bellum var. *luteum*	CBS 141.54^{T}	OP455362	OP455469
Chloridium caudigerum	CBS 145489	OP455379	OP455486
Chloridium caudigerum	CBS 145433	OP455377	OP455484
Chloridium guttiferum	CBS 126073	MH864068	MH875524
Chloridium jilinense	NN046507	OL627659	OL655058
Chloridium setosum	CBS 263.76A	OP455427	OP455534
Chloridium setosum	**KNUF-23-314**	**PP727493**	**PP727510**
Chloridium virescens	CBS 145487	OP455441	OP455549
Chloridium virescens	CBS 138683	OP455431	OP455539
Cladophialophora boppii	CBS 126.86^{T}	EU103997	NG_058762
Cladophialophora chaetospira	CBS 491.70	EU035405	EU035405
Cladophialophora chaetospira	CBS 514.63	MH858340	KF928513
Cladophialophora floridana	**KNUF-23-321L**	**PP727494**	**PP727511**
Cladophialophora floridana	SR1004	AB986344	AB986344
Cladophialophora floridana	SR3028^{T}	AB986343	AB986343
Cladophialophora multiseptata	FMR 10591^{T}	HG003668	HG003671
Cladophialophora potulentorum	CBS 112222	EU03540	EU03540
Cladophialophora potulentorum	CBS 114772	EU035410	EU035410
Cladophialophora tortuosa	BA4b006^{T}	AB986424	AB986424
Exophiala oligosperma	CBS 725.88^{T}	AY163551	NG_059201
Menispora ciliata	CBS 984.70	MH860017	MH871802

ITS: internal transcribed spacer regions; LSU: 28S rDNA large subunit gene.
^{T}Type strain.
The strains identified in this study are indicated in bold.

RESULTS AND DISCUSSION

Cladophialophora floridana Obase, Douhan, Y. Matsuda & M.E. Sm., Mycoscience 57:1 (2015) [MB#812352]

Morphology of strain KNUF-23-321L

On PDA, the colonies achieved a growth of 32–34 mm in diameter after 28 days at 25°C, with the colonies appearing olivaceous-black with a dry, velvety, and ovoid shape (Fig. 1A). On MEA, the strain achieved a diameter of 36–37 mm after 28 days at 25°C. The colonies on MEA appeared olivaceous-black and were velvety with entire margins (Fig. 1B). The conidiophores were ovoid to ellipsoidal, oblong, and apically branched, with either aseptate or 1-septate hyphal cells that were constricted at the septa and brown to pale olivaceous in color (Fig. 1C and 1D). The terminal conidia frequently appeared ovoid and smaller than the other conidia within the chains, suggesting blastic, acropetal conidiogenesis (Fig. 1E). Conidia were 3.2–5.8 × 2.0–3.0 µm (n=30) in diameter, pale olivaceous to brown in color, oblong to subglobose and

ellipsoidal in shape, smooth, and mostly aseptate, with the conidial chains infrequently branched (Fig. 1F). The cultural and morphological characteristics of the isolated strain KNUF-23-321L indicated that it is most likely related to *C. floridana* strain SR3028^{T} (Table 2) [6].

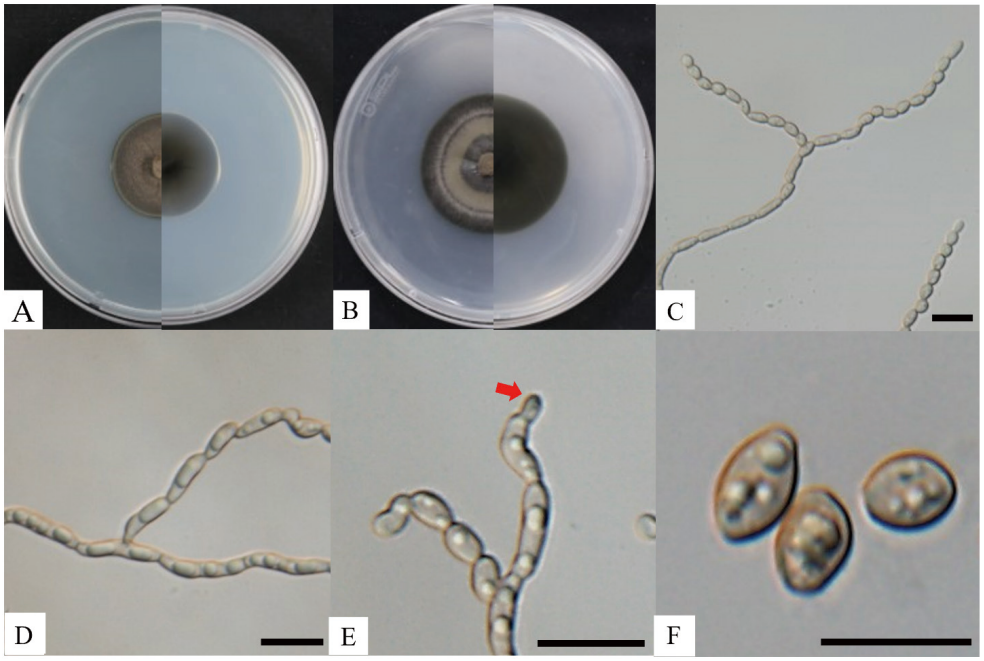

Fig. 1. Cultural and morphological characteristics of *Cladophialophora floridana* KNUF-23-321L. Cultures were grown at 25°C for 28 days. A: front and reverse view of the colony on PDA; B: front and reverse view of the colony on MEA; C: aseptate or 1-septate conidiophores; D: chain conidia branched from conidiophore; E: arrow points to terminal conidia exhibiting an ovoid shape, suggesting blastic, acropetal conidiogenesis; F: conidia. Scale bars = 10 μm.

Table 2. Comparison of the cultural and morphological characteristics of strains KNUF-23-321L and *Cladophialophora floridana* SR3028^{T}

Characteristic	*Cladophialophora floridana* KNUF-23-321L[a]	*Cladophialophora floridana* SR3028^{T}[b]
Colony on PDA	32–34 mm, velvety, dry, margin entire, radially folded, olivaceous-black	22–23 mm, velvety, dry, margin entire, radially folded, olivaceous-black
Colony on MEA	36–37 mm, velvety, dry, margin entire, radially folded, olivaceous-black	22–23 mm, velvety, dry, margin entire, radially folded, olivaceous-black
Conidiophores	Ovoid, ellipsoidal, oblong, and apically branched, aseptate or 1-septate, born terminally on hyphae, brown to pale olivaceous	Oblong to cylindrical, aseptate or 1-septate, born terminally on hyphae, brown to pale olivaceous
Conidia	3.2–5.8 × 2.0–3.0 μm, smooth, thick walled, oblong to subglobose, conidial chains sparsely branched, mostly aseptate, pale olivaceous to brown	3.5–7.9 × 2.0–3.2 μm, smooth, thick walled, oblong to subglobose, conidial chains sparsely branched, mostly aseptate, pale olivaceous to brown

PDA: potato dextrose agar; MEA: malt extract agar.
^{T} Type strain; [a] Fungal strain used in this paper; [b] Source of descriptions [6].

Molecular phylogeny of strain KNUF-23-321L

The ITS regions and LSU gene sequences were amplified to obtain 573 bp and 815 bp, respectively, for the sequencing analyses of strain KNUF-23-321L. The ITS region showed a high similarity of 98.6% with *C. floridana* SR3028^{T} and a similarity of 94.3% with *C. tortuosa* BA4b006. The LSU gene sequence was identical (100% similarity) with the *C. floridana* SR3028^{T} and showed a similarity of 99.4% with *C. tortuosa* BA4b006. Based on the above results, the ITS region and LSU gene sequences were combined to create an NJ phylogenetic tree (Fig. 2), in which strain KNUF-23-321L clustered together with *C. floridana* SR3028^{T}. Based on the morphological characteristics and phylogenetic analysis, strain KNUF-23-321L was identified as *C. floridana*, an unreported species in Korea.

The genus *Cladophialophora* was established to unite fungal species that exhibit most of the characteristics of *Cladosporium* but *Phialophora*-like conidiogenesis [6]. Although *C. floridana* KNUF-23-321L is closely related to *C. tortuosa* phylogenetically, it can be differentiated by the conidia and conidiophores. In *C. floridana*, the apical cells of conidiophore are straight, while those of *C tortuosa* are distinctly bent [6]. To date, *Cladophialophora* has 45 recorded species with diverse habitats. While at least 11 *Cladophialophora* species are considered opportunistic pathogens that can be found infecting humans and other mammals, the species are also known to be sampled from polluted environmental sources, such as hydrocarbon-rich soil [6,19]. However, some species of the genus *Cladophialophora* have also been reported to exist as plant endophytes [11,20]. Though there are no reports of pathogenicity from *C. floridana*, it is thought that other species in the genus *Cladophialophora* may also be pathogenic, and thus further research on this genus is necessary.

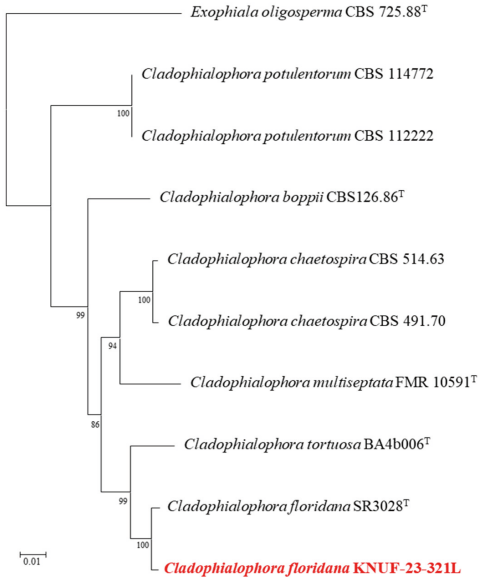

Fig. 2. Neighbor-joining phylogenetic tree based on a combined dataset of partial sequences of internal transcribed spacer (ITS) regions and large subunit (LSU) gene showing the phylogenetic position of strain *Cladophialophora floridana* KNUF-23-321L among *Cladophialophora* species and its closest relationship with *C. floridana*. Bootstrap values greater than 80% (percentage of 1,000 replications) are shown at branching points. The strain isolated in this study is in bold and red. The tree was rooted using *Exophiala oligosperma* CBS 725.88^{T} as an out-group. Bar, 0.01 substitutions per nucleotide position.

Chloridium setosum W.P. Wu & Y.Z. Diao, Fungal Diversity 116:1 (2022) [MB#841601]

Morphological analyses of strain KNUF-23-314

On PDA, the colonies grew to 16–22 mm in diameter after 20 days at 25°C. The colonies appeared grey to grey-brown on the obverse side and pale brown on the reverse side, with wavy pale margins (Fig. 3A). Setae were observed to be erect, cylindrical, branched, straight, and brown in color, with a length of 59.9–75.9 × 2.2–2.8 μm (Fig. 3B). Chlamydospores were dark brown in color and thick-walled (Fig. 3C). Conidia were 2.8–4.0 × 2.3–3.4 μm (n = 30) in diameter, hyaline, ellipsoidal, and aseptate (Fig. 3D). The cultural and morphological characteristics of the isolated strain KNUF-23-314 indicated that it is most likely related to *Ch. setosum* CGMCC 3.20741^{T} (Table 3).

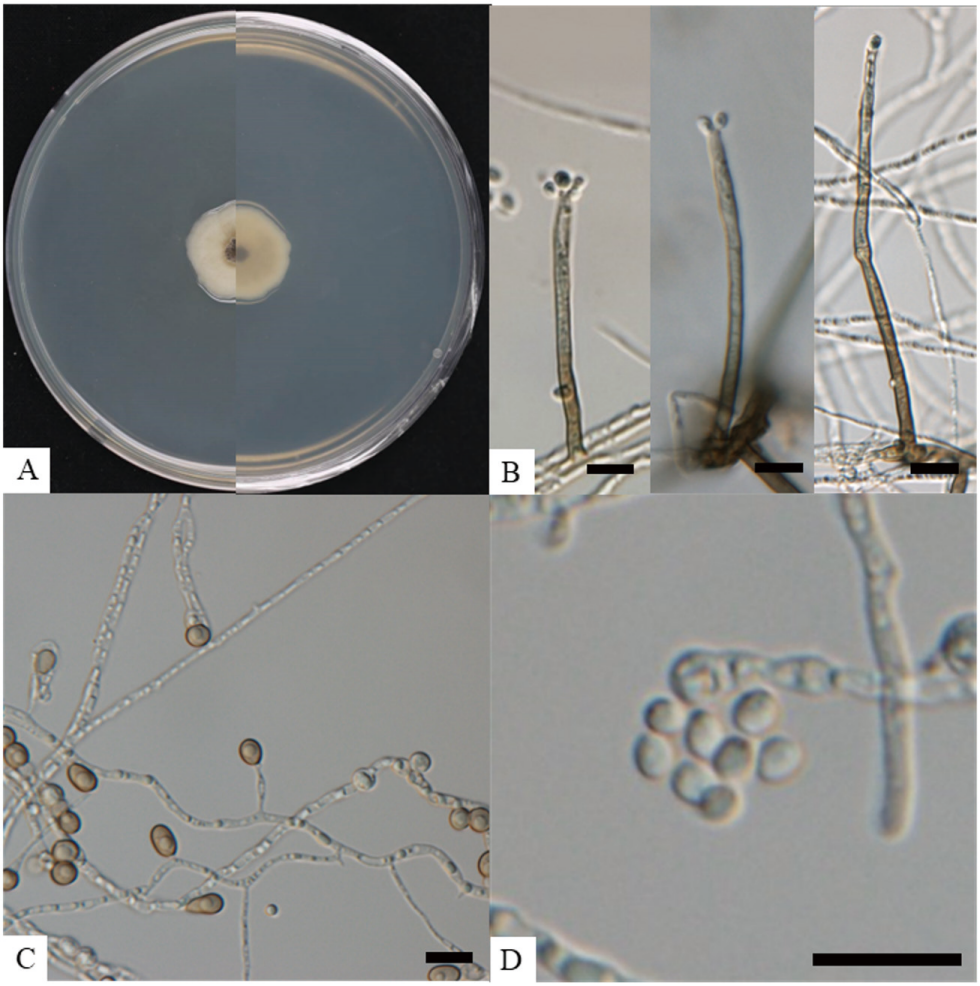

Fig. 3. Cultural and morphological characteristics of *Chloridium setosum* KNUF-23-314. Cultures were grown at 25°C for 20 days. A: front and reverse view of the colony on PDA; B: various lengths of setae; C: terminal chlamydospores; D: conidia accumulating in slimy whitish heads. Scale bars = 10 μm.

Table 3. Comparison of the cultural and morphological characteristics of strains KNUF-23-314 and *Chloridium setosum* CGMCC 3.20741[T]

Characteristic	*Chloridium setosum* KNUF-23-314[a]	*Chloridium setosum* CGMCC 3.20741[Tc]
Colony on PDA	16–22 mm, grey to grey-brown, with pale margins, reverse pale brown	15–20 mm, grey to grey-brown, with pale margins, reverse pale brown to dark brown
Conidia	2.8–4.0 × 2.3–3.4 µm, hyaline, ellipsoidal, aseptate	3–3.8 × 2–2.5 µm, hyaline, ellipsoidal, subglobose, aseptate
Setae	59.9–75.9 × 2.2–2.8 µm, cylindrical, branched, erect, straight, brown	80–137 × 2.5–3.7 µm, cylindrical, branched, erect, straight, brown to dark brown

PDA: Potato dextrose agar.
[T]Type strain; [a]Fungal strain used in this paper; [c]Source of descriptions [10].

Molecular phylogeny of strain KNUF-23-314

The ITS regions and LSU gene sequences were amplified to obtain 513 bp and 788 bp, respectively, for the sequencing analyses of strain KNUF-23-314. The ITS region showed a 100% similarity with that of *Chloridium setosum* CBS 263.76A, a similarity of 98.2%. with *Ch. virescens* CBS 145487, and a similarity of 96.9% with *Ch. bellum* var. *bellum* CBS 709.73A. The LSU gene sequence of the strain was also identical to that of *Ch. setosum* CBS 263.76A, confirming its position in the genus *Chloridium*. Based on the above results, the concatenated ITS region and LSU gene sequences were used to generate an NJ phylogenetic tree (Fig. 4). The tree shows strain KNUF-23-314 clustering with *Ch. setosum* CBS 263.76A. Based on the morphological characteristics and phylogenetic analysis, strain KNUF-23-314 was identified as *Ch. setosum*, a species previously unreported in Korea.

The genus *Chloridium* has been isolated from various parts of the world. Most species are found in temperate, subtropical, and tropical regions, with only a few species inhabiting boreal and tundra regions [10]. As such, the genus *Chloridium* is temperature sensitive, with temperature determining its habitat and geographical distribution. In the case of *Ch. setosum*, since it was first discovered in tree remnants in China, it has been found worldwide in a variety of environments, including forests, grasslands, woodlands, and agricultural areas, but not in boreal or tundra regions [11]. Fungi of the genus *Chloridium* form a secondary metabolite, javacin, which is known to have antagonistic effects on soilborne plant pathogens such as *Rhizoctonia solani* and *Verticillium dahliae* [16]. Javacin can also be used to control diseases caused by *Pseudomonas* spp. in humans, animals, and various plants [10] and has been reported to control diseases caused by *R. solani, V. dahliae,* and *Cercospora arachidicola* [21].

Given that only two species of the genus *Chloridium*, namely *Ch. virescens* and *Ch. apiculatum*, have been documented in Korea from soil samples, the isolation of *Ch. setosum* for the first time holds particular importance as it has not been previously reported in Korea. Consequently, this research endeavor has the potential to enrich the fungal biodiversity in Korea by adding a new species to the existing species inventory.

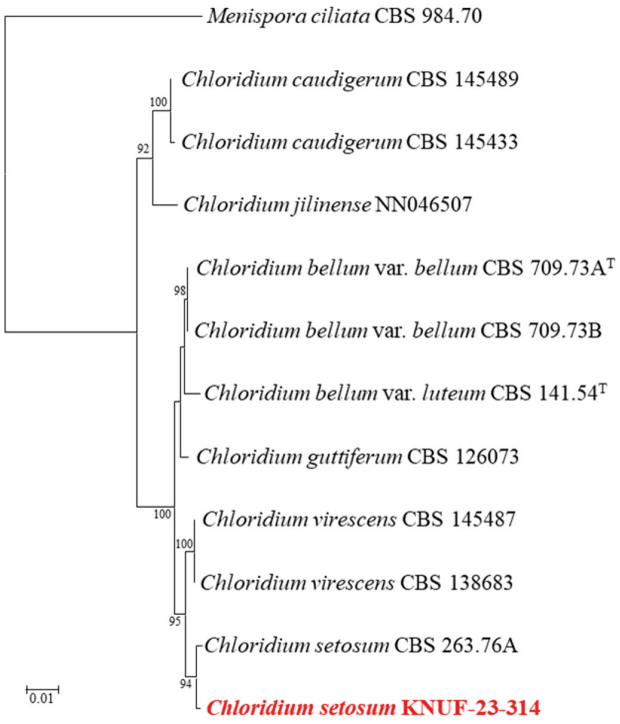

Fig. 4. Neighbor-joining phylogenetic tree based on a combined dataset of partial sequences of internal transcribed spacer (ITS) regions and large subunit (LSU) gene showing the phylogenetic position of strain *Chloridium setosum* KNUF-23-314 among *Chloridium* species and its closest relationship with *Ch. setosum*. Bootstrap values greater than 90% (percentage of 1,000 replications) are shown at branching points. The strain isolated in this study is in bold and red. The tree was rooted using *Menispora ciliata* CBS 984.70 as an out-group. Bar, 0.01 substitutions per nucleotide position.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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