RESEARCH ARTICLE

Morphological and Phylogenetic Analyses of Two Unreported Species of Entomogenous Fungi *Blackwellomyces calendulinus* and *Neoaraneomyces araneicola* in Korea

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ABSTRACT

This study reports the isolation and characterization of two previously unrecorded fungal strains, designated KNUF-23-232 and KNUF-23-344. The initial classification was based on an examination of their morphological and cultural characteristics. To further confirm their identities and elucidate their evolutionary relationships, molecular phylogenetic analyses were conducted using sequences from the internal transcribed spacer (ITS) region, the large subunit of 28S rRNA (LSU), the second largest subunit of RNA polymerase II (*RPB2*), and translation elongation factor-1α (*TEF1*). The largest subunit of RNA polymerase II (*RPB1*) was also amplified for strain KNUF-23-344. The cultural and morphological traits of strain KNUF-23-232 were consistent with those of *Blackwellomyces calendulinus* BCC 68502^{T}, whereas those of strain KNUF-23-344 corresponded to *Neoaraneomyces araneicola* DY101711^{T}. The integration of morphological and phylogenetic analyses led to the identification of KNUF-23-232 as *B. calendulinus* and KNUF-23-344 as *N. araneicola*. To the best of our knowledge, this study represents the first documentation of *B. calendulinus* and *N. araneicola* in Korea.

Keywords: *Blackwellomyces calendulinus*, Entomogenous fungi, Morphology, *Neoaraneomyces araneicola*, Phylogeny

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INTRODUCTION

Entomogenous fungi are typically associated with insects, other arthropods, and even non-arthropod microinvertebrates, primarily functioning as pathogens or parasites [1]. Although these fungi and their related species exhibit a wide range of interactions with their hosts, they follow similar biological principles in the diseases they cause. Infection typically occurs through the penetration of the host's cuticle following a series of competitive interactions, with successful infection requiring the fungi to overcome or evade the host's defenses [2]. Recent advancements in the phylogenetic classification of fungi have enhanced the understanding of various entomopathogenic fungal taxa, potentially facilitating their unconventional

applications and encouraging the use of novel fungal entomopathogens as biocontrol agents. Among these, the genus *Blackwellomyces*, belonging to the order Hypocreales and family Cordycipitaceae, was first isolated from the larvae of coleopteran and lepidopteran insects, with eight species have been reported worldwide [3,4]. The genus *Blackwellomyces* was initially established to accommodate *B. cardinalis* and has *Acremonium*-like, *Evlachovaea*-like, and *Mariannaea*-like conidial arrangements, with multiple forms observed within a single species [4]. Additionally, the genus *Neoaraneomyces*, belonging to the order Hypocreales and family Clavicipitaceae, was isolated from a dead spider carcass in China and exhibits a conidial chain resembling that of *Paecilomyces* [5]. Although spiders are not classified as arthropods, a study conducted in 2023 classified the spider-pathogenic fungus *Neoaraneomyces* as entomogenous [6]. The etymology of the genus reflects its characterization as a new genus of fungi parasitic to spiders, with the type species being *Neoaraneomyces araneicola* [5]. This study aimed to isolate fungi from domestic soil samples and identify the isolated fungi based on morphological and molecular biological characteristics, with the objective of securing domestic fungal resources and reporting potential endemic species.

MATERIALS AND METHODS

Sample collection and fungal isolation

Soil samples were collected from Chungnam (36°11'44.1"N, 127°16'24.0"E) and Jeonbuk Province (35°58'56.3"N, 126°41'46.1"E) in Korea, and used as the source of fungal isolates in this study. The isolation of fungi from the soil samples using the serial dilution technique, as previously described [7]. Colonies showing signs of germination were subsequently transferred to fresh potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates and incubated at 25°C. The fungal strains KNUF-23-232 and KNUF-23-344 were selected for further molecular analyses, as well as cultural and morphological evaluations. These isolates are preserved at the National Institute of Biological Resources (NIBR) under the accession numbers NIBRFGC000510717 and NIBRFGC000510720, respectively.

Cultural and morphological characterization

The isolates were cultured on PDA for 10 days at 25°C for KNUF-23-232, and for 14 days at 25°C for KNUF-23-344 for the cultural and morphological characterization. Additionally, KNUF-23-232 was also cultured on oatmeal agar (OA; Difco, Detroit, MI, USA) for 10 days at 25°C. The cultures were maintained in darkness, and various characteristics were observed, including the size, color, and shape of the mycelium, as well as morphological features such as phialides, conidia, and the arrangement of conidia. Morphological properties were examined using a light microscope (BX-50; Olympus, Tokyo, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

For molecular identification, total genomic DNA was extracted from strains KNUF-23-232 and KNUF-

23-344 using the HiGene™ Genomic DNA Prep Kit for fungi (Biofact, Daejeon, Korea). The internal transcribed spacer (ITS) region, the large subunit of 28S rRNA (LSU), the second largest subunit of RNA polymerase II (*RPB*2), and translation elongation factor-1α (*TEF*1) were amplified for both strains. Additionally, the largest subunit of RNA polymerase II (*RPB*1) was specifically amplified for strain KNUF-23-232. The primers used for amplifying the molecular phylogenetic markers were ITS1F/ITS4 for ITS, LROR/LR5 for LSU, RPB2-5F2/RPB2-7cR for *RPB*2, EF1-983F/EF1-2218R for *TEF*1, and cRPB1A/RPB1Cr for *RPB*1 [8-16]. Successful amplification was confirmed by electrophoresis on 1.0% HP Agarose gels (BIOPURE, Cambridge, USA). The amplified products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently submitted to Macrogen (Seoul, Korea) for sequencing.

Phylogenetic analyses

The sequences obtained were analyzed for similarity using the Basic Local Alignment Search Tool (BLAST) within the National Center for Biotechnology Information (NCBI) database (Table 1). Phylogenetic trees were constructed from the concatenated sequences of the ITS regions, LSU, *RPB*1, *RPB*2, and *TEF*1, employing the neighbor-joining (NJ) method in MEGA 11 [17,18]. Evolutionary distance matrices for the NJ analysis were calculated using Kimura's two-parameter model, with bootstrap values based on 1,000 replications [19].

Table 1. List of species used in this study and their corresponding GenBank accession numbers for phylogenetic analysis (continued)

Species	Strain number	GenBank accession number				
		ITS	LSU	*RPB1*	RPB2	*TEF1*
Aciculosporium oplismeni	MAFF 246966	LC571760	LC571760	-	LC572054	LC572040
Aciculosporium take	MAFF 241224	LC571753	LC571753	-	LC572048	LC572034
Aciculosporium take	TNS-F-60465	LC571755	LC571756	-	LC572049	LC572035
Balansia henningsiana	A.E.G. 96-27a	JN049815	AY545727	-	DQ522413	AY489610
Blackwellomyces aurantiacus	BCC 85060^{T}	MT000692	MT003028	MK411600	MT017819	MK411598
Blackwellomyces aurantiacus	BCC 85061	MT000693	MT003029	MK411601	MT017820	MK411599
Blackwellomyces cardinalis	OSC 93610	JN049843	AY184963	EF469088	EF469106	EF469059
Blackwellomyces calendulinus	BCC 68502^{T}	MT000695	MT003031	MT017803	MT017822	MT017843
Blackwellomyces calendulinus	BCC 68500	MT000694	MT003030	MT017802	MT017821	MT017842
Blackwellomyces calendulinus	**KNUF-23-232**	**PQ144579**	**PQ144577**	**PQ152981**	**PQ148394**	**PQ148396**
Blackwellomyces roseostromatus	BCC 91358^{T}	MT000697	MT003033	MT017805	MT017824	MT017845
Blackwellomyces roseostromatus	BCC 91359	MT000698	MT003034	MT017806	MT017825	MT017846
Blackwellomyces kaihuaensis	HMAS 285455^{T}	OQ981975	OQ981968	OQ980409	OQ980408	OQ980401
Blackwellomyces lateris	MFLU 18-0663^{T}	MK086059	MK086061	MK084615	MK079354	MK069471
Blackwellomyces minutus	BCC 88269^{T}	MT000696	MT003032	MT017804	MT017823	MT017844
Claviceps purpurea	GAM 12885	U57669	AF543789	-	DQ522417	AF543778
Epichloë typhina	ATCC 56429	JN049832	U17396	-	DQ522440	AF543777
Neoaraneomyces araneicola	DY101711^{T}	MW730520	MW730609	-	MW753026	MW753033
Neoaraneomyces araneicola	DY101712	MW730522	MW730610	-	MW753027	MW753034
Neoaraneomyces araneicola	**KNUF-23-344**	**PQ144580**	**PQ144578**	**-**	**PQ148395**	**PQ148397**
P araneoaraneomyces sinensis	ZY 22.006^{T}	OQ709254	OQ709260	-	OQ719621	OQ719626

Table 1. List of species used in this study and their corresponding GenBank accession numbers for phylogenetic analysis

Species	Strain number	GenBank accession number				
		ITS	LSU	*RPB1*	RPB2	*TEF1*
Paraneoaraneomyces sinensis	ZY 22.007	OQ709255	OQ709261	-	OQ719622	OQ719627
Paraneoaraneomyces sinensis	ZY 22.008	OQ709256	OQ709262	-	OQ719623	OQ719628
Pleurocordyceps aurantiaca	MFLUCC 17-2113	MG136916	MG136910	-	MG136870	MG136875
Pleurocordyceps marginaliradians	MFLU 17-1582	MG136920	MG136914	-	MG271931	MG136878
Purpureocillium lilacinum	CBS 284.36^{T}	MH855800	FR775484	EF468898	EF468941	EF468792
Ustilaginoidea virens	MAFF 240421	JQ349068	JQ257011	-	JQ257017	JQ257024

ITS: Internal transcribed spacer regions; *LSU*: 28S rRNA large subunit; *RPB*1: the largest subunit of RNA polymerase II; *RPB*2: the second largest subunit of RNA polymerase II ; *TEF*1: translation elongation factor-1α.

^{T}Type strain.

Strains used in this study are indicated in bold.

RESULTS AND DISCUSSION

Blackwellomyces calendulinus Mongkolsamrit, Noisripoom, Khonsanit & Luangsa-ard, Mycological Progress 19:967 (2020) [MB#835360]

Cultural and morphological characteristics of KNUF-23-232

When cultured on PDA at 25°C for 10 days, the colonies reached a diameter of 24–26 mm. The obverse side appeared white and cream with some aerial hyphae, slightly wrinkled, and ciliate hyphae were observed at the edges along with reddish pigmentation (Fig. 1A). The reverse side of the colony exhibited a dark burgundy center, transitioning to scarlet toward the edges, with a white border (Fig. 1A). On OA, the colonies grew to a diameter of 22–24 mm after 10 days at 25°C. The obverse side was white, with cottony aerial hyphae and a fringe-like edge, and the center of the colony was umbonate (Fig. 1B). The reverse side was light brown with no pigmentation (Fig. 1B). The phialides gradually tapered from a swollen basal portion to the apex, appearing solitary or in groups of two to three on each branch, arising from aerial hyphae (Fig. 1E-G). The phialides measured 12.5–22.4 × 1.3–2.5 μm (n=20). Conidia were hyaline, unicellular, elliptical to oblong-elliptical, with a smooth surface (Fig. 1H). The conidia measured 3.2–5.3 × 1.6–2.6 μm (n=50) and were produced at the tips of the hyphae in a *Mariannaea*-like conidial arrangement (Fig. 1C, 1D). A comparison of the morphological features of strain KNUF-23-232 with those of *Blackwellomyces calendulinus* BCC 68502^{T} is provided in Table 2 [4]. While the reported *B. calendulinus* BCC 68502^{T} exhibits both *Mariannaea*-like and *Acremonium*-like conidial arrangements, strain KNUF-23-232 demonstrates only the *Mariannaea*-like arrangement. Other morphological and cultural characteristics observed indicate that strain KNUF-23-232 is most closely related to *B. calendulinus* BCC 68502^{T}.

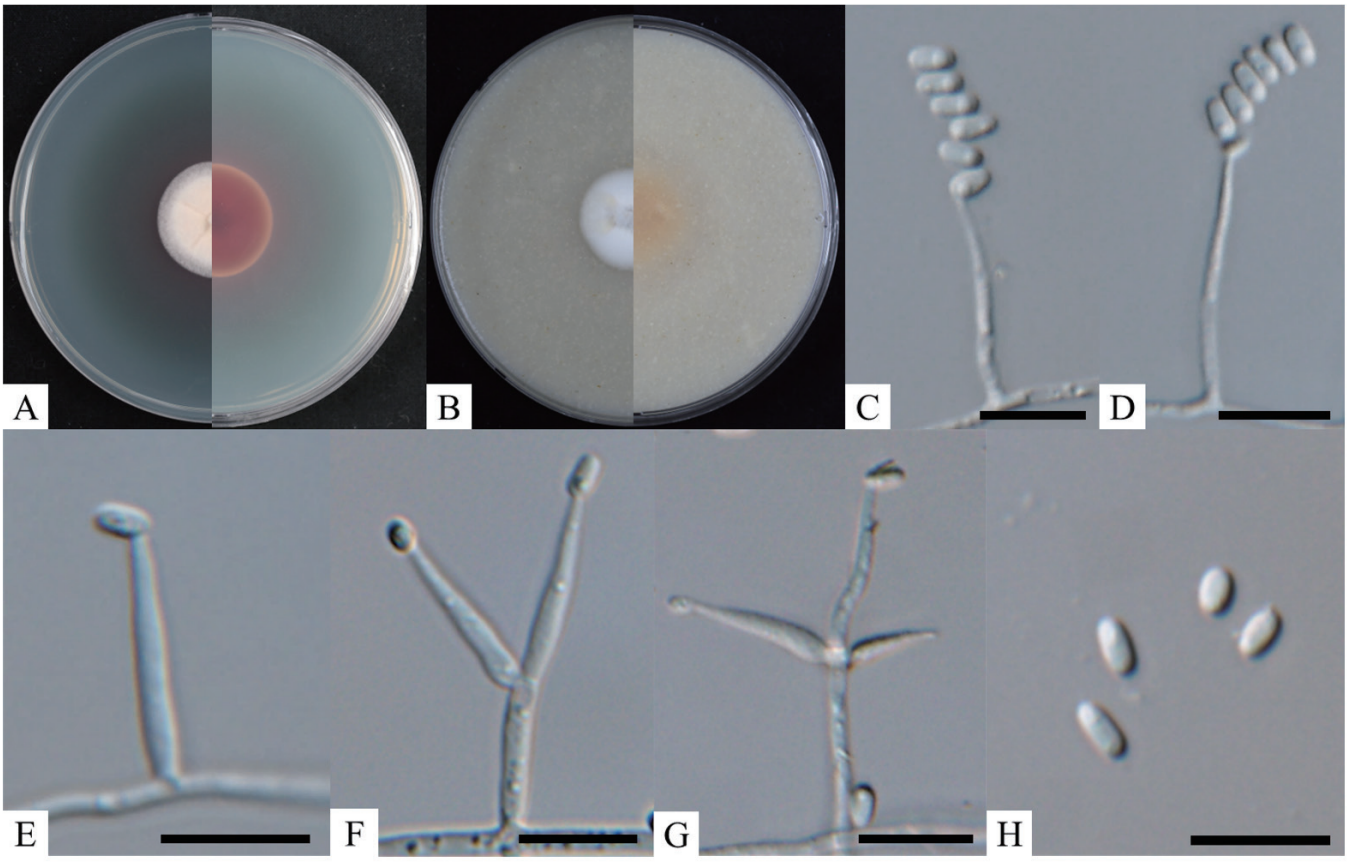

Fig. 1. Cultural and morphological characteristics of *Blackwellomyces calendulinus* KNUF-23-232. A, B: Obverse and reverse views of the colony at 25°C after 10 days on potato dextrose agar (PDA) and oatmeal agar (OA), respectively; C, D: *Mariannaea*-like conidial arrangement; E-G: Phialides with 1-3 branches arising directly from aerial hyphae; H: Conidia. Scale bars = 10 μm.

Table 2. Morphological characteristics of *Blackwellomyces calendulinus* KNUF-23-232 compared with a previous report on *B. calendulinus*

Characteristics		*Blackwellomyces calendulinus* KNUF-23-232[a]	*Blackwellomyces calendulinus* BCC 68502[Tb]
Colony on PDA	Size (mm)	24–26	30
	Color	White to creamy, reverse center burgundy and margin scarlet, white fringe	White
	Shape	Circular, ciliate margin, slightly raised and wrinkled, cottony	Circular, ciliate margin, crateriform, cottony with high mycelium density
Colony on OA	Size (mm)	22–24	25
	Color	White, reverse light brown	White
	Shape	Circular, ciliate margin, umbonate, cottony with high mycelium density	Circular, ciliate margin, umbonate, cottony with high mycelium density
Conidia	Size (μm)	3.2–5.3 × 1.6–2.6	3.5–4.5 × 1.5–2.0
	Shape	Hyaline, oblong-elliptical or ellipsoidal, smooth surface, one-celled	Hyaline, oblong-elliptical or ellipsoidal, smooth, one-celled
Phialide	Size (μm)	12.5–22.4 × 1.3–2.5	7.0–12.0 × 1.0–2.0
	Shape	Solitary or in whorls of two to three on each branch, gradually tapering from the base to the apex	Solitary or in whorls of two to five on each branch, gradually tapering from the base to the apex

PDA: potato dextrose agar; OA: oatmeal agar.
[T] Type strain; [a] Fungal strain used in this paper; [b] Source of descriptions [4].

Molecular phylogeny of strain KNUF-23-232

For the molecular identification of the isolated fungal strain KNUF-23-232, total genomic DNA was amplified to obtain sequences for the ITS region, LSU, *RPB*1, *RPB*2, and *TEF*1, yielding lengths of 500, 811, 637, 880, and 879 bp, respectively. The ITS regions showed 98.2% similarity with *B. calendulinus* BCC 68502^{T}, whereas the LSU sequence displayed 99.4% similarity with *B. calendulinus* BCC 85060^{T}. The *RPB*1 sequence demonstrated 98.7% with *B. calendulinus* BCC 68502^{T} and 98.0% similarity with *B. lateris* MFLU 18-0663^{T}. The *RPB*2 sequence revealed 99.8% with *B. calendulinus* BCC 68502^{T} and 97.0% with *B. roseostromatus* BCC 91358^{T}. The *TEF*1 sequences showed 99.9% similarity with *B. calendulinus* BCC 68502^{T}. A phylogenetic tree was constructed using the NJ method based on the concatenated sequences of the ITS region, LSU, *RPB*1, *RPB*2, and *TEF*1 (Fig. 2). In this phylogenetic tree, strain KNUF23-232 clustered closely with *B. calendulinus* BCC 68502^{T}. Based on the cultural, morphological, and phylogenetic analyses, strain KNUF-23-232 was identified as *B. calendulinus*.

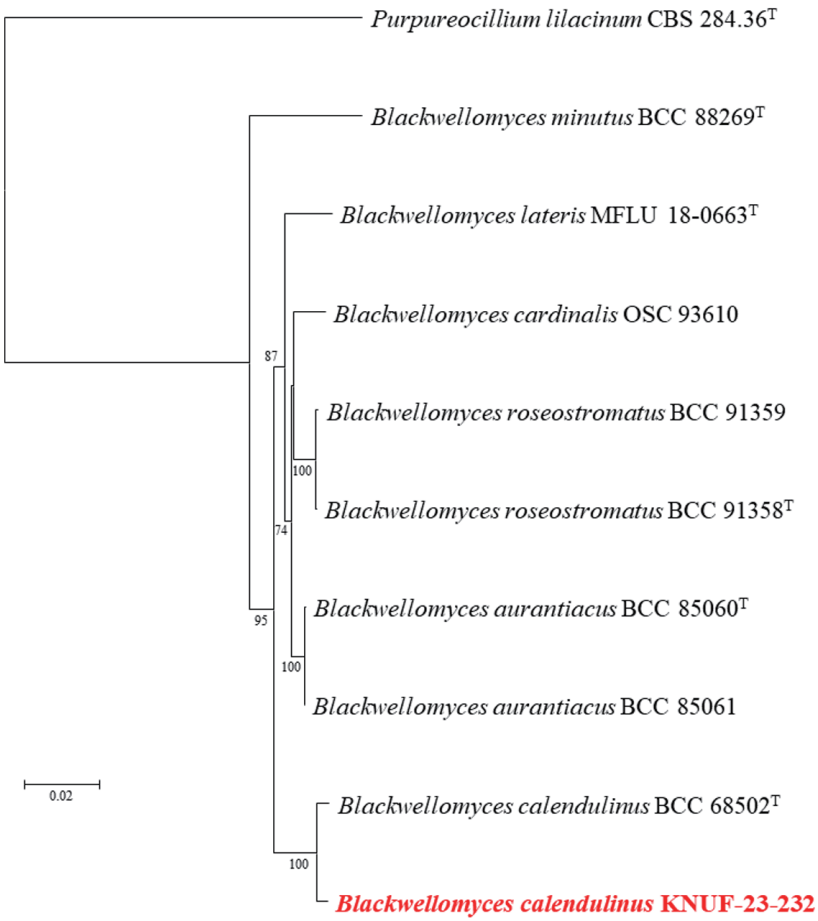

Fig. 2. Neighbor-joining phylogenetic analysis of KNUF-23-232 based on concatenated sequence data from the internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (LSU), the largest subunit of RNA polymerase II (*RPB*1), the second largest subunit of RNA polymerase II (*RPB*2) and translation elongation factor-1α (*TEF*1), illustrating the phylogenetic position of the closest species within the genus *Blackwellomyces*. Bootstrap values greater than 70% (based on 1,000 replications) are indicated at branch points. *Purpureocillium lilacinum* CBS 284.36^{T} was used as an outgroup. The strain isolated in this study is highlighted in bold red. Bar=0.02 substitutions per nucleotide position.

The genus *Blackwellomyces* comprises eight species reported globally, including *B. cardinalis*, *B. pseudomilitaris*, *B. aurantiacus*, *B. roseostromatus*, *B. lateris*, and *B. kaihuaensis*, which have been isolated from lepidopteran larvae, and *B. calendulinus* and *B. minutus* from coleopteran larvae [20]. While all species within this genus have been isolated from insect hosts, there is a notable lack of studies assessing their pathogenicity or investigating their potential as insect pathogens. Genetic analysis of *B. cardinalis* NBRC 103832 has revealed that the red pigment produced in the culture medium is oosporein, a secondary metabolite known to inhibit bacterial growth [21]. This finding suggests the potential for entomopathogenic characteristics within the genus *Blackwellomyces*. Although no studies have specifically examined the secondary metabolites or entomopathogenicity of *B. calendulinus*, it is posited that this species may possess significant potential as an entomopathogenic fungus, warranting further investigation.

Neoaraneomyces araneicola W.H. Chen, Y.F. Han, J.D. Liang & Z.Q. Liang, MycoKeys 91:58 (2022) [MB#842645]

Cultural and morphological characteristics of KNUF-23-344

When cultured on PDA at 25°C for 14 days, the colonies reached a diameter of 24–28 mm. The obverse side appeared white to light yellow, with an umbonate shape and wrinkles radiating from the center to the edges, which were irregularly undulate (Fig. 3A). The reverse side exhibited similar characteristics, with wrinkles extending from the center to the edges and a yellowish color (Fig. 3A). The conidiophores were observed to be mononematous, arising laterally from the aerial hyphae. The phialides were observed to be cylindrical to ellipsoidal, tapering from the base towards the apex (Fig. 3B), with dimensions ranging from 12.0–31.2 × 1.0–1.9 μm (n=20). The conidia, produced at the tip of the phialides, were cateniferous, resembling those of *Paecilomyces* (Fig. 3C). These conidia were hyaline, fusiform to ellipsoidal or semiorbicular, unicellular, and measured 2.8–4.8 × 1.1–2.0 μm in length (n=50) (Fig. 3D). A comparative analysis of the morphological features of strain KNUF-23-344 with *Neoaraneomyces araneicola* DY101711^{T} and *Paraneoaraneomyces sinensis* ZY 22.006^{T} is presented in Table 3, indicating that strain KNUF-23-344 is closely related to *N. araneicola* DY101711^{T}.

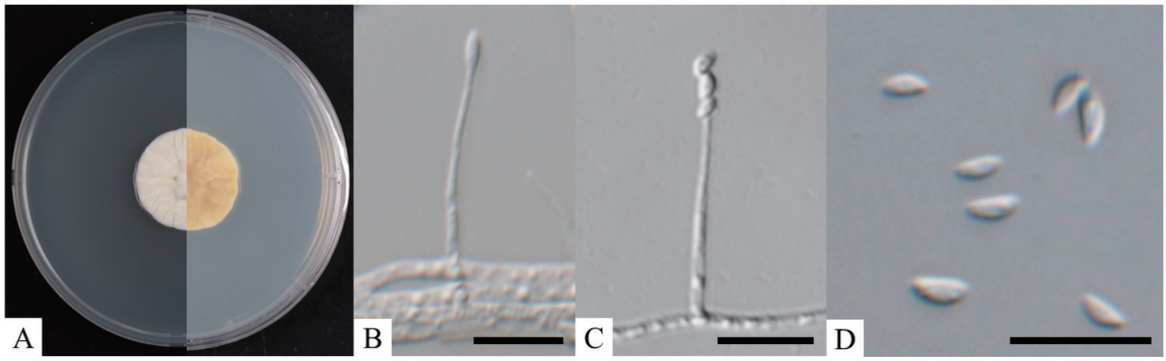

Fig. 3. Cultural and morphological characteristics of *Neoaraneomyces araneicola* KNUF-23-344. A: Obverse and reverse views of the colony at 25°C after 14 days on potato dextrose agar (PDA); B: Phialide; C: Phialide with conidia in chain; D: Conidia. Scale bars=10 μm.

Table 3. Morphological characteristics of *Neoaraneomyces araneicola* KNUF-23-344 compared with a previous report on *N. araneicola*

Characteristics		*Neoaraneomyces araneicola* KNUF-23-344[a]	*Neoaraneomyces araneicola* DY101711[Tb]
Colony on PDA	Size (mm)	24–28	30–32
	Color	White to light yellow, reverse yellowish	White to pale grey, reverse yellowish
	Shape	Irregular and undulate margin, umbonate, wrinkled	Irregular and undulate margin, umbonate, wrinkled, consisting of a basal felt
Phialide	Size (μm)	12.0–31.2 × 1.0–1.9	8.9–23.8 × 1.1–1.6
	Shape	Mononematous, arising from the lateral, aerial hyphae	Mononematous, arising from the lateral, aerial hyphae
Conidia	Size (μm)	2.8–4.8 × 1.1–2.0	2.9–4.4 × 1.3–2.0
	Shape	Hyaline, fusiform to ellipsoidal or semiorbicular, cateniferous, one-celled	Cateniferous, hyaline, fusiform to ellipsoidal, one-celled

PDA: potato dextrose agar; N/A: not available.
[T] Type strain; [a] Fungal strain used in this paper; [b] Source of descriptions [5].

Molecular phylogeny of KNUF-23-344

For the molecular identification of the isolated fungal strain, the total genomic DNA of KNUF-23-344 was amplified to obtain the ITS region, LSU, *RPB*2, and *TEF*1 (343, 416, 594, and 780 bp, respectively). The ITS regions exhibited a high 99.5% similarity to *N. araneicola* DY101711[T], whereas the LSU sequences demonstrated 100% similarity with both *N. araneicola* DY101711[T] and DY101712. The *RPB*2 sequences exhibited a 99.0% similarity with *N. araneicola* DY101711[T] and a 98.7% similarity with *N. araneicola* DY101712. The *TEF*1 sequences showed a 99.8% similarity with both *N. araneicola* DY101711[T] and DY101712. A phylogenetic tree was constructed using the NJ method with the concatenated sequences of the ITS regions, LSU, *RPB*2, and *TEF*1 (Fig. 4). Based on the NJ phylogenetic tree, strain KNUF-23-344 clustered closely with *N. araneicola* DY101711[T] and DY101712. The cultural, morphological, and phylogenetic analyses collectively identified strain KNUF-23-344 as *N. araneicola*.

The genus *Neoaraneomyces* was established by Chen et al. in 2022, with only one species, *N. araneicola*, reported, making it a highly rare taxon [5]. Among the 13 recognized genera of araneogenous fungi, only the genus *Gibellula* is documented to exclusively parasitize spiders. However, *N. araneicola* exhibits distinct characteristics that differentiate it from *Gibellula*, particularly in its conidial structure, which resembles that of *Paecilomyces*, with chain-like conidia observed at the apex of the hyphae. [5,22]. To date, no studies have addressed the pathogenicity of *N. araneicola*. Therefore, the KNUF-23-344 strain isolated in this study warrants further research on the pathogenicity of *N. araneicola* and its metabolites, with potential future applications as an acaricide in the future. To the best of our knowledge, this is the first report of these two species in Korea. The unreported species, *B. calendulinus* and *N. araneicola*, isolated in this study, will contribute to the understanding and conservation of domestic biodiversity. Additionally, they provide valuable data that may enhance the potential utilization of their entomopathogenic properties.

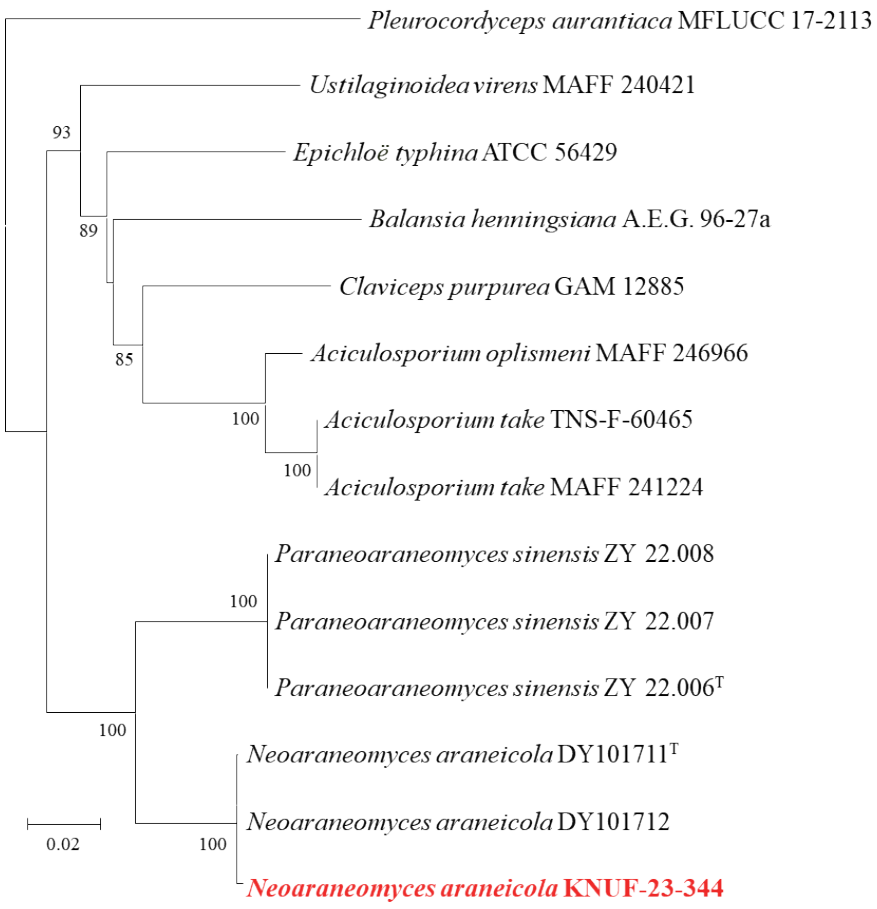

Fig. 4. Phylogenetic analysis of KNUF-23-344 using the neighbor-joining method based on concatenated sequence data from the internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (LSU), the second largest subunit of RNA polymerase II (*RPB2*) and translation elongation factor-1α (*TEF*1), showing the phylogenetic position of the closest genus within the family Clavicipitaceae. Bootstrap values greater than 80% (based on 1,000 replications) are indicated at branch points. *Pleurocordyceps aurantiaca* MFLUCC 17-2113 was used as an outgroup. The strain isolated in this study is highlighted in bold red. Bar=0.02 substitutions per nucleotide position.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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