




Taxonomy of *Botryotrichum luteum* sp. nov. based on Morphology and Phylogeny Isolated from Soil in Korea

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ABSTRACT

In this study, a fungal strain KNUF-22-025 belonging to the genus *Botryotrichum* was isolated from the soil in Korea. The cultural and morphological characteristics of this strain differed from those of closely related species. On malt extract agar, strain KNUF-22-025 showed slower growth than most of the related species, except *B. domesticum*. The conidia size (9.6–21.1 × 9.9–18.4 μm) of strain KNUF-22-025 was larger than those of *B. piluliferum*, *B. domesticum*, and *B. peruvianum* but smaller than those of *B. atrogriseum* and *B. iranicum*. Conidiophores in strain KNUF-22-025 (137 μm) were longer than those in other closely related species but shorter than those in *B. atrogriseum*. Multi-locus analysis of molecular markers, such as ITS, 28S ribosomal DNA, *RBP2*, and *TUB2* revealed that strain KNUF-22-025 was distinct from other *Botryotrichum* species. Thus, this strain is proposed as a novel species based on morphological characteristics along with molecular phylogeny and named *Botryotrichum luteum* sp. nov.

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1. Introduction

Sordariomycetes is the second largest group in the phylum Ascomycota and has considerable importance [1]. Currently, 47 orders, 127 families, 1975 genera, and 23,187 species have been recognized in this phylum [2]. Fungi of this phylum span various ecologies, including mycoparasites, animal and plant pathogens, and endophytes [3]. The order Sordariales is a diverse group of fungi within the class Sordariomycetes. Numerous saprotrophic fungi found in soil, plant materials, and dung from wild animals are included in this group [4]. Winter (1885) established the family Chaetomiaceae, which is characterized by *Chaetomium*, and the family Chaetomiaceae is included in Sordariales, which comprises 25 genera [3–8]. The Chaetomiaceae, genus *Botryotrichum* is characterized by thick-walled conidia with setose, branching, and macronematous conidiophores. Owing to the sexual state of some *Botryotrichum* species, particularly that of *Botryotrichum piluliferum* (Type strain), which was previously regarded as *Chaetomium*, a connection between the two genera was discovered [8,9]. Daniels (1961) collected the *B. piluliferum* strain from cellulose films that were incubated in soil. The

collected strain showed unbranched ascomatal hairs with circinate tips and ellipsoid ascospores, which are the characteristics of *Chaetomium murorum*. As a result, *Chaetomium piluliferum* was assigned a new holomorphic name [9]. Wang et al. (2016), demonstrated that *C. murorum*, *Emilmuelleria spirotricha*, and *Thermomyces verrucosus* were related to *Botryotrichum* species, and new combinations, such as *B. murorum*, *B. spirotrichum*, and *B. verrucosum*, were proposed based on phylogenetic analyses [8,10]. Currently, 23 species in *Botryotrichum* have been listed in the Index Fungorum (www.indexfungorum.org). Of the species of *Botryotrichum*, seven are anamorphically characterized, including *B. piluliferum*, *B. atrogriseum*, *B. verrucosum*, *B. peruvianum*, *B. domesticum*, *B. foricae*, and *B. iranicum* [8–12]. Recently in 2022, new species of *B. inquinatum*, *B. retardatum*, *B. trichorobustum*, *B. vitellinum*, and *B. geniculatum* were included in *Botryotrichum* [13].

This study aimed to explore and classify the novel species of fungi isolated from soil in Korea. The novelty of the strain was confirmed *via* cultural, morphological, and molecular characteristics. In this study, a new species of fungus was discovered in Korea and given a comprehensive description as well as an illustration.

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2. Materials and methods

2.1. Sample collection and isolation of fungal strain

The soil sample was collected from Subuksan, Okcheon-gun, Chungcheongbuk-do (36°23'45"N, 127°39'19"E), Korea. Isolation of the strain was performed using the standard serial dilution technique [14]. Sample soil (1 g) was added to 10 ml of sterile distilled water, and the soil suspension was diluted serially and spread on potato dextrose agar plates (PDA; Difco, Detroit, MI). The plates were incubated for 3–4 days at 25 °C until single colonies were observed. Further, single colonies were transferred to new PDA plates and incubated at 25 °C. The strain KNUF-22-025 was selected based on cultural, morphological, and phylogenetic analyses.

2.2. Morphological characterization of strain KNUF-22-025

The cultural and morphological characteristics of the strain KNUF-22-025 were observed using four different media viz. PDA, oatmeal agar (OA), malt extract agar (MEA), and potato carrot agar (PCA) [15]. The colony characteristics of the strain were recorded, and the mycological characteristics were observed using a light microscope (BX-50; Olympus, Tokyo, Japan).

2.3. Genomic DNA extraction, PCR amplification, and sequencing

The total genomic DNA from fungal mycelia of the strain KNUF-22-025 was extracted using a HiGene Genomic DNA prep kit (BIOFACT, Daejeon, Korea) according to the manufacturer's instructions. The following combined datasets were used according to previous studies: internal transcribed spacer (ITS) regions, 28S ribosomal DNA (LSU), the

second largest subunit of RNA polymerase II (*RPB2*) genes, and β -tubulin (*TUB2*) [10,12]. The primer pairs of ITS1F/ITS4, NL1/NL4, RPB2AM-1gf/RPB2AM-7R, and T1/T2 were used for ITS, LSU, *RPB2*, and *TUB2*, respectively [16–18]. The thermal conditions for PCR amplification were used as described in previous studies [12, 19]. PCR products were confirmed by running the amplified DNA samples in 1.2% agarose gel stained with ethidium bromide. The purification of the PCR products was determined using EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced at Solgent Co., Ltd., Korea. The DNA sequence data were analyzed using SeqMan Lasergene software (DNASTar Inc., Madison, Wisconsin, USA).

2.4. Phylogenetic analyses

Using the Basic Local Alignment Search Tool (BLAST), four molecular markers (ITS, LSU, *RPB2*, and *TUB2*) of strain KNUF-22-025 were compared with reference sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) (Table 1). Based on Kimura's neighbor-joining algorithm, the evolutionary distance matrices were generated [20]. To determine the precise taxonomic position of the strain, neighbor-joining [21], maximum likelihood [22], and maximum parsimony [23] trees were constructed. The MEGA 7.0 software program was used for phylogenetic tree analysis, and bootstrap values were based on 1000 replications [24].

3. Results

3.1. Taxonomical analysis of *Botryotrichum luteum* sp. nov.

Strain KNUF-22-025 was identified as a novel species based on differences in morphological and

Table 1. GenBank accession numbers of fungal strains used in this study for the phylogenetic analysis.

Species name	Strain numbers	Accession numbers			
		ITS	LSU	<i>RPB2</i>	<i>TUB2</i>
<i>Botryotrichum luteum</i>	KNUF-22-025^T	LC731694	LC731695	LC731696	LC731697
<i>B. atrogriseum</i>	CBS 130.28 ^T	KX976589	KX976714	KX976813	KX976931
<i>B. atrogriseum</i>	CBS 604.69	KX976590	KX976715	KX976814	KX976932
<i>B. domesticum</i>	UAMH 11929 ^T	MH899168	MH899169	MH899171	MH899172
<i>B. geniculatum</i>	CBS 144475 ^T	MZ334719	MZ351422	MZ342972	MZ343011
<i>B. inquinatum</i>	CBS 155.80 ^T	MK919289	MK919289	MK919345	MK919403
<i>B. iranicum</i>	ABRIICC 10152 ^T	MN134583	MN134576	MN128437	MN128435
<i>B. iranicum</i>	ABRIICC 10153	MN134584	MN134577	MN128438	MN128436
<i>B. murorum</i>	CBS 163.52	KX976591	KX976716	KX976815	KX976933
<i>B. peruvianum</i>	CBS 460.90	KX976595	KX976720	KX976819	KX976937
<i>B. peruvianum</i>	CBS 421.93	KX976596	KX976721	KX976820	KX976938
<i>B. piluliferum</i>	CBS 105.14	KX976598	KX976723	KX976822	KX976940
<i>B. spirotrichum</i>	CBS 211.55 ^T	KX976601	KX976726	KX976825	KX976943
<i>B. verrucosum</i>	CBS 116.64 ^T	LT993567	LT993567	LT993486	LT993648
<i>B. vitellinum</i>	CBS 180.84 ^T	MZ334725	MZ351421	MZ342979	MZ343018
<i>Chaetomium globosum</i>	CBS 160.62 ^T	KT214565	KT214596	KT214666	KT214742

The identified strain is indicated in bold in this study. ^TType strain.

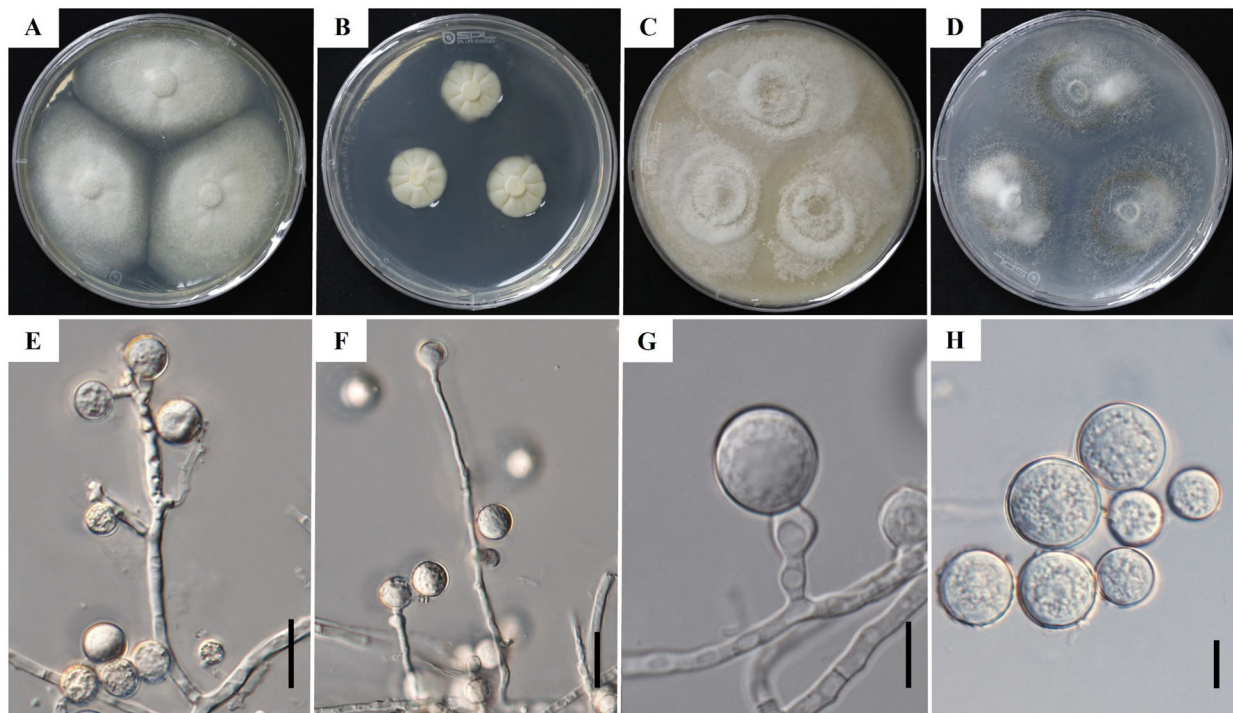


Figure 1. Cultural and morphological characteristics of *Botryotrichum luteum* (KNUF-22-025^T). Colonies on potato dextrose agar (A), malt extract agar (B), oatmeal agar (C), and potato carrot agar (D), after 14 days incubation at 25 °C. Conidiophores and conidiogenous cells (E-G); Conidia (H). Scale bars: E, F = 20 µm; G, H = 10 µm.

molecular characteristics from closely related *Botryotrichum* species.

***Botryotrichum luteum* J. J. Ryu, S.Y. Lee, and H.Y. Jung, sp. nov.** (Figure 1)

Mycobank: MB 845982

Etymology: The specific name “*luteum*” represents yellow color colonies.

Typus: Isolated from mountain soil containing plant debris from Subuksan, Okcheon-gun, North Chungcheong Province (36°23′45″N, 127°39′19″E), Korea. The metabolically inactive stock culture of the strain (NIBRFGC000509835) was deposited at the culture center of National Institute of Biological Resources (NIBR).

Ecology and distribution: Several species have been isolated from mountains and field soils, the dung of animals such as rabbits and deer, etc. Additionally, members of this genus were isolated from the air and indoor environments, such as inside villas and ceiling tiles. In the present study, the proposed novel species *Botryotrichum luteum* was isolated from the forest soil in South Korea.

Cultural characteristics: The colonies were smooth, round, and flat with lobate margins spreading moderate aerial mycelium. Colonies were 63.5–66.3 mm in diameter after culturing for 14 days at 25 °C and the reverse colonies were yellow to light beige on PDA (Figure 1(A)). On MEA, the colonies grew very slowly, the surface produced floccose white

colonies, and attained 21.5–24.0 mm in diameter within 14 days of growth at 25 °C with reverse pale yellow to light beige (Figure 1(B)). On OA media, the colony surface was floccose to funiculose, and the mycelium color was white with dark brown to black spots. Mycelium diameter was 74.5–80.0 mm after 14 days of growth at 25 °C (Figure 1(C)). On PCA, the fungal colonies were white to dirty white with 81.5–83.0 mm diameter after a growth period of 14 days at 25 °C. The color of the reverse colonies was pale yellow to light beige (Figure 1(D)).

Morphological characteristics: The conidiophores were produced laterally from hyphae near the base with a diameter of 2.5–5.3 µm and a length of upto 137 µm. The mycelium was immersed, colorless, and 1.8–2.2 µm thick (Figure 1(E and F)). Conidiophores were micronematous, present on both submerged and aerial hyphae, reduced to conidiogenous cells when multiple-celled, unbranched or branching, and colorless to light brown. Conidiogenous cells were solitary, monoblastic, hyaline, smooth, terminal, intercalary, or occasionally integrated into the hyphae. The cells were barrel-shaped, cylindrical, or T-shaped (Figure 1(G)). Conidia were solitary, partial hyaline when young, one-celled, globose to subglobose, rarely in chains of coupled spores, smooth to slightly roughened, 9.6–21.1 × 9.9–18.4 µm (\bar{x} = 15.5 × 14.6 µm, n = 50) with a length/width ratio of 1.05 and visible hilum, 3–6.4 µm wide, and 2.7–4.2 µm high (Figure 1(H)).

Table 2. Morphological characteristics of *Botryotrichum luteum* (KNUF-22-025^T) and comparison with closely related species grown on MEA.

Characteristics	Colony	Conidiophores	Conidia	Ref.
<i>Botryotrichum luteum</i> (KNUF-22-025 ^T)	White with reverse pale yellow to light beige; 21.5–24.0 mm in diameter in 14 days at 25 °C	Up to 137 µm long	Smooth to slightly roughened, 9.6–21.1 × 9.9–18.4 µm	This study
<i>B. atrogriseum</i> (CBS 130.28 ^T)	Dark gray colonies	Up to 150 µm long	Spherical, verrucose, 10–25 µm	[25]
<i>B. domesticum</i> (UAMH 11929 ^T)	Colorless; 5.5 mm in diameter in a month	N/A	Thick-walled, 15.1–20.3 × 14.8–19.8 µm	[12]
<i>B. foricae</i> (CCF 5752 ^T)	Mustard yellow; 21 mm diam. In 7 days at 25 °C	Up to 35 µm long	Smooth, rarely slightly roughened, 8–13 µm	[11]
<i>B. iranicum</i> (ABRIICC 10152 ^T)	Mycelium white to pinkish buff, reverse light ochraceous-buff; 22–23 mm diam. In 7 days at 25 °C	Up to 70 µm long	Roughened, 9.5–23.5 × 9.5–17 µm	[19]
<i>B. peruvianum</i> (CBS 460.90)	Pale ochraceous, grayish sepia, reverse ochraceous to umber; 21–28 mm diam. In 7 days at 25 °C	Up to 60 µm long	Conspicuously roughened 12–16 µm	[10]
<i>B. piluliferum</i> (CBS 654.79)	White to rosy buff, reverse ochraceous; 25–31 mm diam. In 7 days at 25 °C	Up to 40 µm long	Smooth to slightly roughened, 11–17.5 µm	[10, 26]
<i>B. verrucosum</i> (CBS 116.64 ^T)	White, mouse gray, reverse dark mouse gray; 17–23 mm diam. In 7 days at 25 °C	Up to 65 µm long	Conspicuously warted 14–15.5 µm	[8]

N/A: not available.

Note: *B. piluliferum*, *B. atrogriseum*, *B. domesticum*, *B. iranicum*, *B. peruvianum*, and *B. verrucosum* are all phylogenetically related to the strain KNUF-22-025. However, several morphological characteristics distinguished it from related species (Table 2). Strain KNUF-22-025 achieved a growth of 21.5–24.0 mm in diameter after culturing for 14 days at 25 °C on MEA, whereas the related strain *B. domesticum* (UAMH 11929^T-^TType strain). Grew 5.5 mm in diameter in 1 month at 25 °C. The other related species, such as *B. iranicum* (ABRIICC 10152^T), *B. piluliferum* (DTO 254-B8), *B. peruvianum* (CBS 421.93), and *B. verrucosum* (CBS 116.64^T), showed growths of 22–23, 25–31, 21–28, and 17–23 mm, respectively, after 7 days at 25 °C (Table 2). The strain KNUF-22-025 produced white colonies with reverse pale yellow to light beige, whereas *B. iranicum* displayed white to pinkish buff, reverse light ochraceous-buff. The conidia produced by KNUF-22-025 were larger in size than those produced by *B. piluliferum* (11.0–17.5 µm), *B. domesticum* (15.1–20.3 14.8–19.8 µm), *B. peruvianum* (12.0–16.0 µm), and *B. verrucosum* (14.0–15.5 µm) and smaller than those produced by *B. atrogriseum* (10.0–25.0 µm) and *B. iranicum* (9.5–23.5 × 9.5–17.0 µm) (Table 2). The *B. iranicum* and *B. atrogriseum* strains closest to strain KNUF-22-025 produced conidia with rough and verrucose surfaces, respectively (Table 2). However, the conidia of strain KNUF-22-025 were mostly smooth with a slight rough surface. The conidiophore of KNUF-22-025 (137 µm) was longer than those of *B. piluliferum* (40 µm), *B. iranicum* (70 µm), *B. verrucosum* (65 µm), and *B. peruvianum* (60 µm) but shorter than those of *B. atrogriseum* (150 µm). Thus, strain KNUF-22-025 is well distinguished from the previously described closest *Botryotrichum* species.

3.2. Molecular phylogeny of strain KNUF-22-025

The obtained lengths of the sequences were 583, 576, 627, and 642 bp for ITS, LSU, *RPB2*, and *TUB2*, respectively. These sequences were deposited in NCBI GenBank with accession numbers LC731694, LC731695, LC731696, and LC731697 for ITS, LSU, *RPB2*, and *TUB2*, accordingly. Strain KNUF-22-025 was found to be 99.83% similar to *B. piluliferum*, (CBS 503.83), 99.82% to *B. atrogriseum* (CCF 5752), and 99.49% to *B. domesticum* (UAMH 11929^T) in the ITS regions. Additionally, in the LSU, it showed 100% similarities to *B. piluliferum* (CBS 503.83), and *B. verrucosum* (CBS 116.64^T) and 99.83%, and 99.65% to *B. atrogriseum* (CCF 5752) and *B. domesticum* (UAMH 11929^T), respectively. Similarly, *RPB2* gene sequence analysis of KNUF-22-025 showed 97.28%, 96.55%, 95.69%, 95.59%, and 95.40% similarities to *B. verrucosum* (CBS 116.64), *B. atrogriseum* (CBS 604.69, CBS 130.28^T), *B. domesticum* (UAMH 11929^T), *B. peruvianum* (CBS 460.90), and *B. piluliferum* (DTO 254-B9), respectively. Moreover, *TUB2* gene sequences of KNUF-22-025 showed 94.58% and 94.28% similarities with *B. domesticum* (UAMH 11929^T) and *B. piluliferum* (CBS 654.79), respectively. Also *B. atrogriseum* (CBS 130.28^T, CCF 5752, and CBS 604.69) displayed 95.47 to 96.14% similarities based on *TUB2* gene. Additionally, ITS, LSU, and *TUB2* sequences of strain KNUF-22-025 showed a high similarity to *B. foricae* with 99.55%, 99.83%, and 94.28% similarities, respectively. Although ITS and LSU sequences of strain KNUF-22-025 showed a high similarity (99.8%–100%) with the strain *B. iranicum*, *TUB2* and *RPB2* sequences of this strain depicted a high similarity to *B. iranicum* (94.94%) and *B. atrogriseum* (96.14%, 96.55%), respectively.

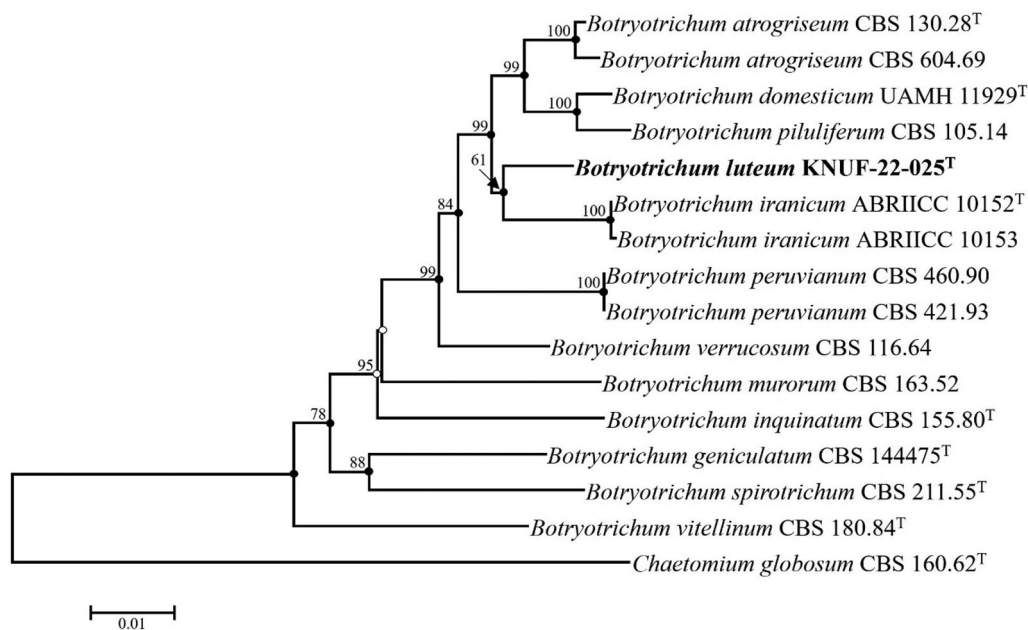


Figure 2. Neighbor-Joining phylogenetic tree based on molecular markers the internal transcribed spacer (ITS) regions, 28S ribosomal DNA (LSU), the second largest subunit of RNA polymerase II (*RPB2*), and β -tubulin (*TUB2*) genes sequences showing the phylogenetic position of the strain KNUF-22-025. *Chaetomium globosum* CBS 160.62^T comprised the outgroup. The neighbor-joining phylogenetic tree, maximum likelihood, and maximum parsimony trees indicated with filled nodes, whereas open circles showed maximum-likelihood or maximum-parsimony. The numbers (>60%) above the branches represent the bootstrap values obtained for 1,000 replicates. The isolated strain is shown in bold. Bar, 0.01 substitutions per nucleotide position.

For more precise identification, we performed multilocus sequence analysis using the combined dataset sequences of the ITS regions, LSU, *RPB2*, and *TUB2* genes of strain KNUF-22-025. The combination of these four molecular markers has shown to be highly effective in the identification of species using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) phylogenetic trees. The neighbor-joining phylogenetic tree node as well as the filled nodes in the maximum parsimony and maximum likelihood trees were used to construct the phylogenetic analysis using the combined dataset of the ITS regions, LSU, *RPB2*, and *TUB2* (Figure 2). As indicated in the open circles, the corresponding nodes were also constructed using the maximum likelihood or maximum parsimony algorithms. The precise taxonomic position of the strain was ascertained by a phylogenetic analysis based on a combination of sequences with maximum parsimony (tree length = 733, consistency index = 0.58, retention index = 0.62, and composite index = 0.45). The results of the phylogenetic tree demonstrated that strain KNUF-22-025 did not cluster with closely related fungal strains. Hence, the strain KNUF-22-025 is proposed as a novel species within the *Botryotrichum* genus.

4. Discussion

Previous studies have reported that the strains belonging to the genus *Botryotrichum* were widely discovered

in the excrement of various animals, including rabbit, herbivore, deer, donkey, and field soil [10]. The strains *B. piluliferum* and *B. murorum* have been discovered from interior substrates, such as plaster walls, villa walls, ceiling tiles, and anthropogenic environments, whereas *B. peruvianum* strain has been found in indoor air. The strain *B. murorum* was identified in the liquor cerebrospinalis of *Homo sapiens* [10]. Secondary metabolites produced by fungi are well-known for their various biological properties, which led to the development of uncounted agrochemicals and life-saving medicines. *B. piluliferum*, the mycotoxin-producing type species of *Botryotrichum*, produces oxisterigmatocystins E, G, and H, which have shown antimalarial activity against *Plasmodium falciparum* and cytotoxic activity against KB, MCF-7, and NCI-H187 cell lines [27]. These two strains also produce altersolanol C, diorcinol, and botryobutenolide A, which have shown antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* [28]. Additionally, the strain of *B. piluliferum* has a wide ecological distribution and has been identified as a chili pepper seed-borne fungus [29]. *B. murorum* has previously been recognized as a human pathogen that causes phaeohyphomycosis [30]. Additionally, strains *B. atrogriseum* and *B. iranicum* (Type strain) were isolated from the cornfield soil in Canada and Iran, respectively [19,25]. In Korea, *Botryotrichum* sp. was identified in the upland soil and cultivated garlic in Chungcheongbuk-do, Korea [31]. *Botryotrichum* sp., along with several other fungi and bacteria, was identified from Nuruk, a Korean cereal

fermentation starter [32]. Recently, *B. iranicum* was identified in soil samples from Korea [33]. So, there are several species of *Botryotrichum* that produce mycotoxins, and metabolites, show anti-malarial activities, and the genus has a wide range of distribution, and pathogenicity which is not been explored thoroughly even in Korea. As an outcome, a novel species (*B. luteum*) was discovered in Korean soil during this current investigation. Thus, the importance of *B. luteum* can be referred to by its potential for medicines or biological use which is needed to do further research.

In conclusion, the genus *Botryotrichum* is broadly distributed and possesses cytotoxic activities, therefore, systematic investigation of strains of these species obtained from natural sources is required to evaluate their bioactive potential. The findings of this study might guide future studies on this strain. Hence, further understanding of the phylogeny and ecology of *Botryotrichum* species is essential.

Disclosure statement

No potential conflict of interest was reported by the authors.

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