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Characterization of three unrecorded yeast species, *Nakaseomyces glabratus*, *Crinitomyces flavificans* and *Kazachstania africana*, isolated from *Batillaria cumingii*, *Batillaria multiformis* and soil in Republic of Korea

Seonyeong Kim[1], Yuna Park[2], Seo Hyun Kim[3], Ju Eun Ko[3], Jae-Hyoung Joo[3], Young Ji Choi[3], Bomi Nam[3], Soyeon Lee[3] and Soohyun Maeng[1,*]

[1]*Department of Bio & Environmental Technology, College of Natural Science, Seoul Women's University, Seoul 01797, Republic of Korea*
[2]*DNA Analysis Division, Busan Institute, National Forensic Service, Yangsan 50612, Republic of Korea*
[3]*Division of Bioresources Bank, Honam National Institute of Biological Resources, Mokpo 58762, Republic of Korea*

**Correspondent: biotech@swu.ac.kr*

The purpose of this study is to isolate and identify wild yeasts from *Batillaria cumingii*, *Batillaria multiformis* and soil samples collected in Mokpo-si and Shinan-gun, Republic of Korea. Out of the fourteen strains identified, ten had been previously reported, whereas four strains were unreported in the Republic of Korea. Identification of wild yeast strains was performed through pairwise sequence comparisons of the D1/D2 region of the 26S rRNA gene using the Basic Local Alignment Search Tool (BLAST) (Fell *et al*., 2000). Cell morphology was examined using a phase contrast microscope, and assimilation tests were conducted with the API 20C AUX kit. Of the four unreported yeast strains, D-7 and 11-2 belong to the genus *Nakaseomyces* (family: *Saccharomycetaceae*, order: *Saccharomycetales*, class: *Saccharomycetes*) and the genus *Kazachstania* (family: *Saccharomycetaceae*, order: *Saccharomycetales*, class: *Saccharomycetes*), respectively. Meanwhile, K-9 and K-22 belong to the genus *Crinitomyces* (family: *Trichomonascaceae*, order: *Dipodascales*, class: *Dipodascomycetes*). The 26S rRNA gene sequences of strains D-7, K-9, K-22, and 11-2 have been deposited in GenBank under the accession numbers PV244027, PV244028, PV244029, and PV243995, respectively. All strains had oval-shaped and multilateral budding cells. This research elucidated the morphological and biochemical properties of these three previously unreported yeast species in Korea.

Keywords: 26s rRNA, *Crinitomyces*, *Kazachstania*, *Nakaseomyces*, unreported yeast, yeast isolation

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Introduction

With the enforcement of the Nagoya Protocol in 2014, access to and utilization of microbial resources among nations have been significantly restricted. Consequently, securing the diversity of domestic microbial resources, studying their characteristics for resource development, and utilizing them as fundamental materials for industrial applications have become essential. Yeasts, as one of the microbial resources, play a crucial role in various industries. However, despite their potential, only 1−5% of yeast species have been identified, and most of their diversity remains unexplored (Choudhary and Johri, 2009). To date, yeasts have primarily been isolated from fermented foods

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and raw materials, with some being applied in the food fermentation industry (Kim *et al*., 1999). However, research on the classification of yeasts from natural habitats and their industrial application potential remains limited. Therefore, isolating and classifying yeasts from various natural environments, along with conducting further research, is essential to gain a deeper understanding of their ecological roles, develop them as resources, and maximize their industrial applications.

In this study, wild yeast strains were isolated from *Batillaria cumingii* and *Batillaria multiformis* samples collected in Mokpo-si, Republic of Korea, as well as from soil samples collected in Shinan-gun, Republic of Korea, to investigate the yeast diversity present in these environments. As a result of isolation and identification, many yeast strains were found to be previously reported species, while some were identified as unreported species. These unreported species were classified into the genera *Kazachstania*, *Nakaseomyces*, and *Crinitomyces*.

As a result, four previously unrecorded yeast strains were discovered in domestic ecosystems. Their phenotypic characteristics were thoroughly investigated to evaluate their taxonomic identities and potential applications.

Materials and Methods

Samples were collected from Mokpo-si and Shinan-gun, Republic of Korea. Each sample was thoroughly crushed, serially diluted in distilled water, and the resulting suspension was spread on Yeast Extract Peptone Dextrose (YPD) agar (Difco, USA). The plates were then incubated at 25°C for 3 days. The strains are preserved in a metabolically inactive state at Honam National Institute of Biological Resources, Republic of Korea.

The cell morphologies of the strains were observed using a LEICA (DM500) microscope, with yeast strains incubated on YPD agar for 3 days. Phase contrast microscope images and colonies of strains D-7, K-9, K-22 and 11-2 are shown in Fig. 1. To characterize the biochemical features, the API 20C AUX (bioMérieux) test was performed according to the manufacturer's instructions.

The genomic DNA was extracted after incubation on YPD agar for 3 days. The D1/D2 region of the 26S rRNA gene sequence was amplified by PCR using NL1 (5′-GCAT ATCAATAAGCGGAGGAAA AG-3′) and NL4 (5′-GGTC CGTGTTTCAAGACGG-3′) primers (Kurtzman and Robnett, 1998). Pairwise sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) to align with sequences of related species retrieved from GenBank. The MYCOBANK (https://www.mycobank.org/) database was used to identify strain types for each species, and closely related strains' gene sequences were obtained from the NCBI (https://www.ncbi.nlm.nih.gov/) for the 26S rDNA in a row.

The phylogenetic trees based on the D1/D2 domain of the LSU rRNA gene sequence were reconstructed using the neighbor-joining method (Saitou and Nei, 1987) with

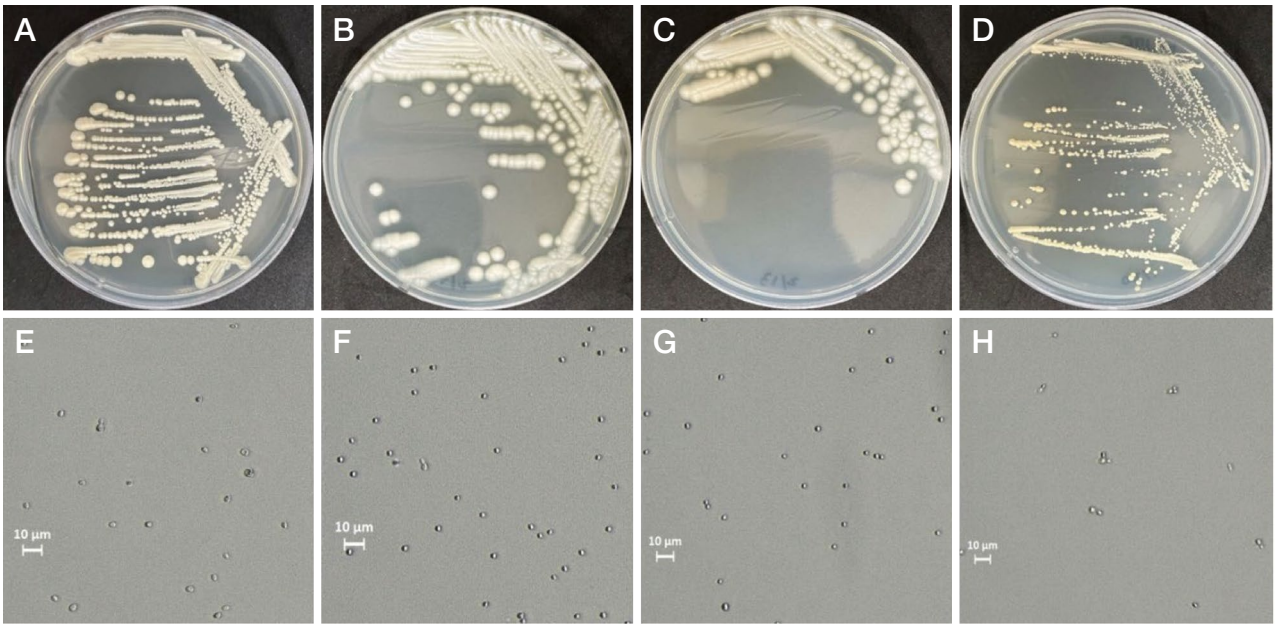

Fig. 1. Morphology of cells from the unrecorded strains D-7, K-9, K-22 and 11-2 incubated at 25ºC. All strains were grown after 3 days on YPD agar. The colonies of *Nakaseomyces glabratus* D-7 (A), *Crinitomyces flavificans* K-9 (B), *Trichosporiella flavificans* K-22 (C) and *Kazachstania africana* 11- 2 (D). The budding cells of of *Nakaseomyces glabratus* D-7 (E), *Crinitomyces flavificans* K-9 (F), *Trichosporiella flavificans* K-22 (G), and *Kazachstania africana* 11-2 (H). Bars, 10 μm.

Table 1. Yeasts isolated strains from *Batillaria cumingii*, *Batillaria multiformis* and soil in Republic of Korea.

Phylum	Class	Order	Family	Strain ID	Sample	Most closely related species	26S rRNA similarity	Record in Korea
Ascomycota	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	11-2	Soil	*Kazachstania africana*	496/496 (100%)	Unreported
				D-7	*Batillaria cumingii*	*Nakaseomyces glabratus*	580/580 (100%)	Unreported
	Dipodascomycetes	*Dipodascales*	*Trichomonascaceae*	K-9	*Batillaria multiformis*	*Crinitomyces flavificans*	362/362 (100%)	Unreported
				K-22	*Batillaria multiformis*	*Crinitomyces flavificans*	511/511 (100%)	Unreported
	Dothideomycetes	*Dothideales*	*Saccotheciaceae*	15-5	Soil	*Aureobasidium leucospermi*	499/500 (99%)	Reported
				19-2	Soil	*Aureobasidium xishuangbannaense*	500/500 (100%)	Reported
Basidiomycota	*Microbotryomycetes*	*Sporidiobolales*	*Sporidiobolaceae*	15-1	Soil	*Rhodotorula graminis*	540/540 (100%)	Reported
	Tremellomycetes	*Tremellales*	*Rhynchogastremaceae*	15-3	Soil	*Papiliotrema flavescens*	560/560 (100%)	Reported
				Y-1	Soil	*Papiliotrema aurea*	516/517 (99%)	Reported
				Y-2	Soil	*Papiliotrema aurea*	514/514 (100%)	Reported
			Trimorphomycetaceae	17-1	Soil	*Saitozyma podzolica*	561/561 (100%)	Reported
		Cystofilobasidiales	*Cystofilobasidiaceae*	16-2	Soil	*Cystofilobasidium macerans*	560/560 (100%)	Reported
		Filobasidiales	*Filobasidiaceae*	15-7	Soil	*Filobasidium magnum*	562/562 (100%)	Reported
				19-3	Soil	*Filobasidium magnum*	562/562 (100%)	Reported

All strains were cultured under 25°C for 3 days.

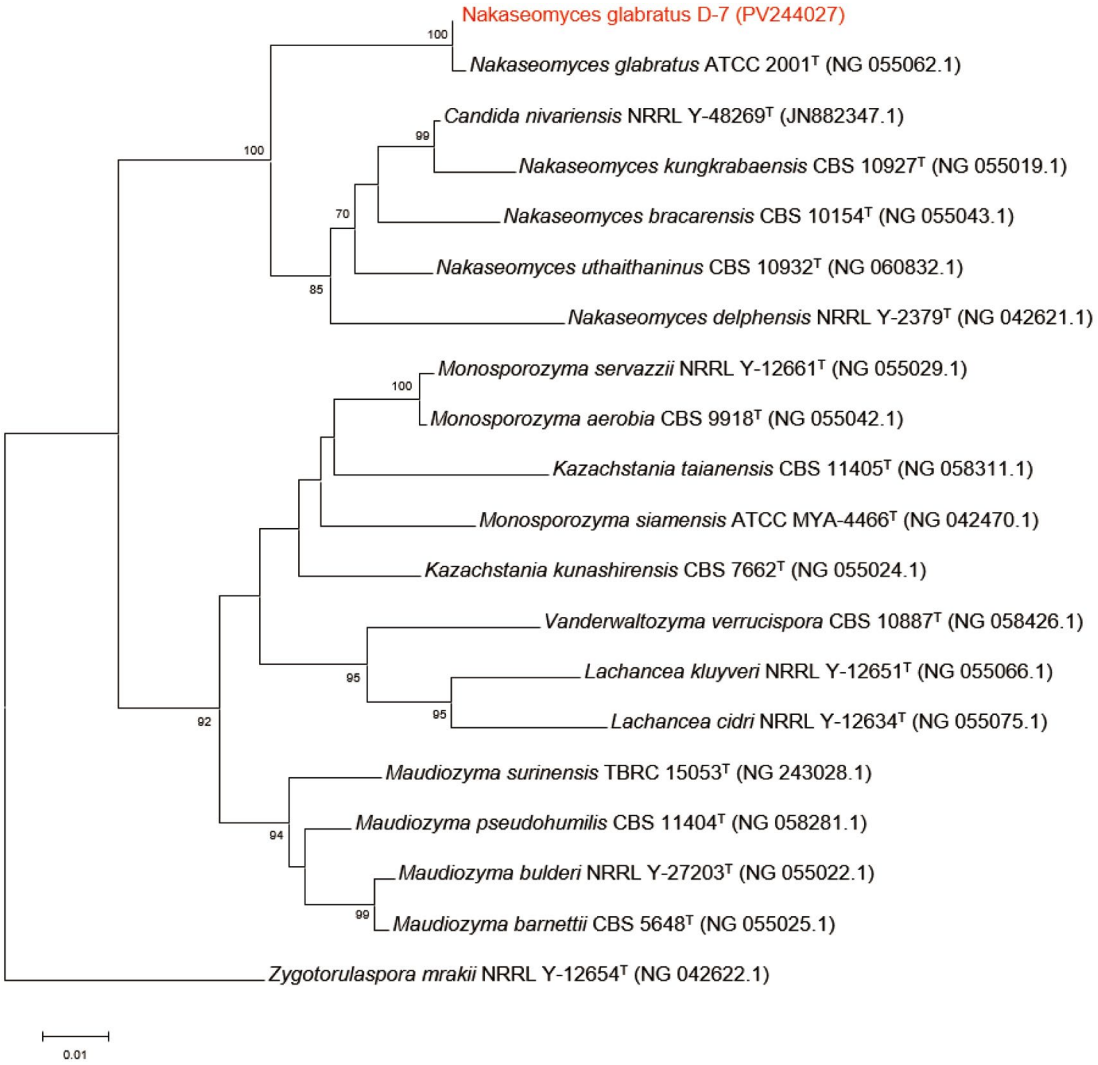

Fig. 2. A Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 26S rRNA gene sequences showing the relationships of strains D-7 with closely related species. Bootstrap values (>70%) based on neighbor-joining methods are shown at the branch nodes. Bar, 0.01 substitutions per nucleotide position.

the MEGA 11 program (Tamura *et al*., 2021). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980) for neighborhood bond analysis, and the confidence levels of the clades were estimated through bootstrap analysis with 1,000 replicates (Felsenstein, 1985). Outgroup species for the phylogenetic tree were selected based on established yeast taxonomy practices, ensuring the inclusion of distantly related species to appropriately root the tree (Guarro *et al*., 1999; Rainey and Oren, 2011).

Results and Discussion

Among the fourteen wild yeast strains analyzed, eleven were isolated from soil samples collected in Shinan-gun, while the remaining three were derived from *Batillaria cumingii* and *Batillaria multiformis* samples obtained in Mokpo-si. The yeast strains were identified by analyzing D1/D2 domain of the 26S rRNA gene sequence similarities, which were calculated using NCBI BLAST. Based on the D1/D2 domain of the 26S rRNA gene sequence, four yeast strains were identified as previously unrecorded species in Korea. Six strains were classified into the families *Saccharomycetaceae* (2 strains), *Trichomonascaceae* (2 strains), and *Saccotheciaceae* (2 strains) within the phylum *Ascomycota*, while eight strains were classified into the families *Sporidiobolaceae* (1 strain), *Rhynchogastremaceae* (3 strains), *Trimorphomycetaceae* (1 strain), *Cystofilobasidiaceae* (1 strain), and *Filobasidiaceae* (2 strains) within the phylum *Basidiomycota*.

The species isolated from soil samples collected in Shin-

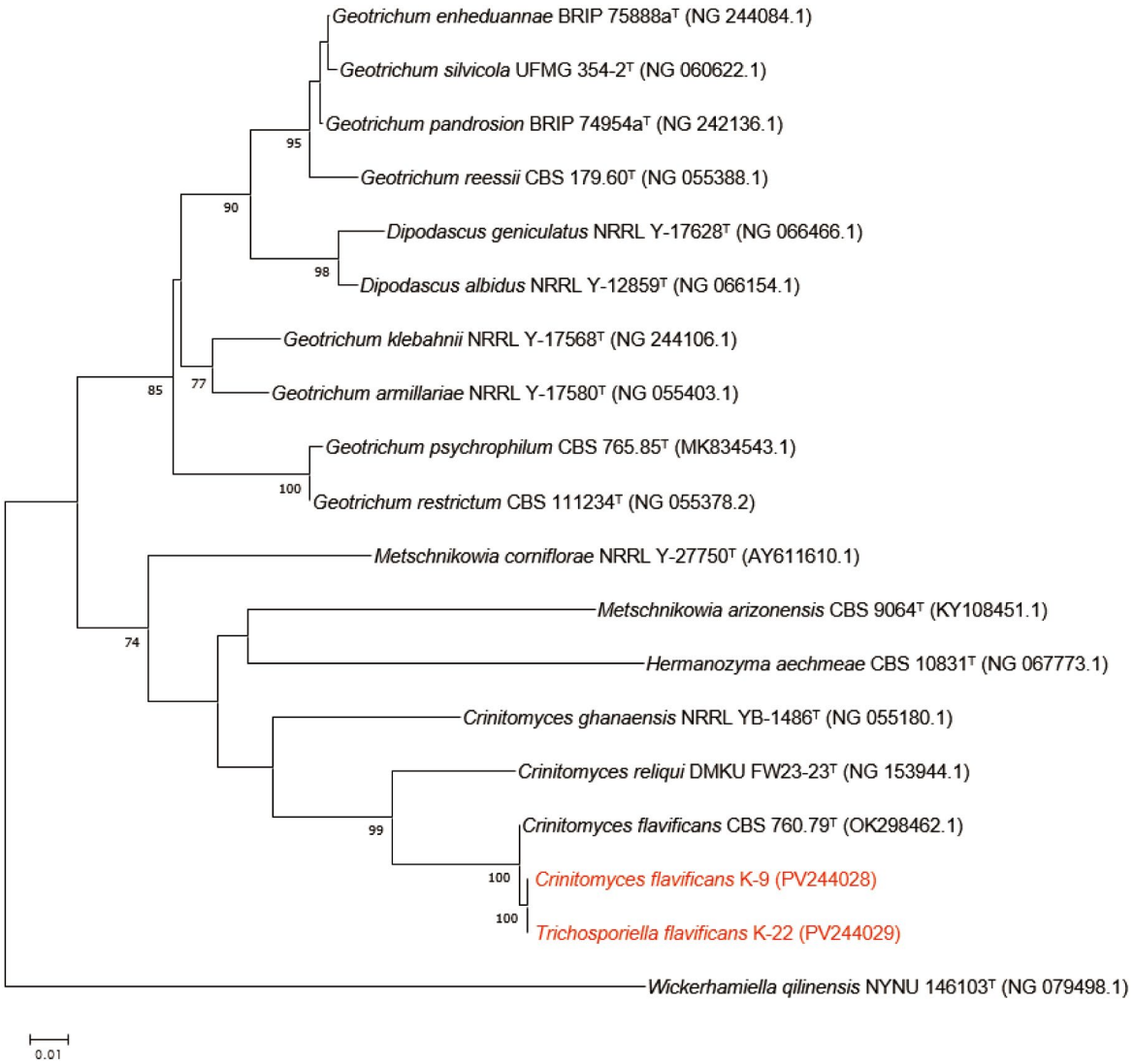

Fig. 3. A Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 26S rRNA gene sequences showing the relationships of strains K-9 and K-22 with closely related species. Bootstrap values (>70%) based on neighbor-joining methods are shown at the branch nodes. Bar, 0.01 substitutions per nucleotide position.

an-gun included 11-2, 15-5, 19-2, 15-1, 15-3, 15-7, Y-1, Y-2, 17-1, 16-2, and 19-3. Among these, 11-2 was identified as a previously unreported species in Korea. Additionally, the yeast strains D-7, isolated from *Batillaria cumingii* in Mokpo-si, and K-9 and K-22, isolated from *Batillaria multiformis* in Mokpo-si, were also identified as other previously unreported species in Korea. The taxonomic composition and identification results are detailed in Table 1.

The unreported yeast strains D-7, K-9, K-22 and 11-2 all belong to the phylum *Ascomycota*. Specifically, D-7 is classified in the genus *Nakaseomyces*, K-9 and K-22 are classified in the genus *Crinitomyces*, and 11-2 is classified in the genus *Kazachstania*. The phylogenetic tree shows that the isolated strains are closely related to the *Nakaseomyces* species, *Crinitomyces* species and *Kazachstania* species with the highest 26S rRNA gene sequence similarity (Figs. 2, 3 and 4), thereby supporting close relationships. Additional taxonomic and morphological characteristics of these three genera are outlined below. The genus *Kazachstania* is a member of the class *Saccharomycetes* within the phylum *Ascomycota*. This genus comprises 56 distinct species, with *Kazachstania viticola* designated as the type species (https://www.mycobank.org). The genus *Nakaseomyces* is a member of the class *Saccharomycetes* within the phylum *Ascomycota*. This genus includes 8 distinct species, with *Nakaseomyces delphensis* as the type species (https://www.mycobank.org). The genus *Crinitomyces* is a member of the class *Dipodascomycetes* within the phylum *Ascomycota*. This genus includes 3 distinct

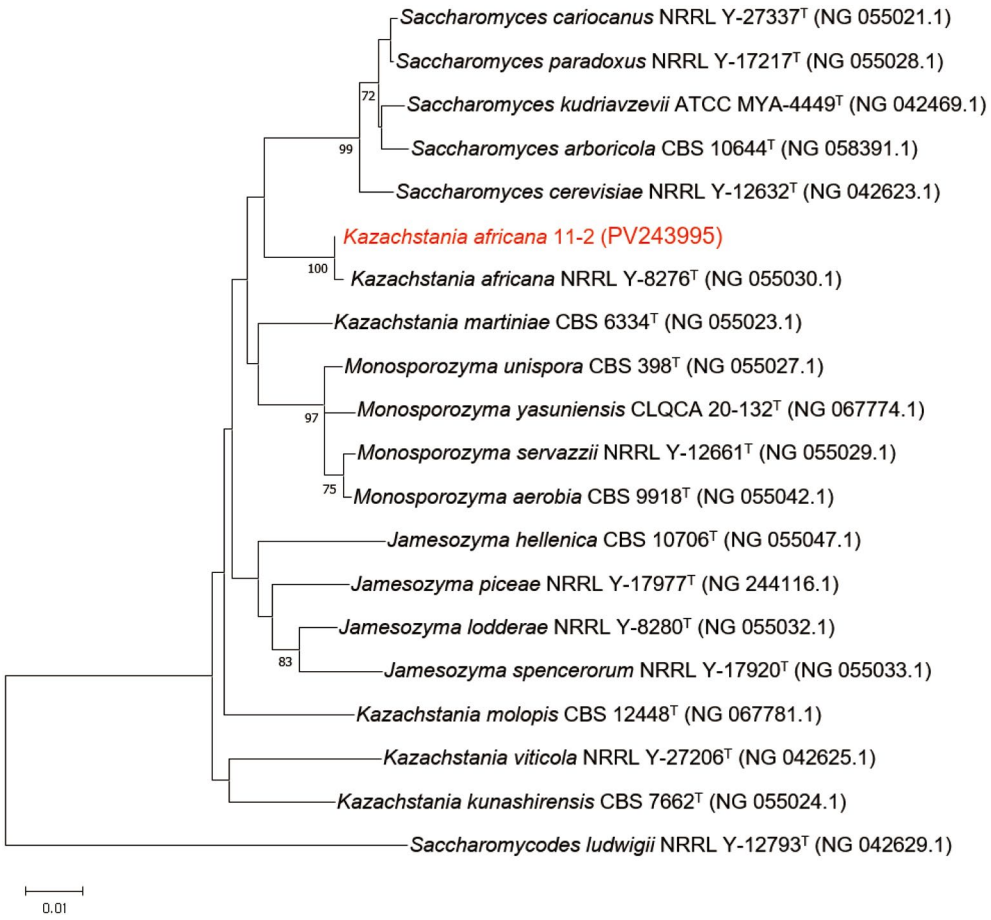

Fig. 4. A Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 26S rRNA gene sequences showing the relationships of strain 11-2 with closely related species. Bootstrap values (>70%) based on neighbor-joining methods are shown at the branch nodes. Bar, 0.01 substitutions per nucleotide position.

species, with *Crinitomyces flavificans* as the type species (https://www.mycobank.org).

The genus *Kazachstania* belongs to the order *Saccharomycetales* and is considered a sister group to the genus *Naumovozyma* (Lachance, 2018). Asexual reproduction occurs via multilateral budding from a narrow base, with cells that can be spherical, ovoid, or elongate. Pseudohyphae may be produced, but true hyphae are not formed, and colonies are generally smooth, displaying colors ranging from white to tan. Sexual reproduction involves the formation of asci through conjugation between independent cells or between a mother cell and its progeny; these asci may persist or dissolve after maturation, and each ascus contains 1−16 ascospores that are spherical, ovoid, or reniform, with spore surfaces that can be either smooth or rough. Standard test reactions for yeast classification are generally limited in *Kazachstania*. Most species ferment glucose and are capable of assimilating galactose; however, nitrogen sources such as cadaverine, L-lysine, and ethylamine are scarcely utilized, and nitrate is not used. In addition, the primary respiratory quinone produced by this genus is Coenzyme Q-6, and the Diazonium Blue B reaction is negative. The type species of the genus is *Kazachstania viticola* (Vaughan-Martini *et al*., 2011).

The genus *Nakaseomyces* was proposed by Cletus P. Kurtzman in 2003 and established as an independent lineage by reclassifying several species formerly included in the genus *Candida* based on molecular phylogenetic analysis (Kurtzman, 2003). Asexual reproduction occurs via multilateral budding from a narrow base, with cells exhibiting spherical, ovoid, or elongate shapes, and neither pseudohyphae nor true hyphae are formed. Sexual reproduction involves the formation of deliquescent asci—produced either by conjugation between independent cells or via an unconjugated process—that contain 1−8 ascospores, which are reniform or bacilliform. *Nakaseomyces* species are capable of fermenting glucose, although galactose is neither fermented nor assimilated; nitrogen sources such as cadaverine, L-lysine, and ethylamine are scarcely utilized, and nitrate is not used. In addition, the primary

respiratory quinone produced by this genus is Coenzyme Q-6, and the Diazonium Blue B reaction is negative. The type species of the genus is *Nakaseomyces delphensis* (Takashima and Sugita, 2022).

The genus *Crinitomyces* belongs to the subphylum *Saccharomycotina* within the order *Saccharomycetales* and was reclassified as an independent genus based on molecular phylogenetic analyses, despite its close relationship with *Trichosporiella flavificans* and *Candida ghanaensis* (Sakpuntoon *et al*., 2022). Asexual reproduction in this genus occurs via multilateral budding from a narrow base, with cells that are either spherical or ellipsoidal in shape. In addition, true hyphae are formed and septate hyphae are observed. In contrast, sexual reproduction has not been observed, as no formation of asci has been detected. *Crinitomyces* species are capable of assimilating a variety of carbon sources and can ferment sugars such as glucose, galactose, and maltose, although nitrate is not utilized as a nitrogen source. Moreover, the primary respiratory quinone produced by this genus is Coenzyme Q-6; the Diazonium Blue B reaction is negative, and neither urea hydrolysis nor the production of starch-like compounds is observed. Members of *Crinitomyces* have been isolated from diverse environments, including rivers, sewage sludge, and wastewater treatment facilities. The type species of the genus is *Crinitomyces flavificans* (Sakpuntoon *et al*., 2022).

Description of *Nakaseomyces glabratus* D-7

Cells are oval-shaped, and budding is multilateral (Fig. 1). Colonies are convex, smooth, and white to light yellow colored after 3 days of incubation on YPD agar at 25°C. In the API 20C AUX test, strain D-7 is positive for glucose and D-trehalose; and negative for glycerol, calcium 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-melezitose and D-raffinose (Table 2).

Strain D-7 (HNIBR-FC132) was isolated from *Batillaria cumingii* collected in Mokpo-si, Jeollanam-do, Republic of Korea. The 26S rRNA gene sequence of strain D-7 has been deposited in GenBank under the accession number PV244027.

Table 2. Phenotypic characteristics of strain D-7 and related species.

	1	2	3	4
Growth on				
Temp (°C)	15−30	19−45	19−42	19−42
Assimilation of:				
Glucose	+	+	+	+
Glycerol	−	w	−	w
Calcium 2-Keto-Gluconate	−	v	ND	−
L-Arabinose	−	−	−	−
D-Xylose	−	−	−	−
Adonitol	−	−	−	−
Xylitol	−	−	−	−
D-Galactose	−	−	w	−
Inositol	−	−	w	−
D-Sorbitol	−	−	−	−
Methyl-α-D-Glucopyranoside	−	−	−	−
N-Acetyl-Glucosamine	−	−	−	ND
D-Cellobiose	−	−	−	−
D-Lactose	−	−	−	−
D-Maltose	−	w	w	−
D-Saccharose	−	−	+	−
D-Trehalose	+	v	w	+
D-Melezitose	−	−	−	−
D-Raffinose	−	−	−	−

Taxa: 1, *Nakaseomyces glabratus* D-7; 2, *Nakaseomyces glabratus* ATCC 2001^T; 3, *Nakaseomyces nivariensis* NRRL Y-48269^T; 4, *Nakaseomyces bracarensis* CBS 10154^T.
+, positive; w, weakly positive; −, negative; v, variable; ND, no data.

Table 3. Phenotypic characteristics of strains K-9 and K-22 and related species.

	1	2	3	4
Growth on				
Temp (°C)	15−30	15−30	15−40	15−37
Assimilation of:				
Glucose	+	+	+	v
Glycerol	−	−	+	+
Calcium 2-Keto-Gluconate	−	−	v	w
L-Arabinose	−	−	+	+
D-Xylose	−	−	+	+
Adonitol	−	−	v	+
Xylitol	−	−	ND	ND
D-Galactose	w	w	+	+
Inositol	−	−	−	w
D-Sorbitol	−	−	v	w
Methyl-α-D-Glucopyranoside	−	−	−	+
N-Acetyl-Glucosamine	+	+	+	+
D-Cellobiose	w	w	+	+
D-Lactose	−	−	−	−
D-Maltose	−	−	−	v
D-Saccharose	−	−	v	w
D-Trehalose	−	−	+	+
D-Melezitose	−	−	v	w
D-Raffinose	−	−	v	w

Taxa: 1, *Crinitomyces flavificans* K-9; 2, *Crinitomyces flavificans* K-22; 3, *Crinitomyces flavificans* CBS 760.79^T; 4, *Crinitomyces reliqui* DMKU FW23-23^T.
+, positive; w, weakly positive; −, negative; v, variable; ND, no data.

Table 4. Phenotypic characteristics of strain 11-2 and related species.

	1	2	3	4
Growth on				
Temp (°C)	15−30	19−37	19−35	19−35
Assimilation of:				
Glucose	−	+	+	+
Glycerol	−	−	−	−
Calcium 2-Keto-Gluconate	−	−	+	w
L-Arabinose	−	−	−	−
D-Xylose	−	−	−	−
Adonitol	−	−	−	−
Xylitol	−	−	−	−
D-Galactose	−	+	+	w
Inositol	−	−	−	−
D-Sorbitol	−	−	−	−
Methyl-α-D-Glucopyranoside	−	−	−	−
N-Acetyl-Glucosamine	−	−	−	ND
D-Cellobiose	−	−	−	−
D-Lactose	−	−	−	−
D-Maltose	−	−	−	−
D-Saccharose	−	−	−	+
D-Trehalose	−	w	+	w
D-Melezitose	−	−	−	w
D-Raffinose	−	−	−	+

Taxa: 1, *Kazachstania africana* 11-2; 2, *Kazachstania* africana NRRL Y-8276^T; 3, *Kazachstania martiniae* CBS 6334^T; 4, *Saccharomyces cariocanus* NRRL Y-27337^T.
+, positive; w, weakly positive; −, negative; v, variable; ND, no data.

Descriptions of *Crinitomyces flavificans* K-9, K-22

Cells are oval-shaped, and budding is multilateral from a narrow base (Fig. 1). Colonies are convex, filamentous, and white colored after 3 days of incubation on YPD agar at 25°C. In the API 20C AUX test, strains K-9 and K-22 are positive for glucose and *N*-acetyl-glucosamine; weakly positive for D-galactose and D-cellobiose; and negative for glycerol, calcium 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, inositol, D-sorbitol, methyl-α-D-glucopyranoside, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose and D-raffinose (Table 3).

Strain K-9 (HNIBR-FC137) and Strain K-22 (HNIBR-FC144) were isolated from *Batillaria multiformis* collected in Mokpo-si, Jeollanam-do, Republic of Korea. The 26S rRNA gene sequences of strains K-9 and K-22 have been deposited in GenBank under the accession numbers PV244028 and PV244029, respectively.

Description of *Kazachstania africana* 11-2

Cells are oval-shaped, and budding is multilateral (Fig. 1). Colonies are convex, butyrous, and white to tan colored after 3 days of incubation on YPD agar at 25°C. In the API 20C AUX test, strain 11-2 is negative for all substrates; specifically, it does not utilize glucose, glycerol, calcium 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose and D-raffinose (Table 4).

Strain 11-2 (HNIBR-FC176) was isolated from soil collected in Shinan-gun, Jeollanam-do, Republic of Korea. The 26S rRNA gene sequence of strain 11-2 has been deposited in GenBank under the accession number PV243 995.

Conflicts of Interest

The authors of this paper have no affiliation with any interests and are solely responsible for the paper.

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