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New Records of *Kazachstania* Species in Taiwan

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ABSTRACT: Seven yeast strains isolated from the soil in Northern Taiwan were identified as *Kazachstania africana*, *Kazachstania exigua* and *Kazachstania yakushimaensis*, based on morphological, physiological, and molecular characteristics. This discovery marked the first finding of this genus, which is represented by these three species, in Taiwan. The DNA sequences of the D1/D2 domain of the large subunit (LSU) rRNA genes were identical to or less than three nucleotides different from that of the respective type strains. In this paper, morphological, physiological and molecular characteristics of the strains representing these three species are described.

KEYWORDS: ***Kazachstania africana, Kazachstania exigua, Kazachstania yakushimaensis,* taxonomy, yeast, new record.**

INTRODUCTION

The genus *Kazachstania* was first described by Zubkova (1971) but was not proposed until 2003 by Kurtzman (2003). The '*Saccharomyces* complex', which includes the genera *Kluyveromyces*, *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces*, are difficult to be distinguished from each other because of the occurrence of intermediate phenotypes (Stelling-Dekker, 1931; Lodder and Kreger-van Rij, 1952; van der Walt, 1970). Kurtzman and Robnett (2003) clarified the phylogenetic relationships of the species of the '*Saccharomyces* complex' by analysis of multigene sequences, classifying them into 11 clades. Each clade was proposed as an individual genus, and clade 2 was proposed as the genus *Kazachstania*, which previously included the genera *Arxiozyma*, *Candida*, *Kluyveromyces*, *Pachytichospora*, and *Saccharomyces* (Kurtzman and Robnett, 2003). At present, more than 20 species of this genus have been described (Kurtzman and Robnett, 2003; Lu et al., 2004; Kurtzman et al., 2005; Wu and Bai, 2005; Limtong et al., 2006; Imanishi et al., 2007; Lee et al., 2008).

During an investigation of yeast diversity in the soil in Taiwan, strains belonging to seven *Kazachstania* species were isolated and identified based on morphological and physiological characteristics, and the sequences of the D1/D2 domain of the large subunit rRNA gene. Of the seven species, three species were undescribed, *K. jiainicus* was proposed as a new species (Lee et al., 2008), and the other three species, *K. africana*, *K. exigua*, and *K. yakushimaensis* referred new records to Taiwan. In this paper, the morphological, physiological, and molecular characteristics of the strains of these three new records of *Kazachstania* species are described.

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MATERIALS AND METHODS

Strains isolation and identification

Three strains of *K. africana* and *K. yakushimaensis*, and one strain of *K. exigua* were isolated from soil samples collected from northern and central Taiwan in 2005 and 2006 (Table 1).

The isolation of the strains was performed by the method described by Lee (2008). To isolate the yeasts from the soil, one gram of soil from each sample was diluted in 9 ml of sterilized water and then vortex-mixed. One tenth of a milliliter of successive decimal dilutions was spread on acidified YMA (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1.5% agar, pH3.5) or DRBC (Dichloran rose Bengal chloramphenicol agar, Merck, Darmstadt, Germany). The plates were incubated at 24℃ for 3 days. Representative colonies were picked, purified, and maintained on YMA or in the freezer at -70℃. All the strains studied in this study have been deposited in National Hsinchu University of Education, Hsinchu, Taiwan.

Examination of morphological and physiological characteristics

The morphological, physiological, and biochemical characteristics of the species were

Table 1. *Kazachstania* strains isolated from soil samples collected from Taiwan in this study.

Species/Strain no.	Source	GenBank Accession no.
K. africana FN9S01	Taian, Miaoli	EF460522
K. africana SA14S05	Dajia, Taichung	EF460640
K. africana SA15S04	Dajia, Taichung	EF460645
K. exigua SA14S01	Dajia, Taichung	EF460637
K. yakushimaensis SA5S10	Sanyi, Miaoli	EF460616
K. yakushimaensis SJ1S01	Yuchih, Nanto	EF460537
K. yakushimaensis SF2S05	Jian, Hualien	EF460571

determined by the methods described by Yarrow (1998). The keys published by Kurtzman and Fell (1998) were used to identify the yeasts.

Phylogenetic analysis

The D1/D2 DNA fragment of the LSU rRNA gene was amplified on genomic DNAs extracted from yeast cells with a Biokit Genome DNA Extraction Kit (Biokit Co., Taiwan) using the primers, NL1 and NL4 (Kurtzman and Robnett, 1998) with a Peltier thermal cycler (PTC-200, MJ Research). The sizes of PCR products were confirmed by agarose gel electrophoresis. Sequencing of the fragments was performed with an automatic sequencer (model CEQ 2000 DNA Analysis System, Beckman, Coulter, Fullerton, CA, USA). Both strands of DNA were sequenced. Sequence data of the strains examined were deposited in GenBank, and their accession numbers are listed in Table 1. A phylogenetic tree was constructed by the neighbor-joining method with the CLUSTAL X 1.83 (Thompson et al., 1997). For sequence analysis, the sequences of the D1/D2 domain of the LSU rRNA genes of the type strains were retrieved from GenBank. Schizosaccharomyces pombe was used as an outgroup. The numbers provided on the branches are bootstrap values based on a sampling of 1,000 replicates (Felsenstein, 1995). All taxa are represented by the type strains of the genus Kazachstania and the strains in this study.

RESULTS AND DISCUSSION

Strain identification and species delineation

All of the strains examined in this paper produced 1-8 spheroidal, oblong, or reniform ascospores per ascus on sporulation agar and revealed similar physiological characteristics to that of the respective type strains. Meanwhile, all of the strains were phylogenetically related to the genus *Kazachstania* based on the D1/D2 sequences of the LSU rRNA genes (Fig. 1). The data previously described showed that all of the strains belong to the genus of *Kazachstania*. The four species *K. africana*, *K. exigua*, *K. unispora*, and *K. yakushimaensis* marked the first finding of the genus in Taiwan, which represents new records to Taiwan. The D1/D2 sequences of the strains were identical to or less than three nucleotides different from that of their related type strains, respectively. We isolated a single strain of *K. exigua* and *K. unispora*, while three more strains were isolated and identified as *K. africana* or *K. yakushimaensis*. In agreement with the nucleotide sequences, strains of *K. africana* or *K. yakushimaensis* showed similarities to the respective type strains in terms of morphological and physiological characteristics, indicating that they are conspecific.

Description of new records

Kazachstania africana (van der Walt) Kurtzman comb. nov. (van der Walt, 1956; Kurtzman, 2003) strains FN9S01, SA14S05, and SA15S04.

Morphology:

After 3 days of growth on GYP broth at 25℃, butyrous sediment was observed, and no pellicles formed along the edges of the tube above the surface of the medium. The cells reproduced by multilateral budding and were spheroidal in shape (4.0 - 8.4 × 3.5 - 5.5 μm). The cells occurred singly, in pairs or in short chains (Fig. 2A).

After 7 days of growth on GYP agar at 25℃, the colonies were cream, smooth, and glistening with a butyrous texture, entire margin, and convex elevation.

After 7 days of slide culture on corn meal agar at 25℃, no pseudomycelium was present.

Regarding the formation of ascospores, one to seven oblong or reniform ascospores per ascus were found (Fig. 2B) on Fowell's acetate agar (Yarrow, 1998) after 7 days at 18℃, The asci were evascent. The ascospores tended to agglutinate after they were liberated from the ascus.

Physiology: The physiological and biochemical tests, including fermentation, carbon assimilation, nitrogen assimilation, antibiotic resistance, growth temperature, and biochemical tests, were examined and the results for these strains are listed in Table 2.

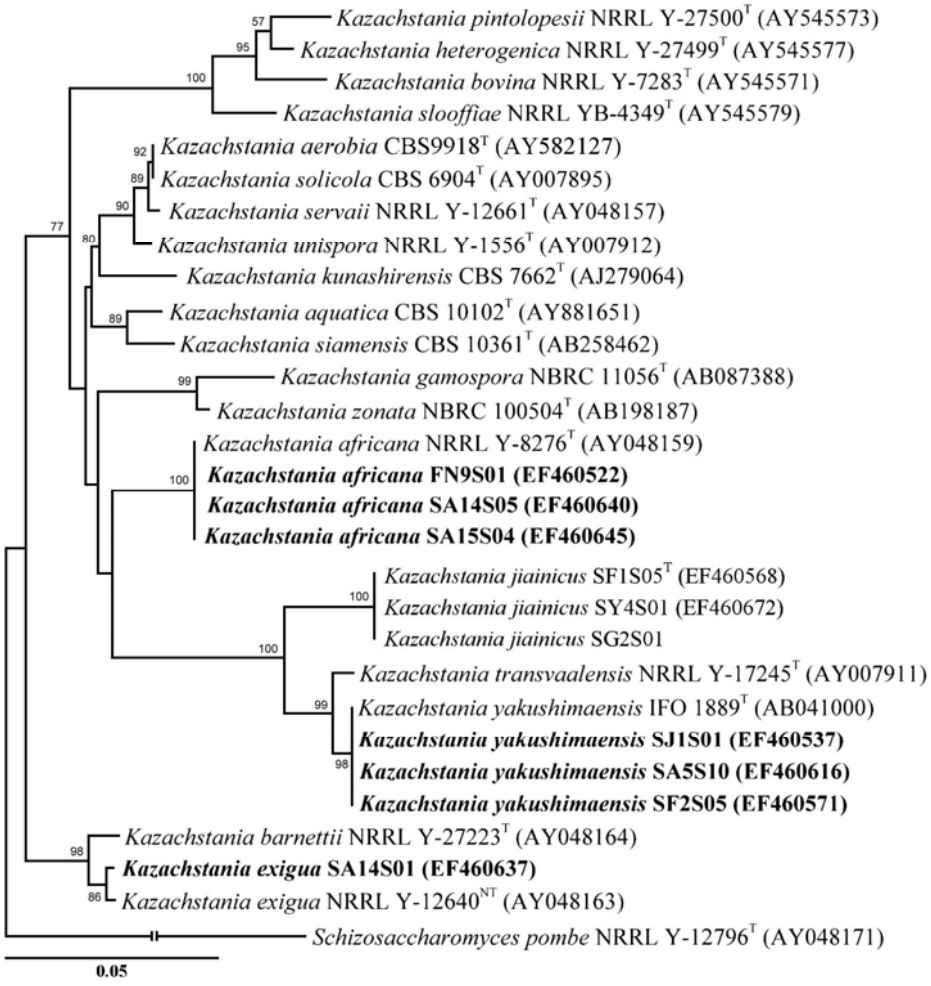

Fig. 1. Neighbor-joining tree for strains of the genus *Kazachstania* based on the D1/D2 domain of the large subunit (26S) rRNA gene. *Schizosaccharomyces pombe* was used as an outgroup. The numbers given on the branches are bootstrap values based on sampling of 1,000 replicates. Frequencies under 50 are not provided. All taxa are represented by the type strains of the genus *Kazachstania* and the strains in this study. The *Kazachstania* strains isolated in this study were marked by broad form. T = type strain; NT = neotype strain. Bar = 0.05 substitutions per nucleotide position.

Sequences of the D1/D2 domain of the LSU rRNA genes: Sequence data for the strains examined were deposited in GenBank, and their accession numbers are listed in Table 1. All of the sequences were identical to that of the type strain of K. africana (Fig. 1).

Comments: Strains FN9S01, SA14S05, and SA15S04 revealed similar morphological, physiological, and molecular characteristics, which indicated conspecificity. The three strains were similar to the type strain, *K. africana* NRRL Y-8276 in their morphological, physiological, and molecular characteristics, indicating that the strains could be identified as *K. africana*. The strains FN9S01, SA14S05, and SA15S04 were different from the strains published previously in assimilation of sucrose, gluconate and glycerol (Table 2; Nakase et al., 1998). *K. africana* was transferred from *Kluyveromyces africanus* which is distinct from other *Kluyveromyces* species in its absence of growth on sucrose, trehalose, and 2-keto-D-gluconate (Nakase, et al., 1998). *K. africana* has been isolated from soil in Kenya, South Africa, and Zimbabwe (Barnett, et al., 2000). In this study, *K. africana* was also isolated from soil in Taiwan.

Kazachstania exigua (Reess ex Hansen) Kurtzman comb. nov. (Reess, 1870; Kurtzman, 2003) strain SA14S01.

Morphology:

After 3 days of growth on GYP broth at 25℃, butyrous sediment was observed, and no pellicles formed along the edges of the tube above the surface

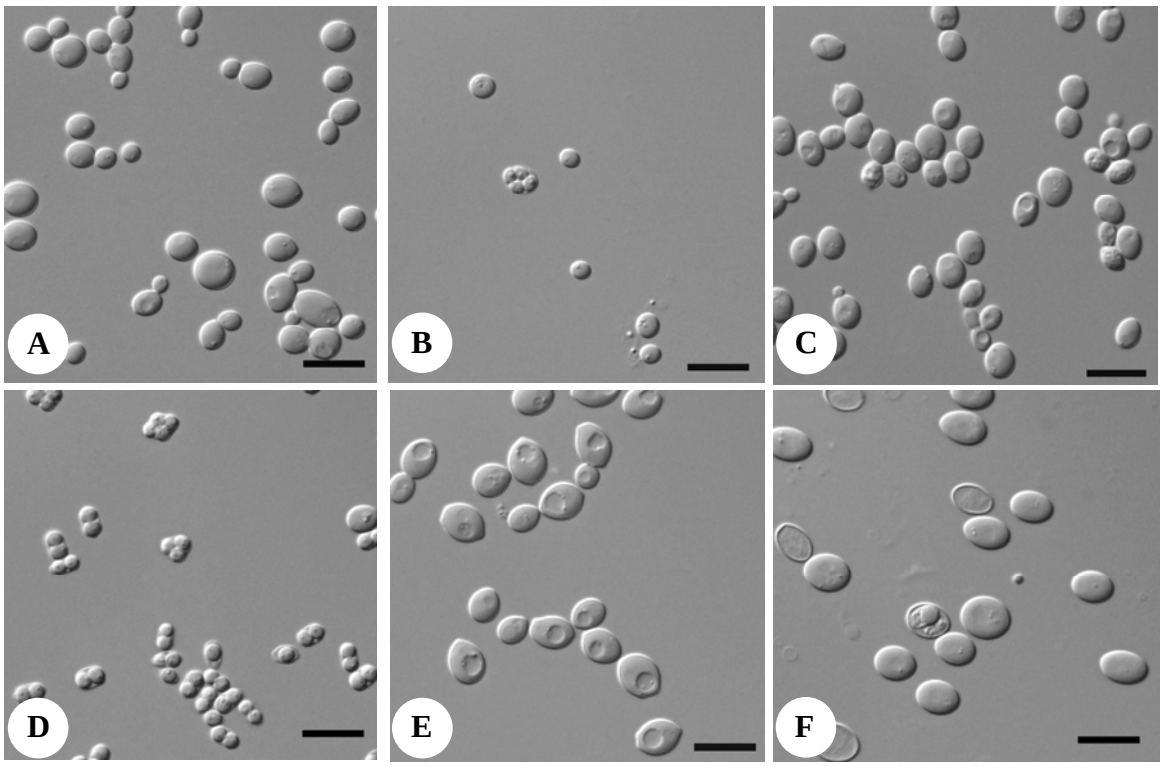

Fig. 2. Morphology of *Kazachstania* species. The morphology of strains belonging to *K. africana* SA15S04 (A-B), *K. exigua* SA14S01 (C-D), and *K. yakushimaensis* SA5S10 (E-F) were determined by light microscopy. A, C, and E: vegetative cells in GYP broth for 3 days at 25℃. B, D, and F: Ascospores on Fowell's acetate agar for 7 days at 18℃. Bar = 10 μm.

of the medium. The cells reproduced by multilateral budding and were subglobose or ellipsoidal in shape (4.6 – 6.9 × 3.4 - 5.7 μm). The cells occurred singly or in pairs (Fig. 2C).

After 7 days of growth on GYP agar at 25℃, the colonies were cream, smooth, and glistening with a butyrous texture, entire margin, and flat elevation.

After 7 days of slide culture on corn meal agar at 25℃, no pseudomycelium was present.

Regarding the formation of ascospores, one or two or in rare cases four globose or ellipsoidal ascospores per ascus were found on Fowell's acetate agar (Yarrow, 1998) after 7 days at 18℃, and the asci of the strain were persistent (Fig. 2D).

Physiology: The physiological and biochemical characteristics of the strain were examined and the results are listed in Table 2.

Sequences of the D1/D2 domain of 26S rDNA: The sequence data for the strain examined was deposited in GenBank, and its accession number is listed in Table 1. Within 545 bp sequence, there is three nucleotide difference to that of the type strain of *K. exigua* (Fig. 1).

Comments: The morphological, physiological, and molecular characteristics of the strain SA14S01 were quite similar to that of the type strain of *K. exigua*. The physiological tests of the strain SA14S01 were identical to those of *K. exigua* strains published previously (Mikata and Ueda-Nishimura, 2001; Kurtzman, 2003). The strain demonstrated a 3-nucleotide sequence difference from the sequence of the type strain *K. exigua* NRRL Y-12640. Based on these evidences, the strain SA14S01 was classified as *K. exigua*. *K. exigua* can be found in different niches, such as on grapes, on strawberries, in sewage, soil, fermenting cucumbers, sour dough, and buttermilk (Barnett et al., 2000). It can also be found in soil in Taiwan.

Kazachstania yakushimaensis (Mikata et Ueda-Nishimura) Kurtzman comb. nov. (Mikata and Ueda-Nishimura, 2001; Kurtzman, 2003) strains SA5S10, SJ1S01, and SF2S05.

Morphology:

After 3 days of growth on GYP broth at 25℃, butyrous sediment was observed, and no pellicles formed along the edges of the tube above the surface of the medium. The cells reproduced by multilateral budding and were spheroidal or ovoid in shape (5.1 – 8.8 × 4.1 – 7.4 μm). The cells occurred singly or in pairs (Fig. 2E).

Table 2. Physiological characteristics of *Kazachstania* strains in this study.

Species	*K. africana*			*K. exigua*	*K. yakushimaensis.*		
	FN9S01	SA14S05	SA15S04	SA14S01	SA5S10	SJ1S01	SF2S05
Fermentation							
D-Glucose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	−	−	−
Maltose	−	−	−	−	−	−	−
Me α-D-glucoside	−	−	−	−	−	−	−
Sucrose	−	−	−	+	−	−	−
α,α-Trehalose	−	−	−	+	−	−	−
Melibiose	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−
Cellobiose	−	−	−	−	−	−	−
Melezitose	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−
Starch	−	−	−	−	−	−	−
D-Xylose	−	−	−	−	−	−	−
Assimilation test							
D-Glucose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
L-Sorbose	−	−	−	−	−	−	−
D-Glucosamine	−	−	−	−	−	−	−
D-Ribose	−	−	−	−	−	−	−
D-Xylose	−	−	−	−	−	−	−
L-Arabinose	−	−	−	−	−	−	−
D-Arabinose	−	−	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−	−
Sucrose	w	w	w	+	−	−	−
Maltose	−	−	−	−	−	−	−
α,α-Trehalose	−	w	w	+	−	−	−
Me α-D-glucoside	−	−	−	−	−	−	−
Cellobiose	−	−	−	−	−	+	+
Salicin	−	−	−	−	−	+	+
Arbutin	−	−	−	−	−	+	+
Melibiose	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−
Raffinose	−	−	−	+	−	−	−
Melezitose	−	−	−	−	−	−	−
Inulin	−	−	−	w	−	−	−
Starch	−	−	−	w	−	−	−
Glycerol	+	+	+	−	−	−	−
Erythritol	−	−	−	−	−	−	−
Ribitol	−	−	−	−	−	−	−
Xylitol	−	−	−	−	−	−	−
L-Arabinitol	−	−	−	−	−	−	−
D-Glucitol	−	−	−	−	−	−	−
D-Mannitol	−	−	−	−	−	−	−
Galactitol	−	−	−	−	−	−	−
myo-Inositol	−	−	−	−	−	−	−
D-Glucono-1,5-lactone	w	w	w	+	−	+	+
2-Keto-D-gluconate	−	−	−	−	−	−	−
5-Keto-D-gluconate	−	−	−	−	−	−	−
D-Gluconate	−	−	−	−	−	−	−
D-Glucuronate	−	−	−	−	−	−	−
D-Galacturonic acid	−	−	−	−	−	−	−
DL-Lactate	−	−	−	+	−	−	−
Succinate	−	−	−	−	−	−	−
Citrate	−	−	−	−	−	−	−

Table 2. Continued.

Species	*K. africana*			*K. exigua*	*K. yakushimaensis.*		
	FN9S01	SA14S05	SA15S04	SA14S01	SA5S10	SJ1S01	SF2S05
Assimilation test							
Methanol	−	−	−	−	−	−	−
Ethanol	−	−	−	+	−	−	−
Propane 1,2 diol	−	−	−	−	−	−	−
Butane 2,3 diol	−	−	−	−	−	−	−
N-Acetylglucosame	−	−	−	−	−	−	−
Nitrogen assimilation							
Nitrate	−	−	−	−	−	−	−
Nitrite	−	−	−	−	−	−	−
Ethylamine	−	−	−	−	+	+	+
L-Lysine	−	−	−	−	+	+	+
Cadaverine	−	−	−	−	−	−	−
Creatine	−	−	−	−	−	−	−
Growth temperature							
30℃	+	+	+	+	+	+	+
35℃	w	+	+	+	−	−	−
40℃	−	−	−	−	−	−	−
Biochemical reaction							
0.01% Cycloheximide	−	−	−	+	−	+	+
0.1% Cycloheximide	−	−	−	−	−	+	+
1% Acetic acid	−	−	−	−	−	−	−
50% D-Glucose	−	−	−	−	−	−	−
60% D-Glucose	−	−	−	−	−	−	−
10% NaCl	−	−	−	w	−	−	−
16% NaCl	−	−	−	−	−	−	−
Additional characteristics							
Starch formation	−	−	−	−	−	−	−
Acetic acid production	−	−	−	+	−	−	−
Urea hydrolysis	−	−	−	−	−	−	−
Diazonium Blue B	−	−	−	−	−	−	−

Species: 1. *Kazachstania africana*. 2. *Kazachstania exigua*. 3. *Kazachstania unispora*. 4. *Kazachstania yakushimaensis*. Scored for response to tests: −, negative; w, weakly positive; +, positive.

After 7 days of growth on GYP agar at 25℃, the colonies were cream, smooth, and glistening with a butyrous texture, entire margin, and flat elevation.

After 7 days of slide culture on corn meal agar at 25℃, no pseudomycelium was present.

Regarding the formation of ascospores, on Fowell's acetate agar (Yarrow, 1998) after 7 days at 18℃, one to four spheroidal ascospores per ascus were found, and the asci were presistent (Fig. 2F).

Physiology: The physiological and biochemical characteristics of the strains were examined and the results are listed in Table 2.

Sequences of the D1/D2 domain of 26S rDNA: Sequence data of the strains examined were deposited in GenBank, which their accession numbers are listed in Table 1. All of the sequences were identical to that of type strain *K. yakushimaensis* .

Comments: Strains SA5S10, SJ1S01, and SF2S05 showed similar morphological, physiological, and molecular characteristics, indicating that they are conspecific. These three strains were similar to the type strain *K. yakushimaensis* IFO 1889 in terms of physiological and morphological characteristics. The strains SA5S10, SJ1S01, and SF2S05 showed identical sequence to the type strain *K. yakushimaensis* IFO 1889 in the D1/D2 domain of the LSU of rRNA gene. The physiological tests of the strains were different from the type strain only in the assimilation of trehalose and D-glucono-1,5-lactone (Mikata and Ueda-Nishimura, 2001). *K. yakushimaensis* was originally classified in the genus *Saccharomyces*. Kurtzman (2003) asserted that *S. yakushimaensis* shared the same branch with *Saccharomyces transvaalensis* and *Kluyveromyces sinensis* in the *Kazachstania clade* and transferred it to the genus *Kazachstania* (Kurtzman and Robnett, 2003).

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臺灣 *Kazachstania* 新紀錄種

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摘　　要

從臺灣北部山區土壤樣品中所分離出 7 株酵母菌株，經形態、生理及 DNA 序列分析結果，分別鑑定為 *Kazachstania africana*、*Kazachstania exigua* 和 *Kazachstania yakushimaensis* 等。這些菌種為台灣之新紀錄屬與三個新紀錄種。此三種菌種之 rDNA 大單元體 DNA 之 D1/D2 區域之核苷酸序列分別與其對應的標準菌株相同或僅差三個核苷酸以內。本文描述此三新紀錄種之生理、形態特徵和 D1/D2 區域序列分析結果。

關鍵詞：*Kazachstania africana*、*Kazachstania exigua*、*Kazachstania yakushimaensis*、分類學、酵母菌、新紀錄種。

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