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Wickerhamomyces kurtzmanii sp. nov. An Ascomycetous Yeast Isolated From Crater Lake Water, Da Hinggan Ling Mountain, China

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Abstract
One novel ascomycetous yeast strain TF5-16-2 was isolated from water samples of Tuofengling crater lake located in Da Hinggan Ling Mountain, in the Inner Mongolia province of China. Morphological, physiological characteristics, as well as phylogenetic analyses of D1/D2 domains of the large subunit rRNA (LSU), ITS region, small subunit rRNA (SSU), and elongation factor-1α (EF-1α) were performed and finally confirmed the phylogenetic placement of strain TF5-16-2 in the genus *Wickerhamomyces*. Sequences analysis revealed that strain TF5-16-2 differed from its most closely related phylogenetic neighbors *'Candida' silvicultrix* CBS 6269^T and *Wickerhamomyces anomalus* CBS 5759^T by 8.0% (including 2.3% gaps), 8.5% (including 2.4% gaps) divergences in D1/D2 domains of LSU, and 11% (including 4.3% gaps) and 13% (including 4.4% gaps) divergences in ITS region, respectively. As the considerable sequence divergence and distinguishable physiological characteristics, strain TF5-16-2 was proposed as a new species of the genus *Wickerhamomyces*, with the name *Wickerhamomyces kurtzmanii* sp. nov. (holotype = CGMCC 2.5597, Mycobank number is MB829959).

Keywords Ascomycetous yeast · Crater lake · *Wickerhamomyces kurtzmanii* · Da hinggan ling mountain

Abbreviations

LSU	Large subunit rRNA
ITS	Internal transcribed spacer;
SSU	Small subunit rRNA
EF-1α	Elongation factor-1α

Introduction

The genus *Wickerhamomyces* was proposed by Kurtzman et al. [1] based on the phylogenetic analysis of concatenated sequences of LSU, SSU, and EF-1α. It consists of a group of species that were earlier assigned to the genus *Pichia* [2]. Up to date, the genus *Wickerhamomyces* accommodates 34 species, embracing the newly published species *Wickerhamomyces xylosivorus* [3] and *Wickerhamomyces menglaensis* [4]. Members of the genus *Wickerhamomyces* occur widely in the natural environments, and have been isolated from different habitats including soil [5–8], phylloplane [9], tree exudates [10], flowers [11, 12], digestive tract of insects [13, 14], larvae of diptera [15], birds [16], natural fermentation of coffee cherries [17], and brined vegetables [18]. However, freshwater environment including crater lake is a special habitat for *Wickerhamomyces* members, as they are rarely discovered in aquatic environment. During our survey of the diversity of culturable yeast in these crater lakes, strain TF5-16-2 isolated from Tuofengling crater lake in Greater Khingan Mountain of China was identified as a novel species of *Wickerhamomyces* based on phylogenetic analyses of LSU, SSU, and EF-1α and morphological and physiological comparison, for which the name *Wickerhamomyces kurtzmanii* sp. nov. was proposed.

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Materials and Methods

Yeasts Isolation

Water samples were obtained from Tuofengling Crater lake (with a height of 1284 m) in Da Hinggan Ling Mountain in September 2015. Samples were taken from five randomly chosen sites on the surface of Tuofengling Lake at the depth of 0.5–1.0 m. The pH value of lake water was 7.5, which was obtained in situ using a portable water quality analyzer (HQ40d, US). Five independent 1 L water samples were collected in sterile bottles, kept at 4 ℃ and transported to the laboratory. Isolation of yeast strains was performed as follows: a tenfold dilution series (10^{-1}–10^{-4}) was prepared for each sample. And 100 µl of each dilution was spread over the YM [0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 1% (w/v) glucose, and 1.5% (w/v) agar] plates supplemented with 0.02% chloramphenicol and then incubated at 20 ℃ for up to 7 days. Three replicated plates were inoculated for each dilution. All emerging yeast colonies on the plates were picked and were purified by repeated streaking on YM agar plates. Subsequently, strains were preserved by lyophilization and in the liquid nitrogen in China General Microbiological Culture Collection Center (CGMCC).

Phenotypic Characterization

Strain TF5-16-2 was characterized morphologically and physiologically using standard methods according to Kurtzman et al. [19]. The assimilation of carbon and nitrogen compounds was examined in liquid medium at 22 ℃. Urease activity was tested with Christensen's urea agar. Dalmau tubes were prepared using Yeast Carbon Base broth supplemented with 0.01% yeast extract to detect the fermentation ability. Cell morphology was observed by light microscopy and scanning electron microscopy (SEM, HITACHI, SU8180) after 3 days of growth in YM broth. Ascospore formation was investigated by incubating strain TF5-16-2 on 5% malt extract agar, YM agar, corn meal agar (CMA), and potato dextrose agar (PDA, BD), at 22 ℃ for 2 weeks.

Phylogenetic Analysis

The sequences were analyzed corresponded to the small subunit of the ribosomal DNA (SSU), the D1/D2 domain of the large submit (LSU or 26S) rDNA, and the translation elongation factor 1-α (TEF1). DNA extraction was performed by using the commercial Eukaryotic Genomic DNA Extraction Kit (Aidlab Biotech, Beijing, China) according to the manufacturer's instructions. The primers for amplification of ITS region were ITS1 and ITS4 [20], for the D1/D2 domains of the LSU were NL1 and NL4 [21], for SSU were P1F and U3R [22], and for the EF-α were EF1-983F and EF1-2218R [23]. These sequences were determined by commercial sequencing facilities (Ruibo Biotech Co., Beijing, China) and then deposited on GenBank (with the accession numbers MK573939, MK573961, MK573960, and MK580818).

The comparisons of the sequences were carried out using BLASTN. Phylogenetic analysis was performed following the previously described methods [24]. Sequences of the closely related species were retrieved from Genbank database (https://www.ncbi.nlm.nih.gov/) and aligned iteratively by using the multiple alignment program CLUSTAL X [25]. The Phylogenetic trees were constructed by both the Maximum-Likelihood and Neighbor-Joining methods in MEGA 7.0 [26]. Single sequence of D1/D2 domains of LSU rDNA and concatenated sequences set of SSU-LSU-EF-1α generated along with the sequences of all *Wickerhamomyces* species and related '*Candida*' species were analyzed. The jModel test was performed in order to select the most appropriate evolution model [27] for the phylogenetic analysis of D1/D2 domains of LSU and the concatenated sequences of SSU-LSU-EF-1α. The Tamura–Nei model was chosen for the D1/D2 partition and the GTR + G + I model was used for the concatenated sequences. Confidence values were estimated from bootstrap analyses of 1000 replicates [28].

Results and Discussion

Yeast Strains Isolated From Water Sample of Crater Lake

A total of 69 yeast strains were isolated from five water samples of Tuofengling lake, belonging to 17 species of 12 genera. *Vishniacozyma victoriae*, *Martiniozyma abiesophila*, and *Kuraishia floccosa* were present as the most abundant species with the number of 19, 17, and 10, respectively. Some species including *Cystobasidium pinicola, Dioszegia butyracea, Vishniacozyma victoriae*, and *Rhodotorula graminis* were present by only one strain.

Species Delineation and Phylogenetic Placement

The results of sequence alignment on NCBI indicated that the D1/D2 domain of strain TF5-16-2 showed the highest similarities to strains '*Candida' silvicultrix* CBS 6269^T and *W. anomalus* CBS 5759^T with the values of 92% (including 53 nt substitutions and 21 gaps out of 926 nt) and 91.5% (including 57 nt substitutions and 22 gaps out of 929 nt), respectively; the divergences in ITS region between strain TF5-16-2 and *'Candida' silvicultrix* CBS 6269^T, *W.*

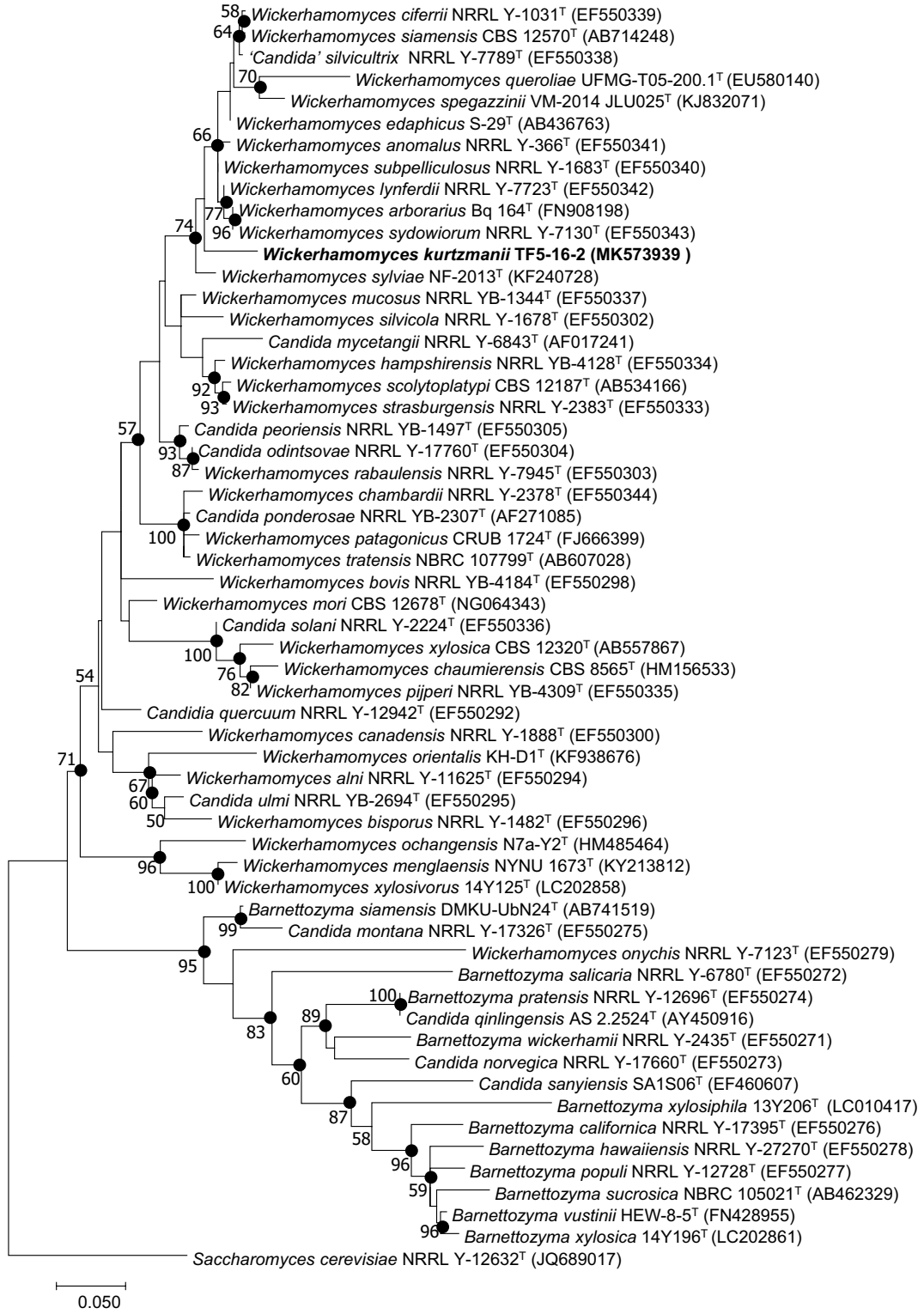

Fig. 1 Phylogenetic tree derived from Maximum-likelihood analysis of the D1/D2 domains of LSU, showing the placement of *Wickerhamomyces kurtzmanii* sp. nov TF5-16-2 and related species in *Wickerhamomyces* clade including *Candida* species. *Saccharomyces cerevisiae* NRRL Y-12632^T was used as outgroup. Bootstrap values (%) over 50% based on 1000 replication are given at nodes. The filled circles indicate nodes recovered using the neighbor-joining method. Bar, 0.05 substitutions per nucleotide position

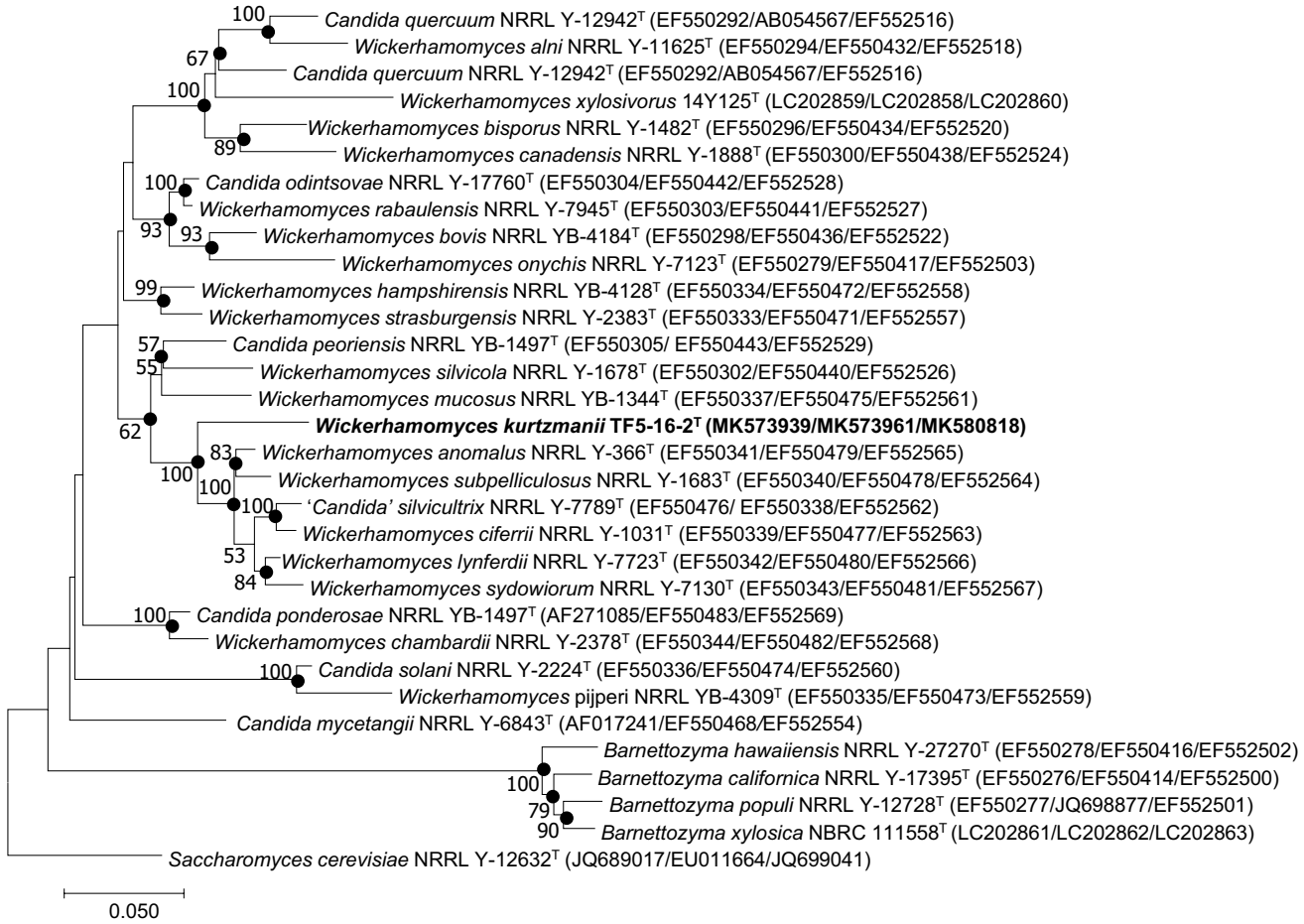

Fig. 2 Phylogenetic tree derived from Maximum-likelihood analysis based on the concatenated sequences of SSU, the D1/D2 domains of LSU and EF-1α showing the placement of *Wickerhamomyces kurtzmanii* sp. nov TF5-16-2 and related species in *Wickerhamomyces* clade including *Candida* species. *Saccharomyces cerevisiae* NRRL Y-12632^T was used as outgroup. Bootstrap values (%) above 50% based on 1000 replication are given at nodes. The filled circles indicate nodes recovered using the neighbor-joining method. Bar, 0.05 substitutions per nucleotide position

anomalus CBS 5759^T were 11% (including 4.3% gaps) and 13% (including 4.4% gaps), respectively.

Maximum-Likelihood analysis based on the D1/D2 domains of the LSU showed that the strain TF5-16-2 located in a *Wickerhamomyces* subclade containing twelve species of *Wickerhamomyces*: *W. anomalus*, *W. sylviae*, *W. edaphicus*, *W. ciferrii*, *W. siamensis*, *W. subpelliculosus, W. lynferdii, W. arborarius, W. spegazzinii, W. queroliae*, *W. sydowiorum*, and *'Candida' silvicultrix* (Fig. 1), indicating that strain TF5-16-2 was a member of the genus *Wickerhamomyces*. Furthermore, the ML-tree based on the concatenated sequences of LSU + SSU + EF-1α showed that strain TF5-16-2 formed a tight clade together with *W. anomalus*, *W. subpelliculosus*, *'Candida' sivicultrix*, *W. ciferrii*, *W. sydowiorum*, and *W. lynferdii* (Fig. 2). The NJ trees showed essentially the same topography as that of the ML trees. The above results indicated that strain TF5-16-2 belonged to the genus *Wickerhamomyces* and is distinguished from any described species of the genus *Wickerhamomyces*.

Phenotypic and Growth Characteristics

The strain TF5-16-2 was examined for sporulation by using CMA, PDA, 5% malt extract, and YM agar at 22℃. Conjugation occurred between two separate cells (Fig. 3b) after 5–7 days growth on PDA or CMA (Fig. 3b). One to four hat-shaped ascospores are formed in each ascus (Fig. 3c, d). Ascospores were released when asci were deliquescent (Fig. 3e). Pseudohyphae were not formed. In sum, strain TF5-16-2 was clearly distinct from the other species of the *Wickerhamomyces* clade by its inability of growth at higher than 30 ℃ (Table 1).

Unfortunately, we have not yet isolated other strain which might be conspecific species of TF5-16-2 in the last four years. We strived to propose this novel species represented

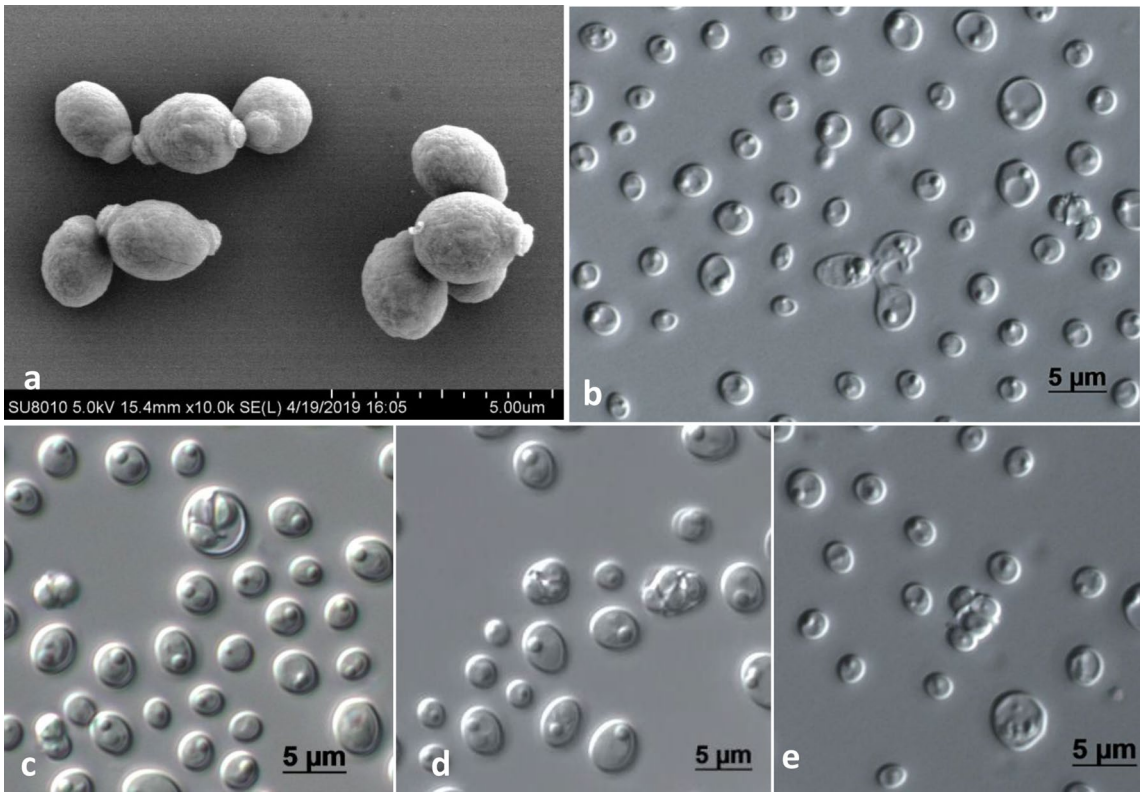

Fig. 3 *Wickerhamomyces kurtzmanii* sp. nov. TF5-16-2. **a** Scanning electron micrographs of *Wickerhamomyces kurtzmanii* sp. nov. TF5-16-2 after 3 days of growth at 22 ℃ in YM broth; formation of asci and ascospores; **b** the conjugated cells; **c** the intact ascus; **d** the deliquesced ascus; **e** the released ascospores. Bar = 5 μm

by single strain based on polyphasic approaches including molecular phylogenetic, morphological, and physiological analyses.

Description of *Wickerhamomyces kurtzmanii* Li, Zhou, and Wang sp. nov.

Wickerhamomyces kurtzmanii (in honor of the prominent yeast taxonomist Cletus P. Kurtzman, who established the genus of *Wickerhamomyces*).

After growth in YM broth for 3 days, cells are ovoid to ellipsoidal with 2.5–4.0 × 3.5–5.5 μm, and occur singly, in pairs or in small clusters (Fig. 3a). Sediment and ring are formed. Budding is multilateral. Growth is cream-white, butyrous, with a smooth surface and an entire margin. Conjugation occurred between two separate cells (Fig. 3b). Asci form one to four hat-shaped ascospores after 5–7 days growth on PDA or CMA agar at 22 ℃. Asci was deliquescent and then ascospores were released (Fig. 3c–e). Pseudohyphae are not formed.

Growth is positive at 4–25 ℃ (with the optimal range of 20–22 ℃), weakly positive at 28 ℃, but negative at 30 ℃. Growth in vitamin-free medium is negative. Growth occurred in 10% Glucose (w/v), but not in media containing 10% NaCl and 50% Glucose (w/v). Urease activity and diazonium blue B reaction are negative.

Fermentation is not observed. Sucrose, melibiose (weak), glycerol, D-mannitol (weak), salicin, DL-lactic acid, and succinic acid were assimilated as sole carbon resource. Galactose, sorbose, maltose, melezitose, cellobiose, trehalose, lactose, raffinose, soluble starch, D-xylose, L-arabinose, α-methyl glucoside, citric acid, inositol, inulin, ribose, L-rhamnose, erythritol, ribitol, galactitol, and D-glucitol were not assimilated. Sodium nitrite, potassium nitrate, cadaverine, ethylamine, and L-lysine were assimilated as sole nitrogen resource, but creatinine was not. Production of starch-like compounds is negative.

The strain TF5-16-2 isolated from the water of crater lake in Da Hinggan Ling Mountain, North of China. CGMCC 2.5597^{T} as the holotype strain was preserved in a metabolically inactive state at China General Microbiological Culture Collection (CGMCC). The ex-type culture was deposited at the Yeast Collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, as CBS 15418, and at Korean Collection for Type Cultures (KCTC) as KCTC 27823. The Mycobank accession number is MB 829959.

Table 1 Physiological characteristics comparison of strain TF5-16-2 and members of other related *Wickerhamomyces* members

	1	2*	3*	4*	5*	6*	7*	8*	9*	10*	11*	12*	13*
Fermentation													
Glucose	-	+	+	+	+	+	+	+	+	+	+	+,l	+
Galactose	-	v	+	-	-	+	+	+	-	-	+	l,lw	+
Sucrose	-	+	+	+	+	+	+	+	+	+	+	+,l	-
Maltose	-	v	v	-	v	v	+,w	s	+	-	+	l,-	+
Raffinose	-	v	+	+	v	+	+,w	+	-	+	+	l,-	-
Assimilation reactions													
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	-,w
Raffinose	-	+	+	+	+	+	+,w	+	-	+	+	+	-
Melibiose	w	-	-	-	-	+	-	+	-	+	+	+	-
Galactose	-	v	+	+	v	+	+	+	-	+	+	+	v
Trehalose	-	+	+	+	+	+	+,w	+	+	n	+	+	+
Maltose	-	+	+	+	+	+	+	+	+	+	+	+	v
Melezitose	-	+	+	+	v	+	+,s	+	-	+	+	+	v
Methyl-α-D-glucoside	-	+	+	+	+	+	+,w	+	n	+	+	+	-,d
Soluble Starch	-	+	+	-	v	v	+,w	+	-	s	+	+	+,w
Cellobiose	-	+	+	+	v	+	+,w	+	-	+	+	v	-,d
Salicin	+	+	+	+	+	+	+,w	+	+	+	+	+,l	v
L-Rhamnose	-	-	+	-	-	+	-	-	+	+	+	+,l	v
D-Xylose	-	v	+	-	v	v	+,s	+	+	+	+	+	d,-
L-Arabinose	-	v	+	-	v	+	-	+	+	n	w	v	+
D-Arabinose	-	-	-	-	v	-	-	+	-	n	n	v	+
D-Ribose	-	v	+	+	v	+	-	+	+	-	+	v	-,d
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	-,d
Erythritol	-	+	+	+	+	+	+,w	+	+	s	+	+	v
Ribitol	-	v	+	+	v	+	-	+	+	-	+	+	-,d
Galactitol	-	-	-	-	-	-	-	-	-	-	+	+	-
D-Mannitol	w	+	+	+	+	+	+,w	+	+	+	+	+	-
D-Glucitol	-	+	+	+	+	+	+,w	+	+	+	+	+	-
DL-Lactate	+	+	+	+	+	+	+	+	v	-	+	+	+
Succinate	+	+	+	+	+	+	+	+	+	n	+	+	+,w
Citrate	-	+	+	+	+	+	-	+	w,s	w	+	+	-
Vitamin-free	-	+	+	+	-	+	-	+	-	+	+	n	+
Nitrate	+	+	+	+	+	+	-	-	+	+	+	+	v
Nitrite	+	n	n	n	n	n	-	-	+	+	+	n	n
Cadaverine	+	n	n	n	n	n	+	+	+	+	+	+	n
Creatinine	-	n	n	n	n	n	n	n	n	-	n	n	n
L-Lysine	+	n	n	n	n	n	+	+	+	+	+	+	n
Ethylamine	+	n	n	n	n	n	+	+	+	-	+	+	n
Other characteristics													
50% Glucose	-	n	n	n	n	n	+	+	-	-	+	+	n
10% NaCl/5% glucose	-	n	+	+	+	+	-	+	-	-	+	+	+
Growth at 30 °C	-	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 35 °C	-	n	n	n	n	n	+	+	+	+	+	n	+
Growth at 37 °C	-	v	v	-	v	-	+	+	+	+	+	-	+

1: TF5-16-2; 2: W. anomalus; 3: W. ciferrii; 4: W. lynferdii; 5: W. subpelliculosus; 6: W. sydowiorum; 7: W. siamensis; 8: 'Candida' silvicultrix; 9: W. queroliae; 10: W. spegazzinii; 11: W. edaphicus; 12: W. arborarius; 13: W. sylviae. *: data were from [1, 5, 9, 13, 15, 16, 29–31]. d: delayed; l: latent; w: weak; s: slow; n: no data

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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