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TAXONOMIC DESCRIPTION

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Wickerhamomyces menglaensis f.a., sp. nov., a yeast species isolated from rotten wood

Chun-Yue Chai, Lin-Na Huang, Han Cheng, Wen-Jing Liu and Feng-Li Hui*

Abstract

Five strains, NUNU 16637, NYNU 16645, NYNU 1673, NYNU 1680 and NYNU 1689, of a novel ascomycetous yeast were isolated from the Xishuangbanna tropical rainforest, Yunnan Province, PR China. The five strains shared identical sequences in both of the D1/D2 domains of the large subunit rRNA gene and the internal transcribed spacer (ITS) regions. Sequence analysis showed that they represent undescribed yeast species belonging to the genus *Wickerhamomyces*. They differed from their closest known species, *Wickerhamomyces xylosivorus* NBRC 111553^T, by 3.4 % sequence divergence (14 substitutions and six gaps out of 584 bp) in the D1/D2 domains and by 9.6 % sequence divergence (28 substitutions and 24 gaps over 543 bp) in the ITS regions, respectively. The five strains of novel species reproduced asexually; no sexual reproduction could be found. In contrast to *W. xylosivorus*, the novel yeast species were able to assimilate L-arabinose, inulin, soluble starch, D-mannitol and citrate, and unable to assimilate trehalose, raffinose, 5-keto-D-gluconate, D-gluconate, ethanol, ethylamine and cadaverine. Growth was observed at 35 °C. The name *Wickerhamomyces menglaensis* f.a., sp. nov. is proposed to accommodate these strains, with NYNU 1673 as the holotype.

The genus *Wickerhamomyces* was proposed by Kurtzman *et al.* with *Wickerhamomyces canadensis* as the type species, this type species was originally transferred from the genus *Pichia* [1]. At the time of writing, 17 species of the genus *Wickerhamomyces* were originally transferred from the genera *Pichia*, *Williopsis* and *Hansenula* following a recent detailed phylogenetic analysis of nucleotide divergence in the genes coding for the large-subunit (LSU) and small-subunit rRNA genes and for elongation factor *1a* [1, 2], and 16 species have subsequently been included [3–18]. Additionally, eight species of the genus *Candida* are known to be members of the *Wickerhamomyces* clade [2]. Species of the genus *Wickerhamomyces* produce persistent or deliquescent, hat- or Saturn-shaped, spherical ascospores. They are characterized by a negative diazonium blue B colour reaction, the inability to assimilate methanol and the presence of Q-7 as the predominant ubiquinone [1, 2].

During a study on yeasts associated with rotten wood in Xishuangbanna tropical rainforest, more than 90 % of the samples used in this study contained yeasts and 145 yeast strains were obtained. Based on the D1/D2 domains of the large subunit (LSU) rRNA gene sequence comparisons, the majority of the yeasts belonged to several major clades in the subphylum Saccharomycotina; some of these species have been identified as novel species in earlier papers, for example *Cyberlindnera xishuangbannaensis* CBS 14692 [19], *Deakozyma yunnanensis* CBS 14688 [20] and *Vanrija jinghongensis* CBS 15229 [21]. Amongst these associates, five novel yeast strains could not be ascribed to any validly known species. Further sequence analysis of the D1/D2 domains of the LSU rRNA gene and the internal transcribed spacer (ITS) regions showed that these strains represent a novel species belonging to the genus *Wickerhamomyces*. In this report, a novel species, *Wickerhamomyces menglaensis* f.a., sp. nov., is described.

More than 50 rotten wood samples were collected from the different locations in the Xishuangbanna tropical rainforest, Yunnan Province, PR China. Three strains, NYNU 16637, NYNU 16645 and NYNU 1673, were isolated from three samples of rotting wood collected in in Menglun, Mengla (approximate GPS coordinates: 21° 41′ N 101° 25′ E). The other two strains, NYNU 1680 and NYNU 1689, were isolated from two samples of rotting wood collected in Galan, Jinghong (approximate GPS coordinates: 21° 27′ N 100° 25′ E). The yeast strains were isolated from rotting wood samples in accordance with the methods described by Hui *et al.* [22]. Each sample (1 g) was added to 20 ml sterile yeast extract–malt extract (YM) broth [1 % glucose (w/v), 0.5 %

Author affiliation: School of Life Science and Technology, Nanyang Normal University, Nanyang 473061, PR China.
***Correspondence:** Feng-Li Hui, fenglihui@yeah.net
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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.
The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domains of the LSU rRNA gene and the ITS regions of *Wickerhamomyces menglaensis* sp. nov. NYNU 1673^T are KY213812 and KY213818, respectively.
One supplementary figure is available with the online version of this article.

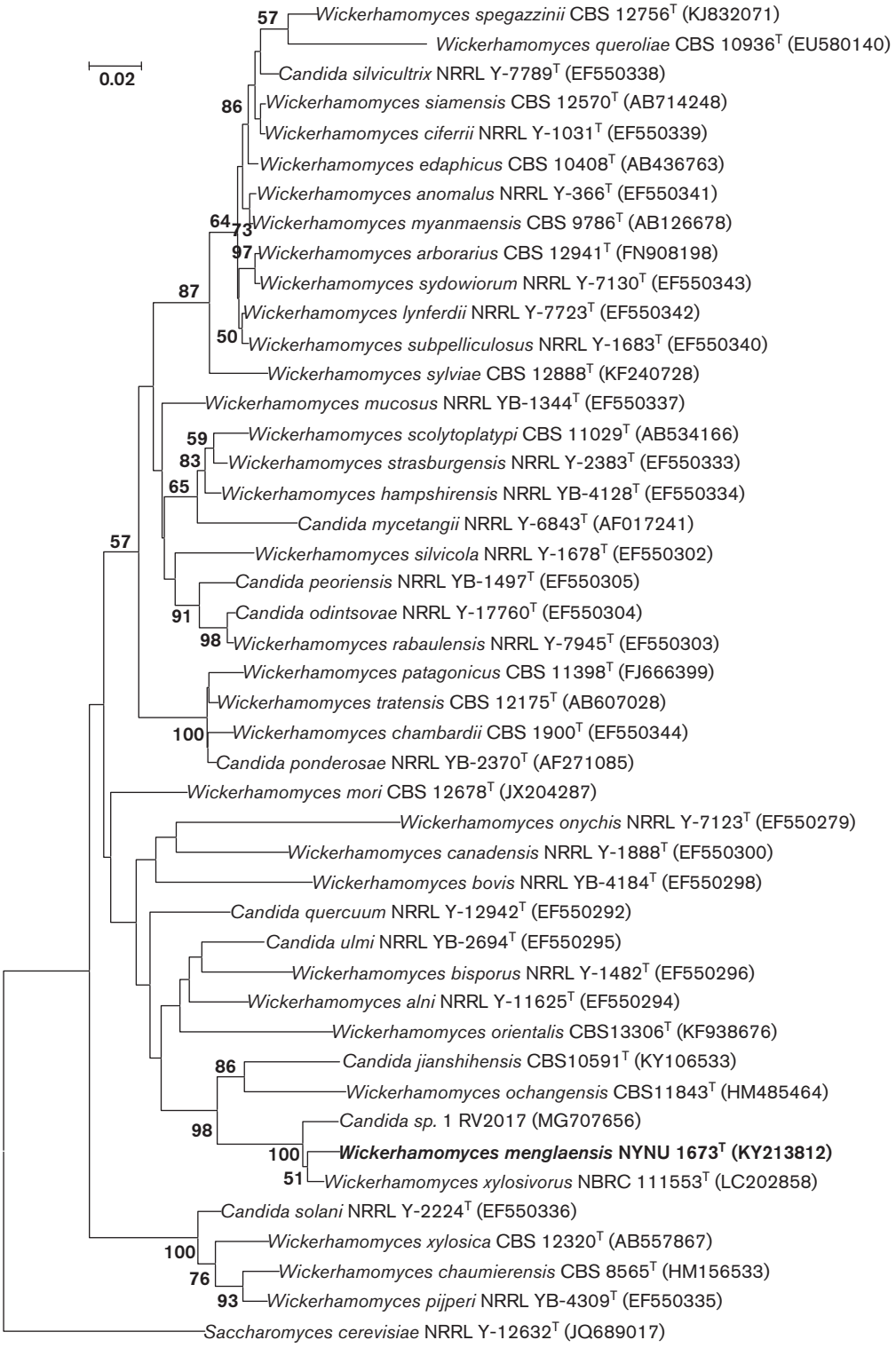

Fig. 1. Phylogenetic tree derived from the neighbour-joining analysys based on sequences of the D1/D2 domains of the LSU rRNA gene, showing the placement of *Wickerhamomyces menglaensis* f.a., sp. nov. in the *Wickerhamomyces* clade. *Saccharomyces cerevisiae* NRRL Y-12632 was used as an outgroup. Sequences were retrieved from the GenBank and CBS (*) databases. Bootstrap values of above 50 % are given at nodes based on 1000 replications. Bar, 0.02 substitutions per site.

peptone (w/v), 0.3 % yeast extract (w/v) and 0.3 % malt extract (w/v); (pH 5.4) supplemented with 0.02 % chloramphenicol (w/v)] in a 150 ml Erlenmeyer flask and then incubated at 25 °C for 3 days on a rotary shaker. Subsequently, 0.1 ml enrichment culture and appropriate decimal dilutions were spread on YM agar plates supplemented with 0.02 % chloramphenicol and then incubated at 25 °C for 3–4 days. Different yeast colony morphotypes were then purified at least twice and then stored on YM agar slants at 4 °C or in 15 % glycerol at −80 °C.

Morphological, physiological and biochemical characteristics were examined according to standard methods by Kurtzman *et al.* [2]. Assimilation tests were performed by replica plating on solid and in liquid media twice, and the results were recorded after 5 and 21 days of incubation. Mating and ascus formation were assessed on YM, 5 % malt extract (MEA), corn meal agar (CEA), V8 and diluted V8, yeast carbon base supplemented with 0.01 % ammonium sulphate (YCBAS) agar (1.1 % yeast carbon base, 0.01 % ammonium sulphate and 1.8 % agar) and Gorodkowa agars (0.5 % sodium chloride, 0.1 % glucose, 2 % agar) individually or by mixing strains in pairs and incubating for up to 4 weeks at 15 and 25 °C.

Genomic DNA was extracted using the Ezup Column Yeast Genomic DNA Purification Kit according to the manufacturer's protocol (Sangon Biotech). The ITS region was amplified with the primers forward ITS1 (5′-TCCGTAGG TGAACCTGCGG-3′) and reverse ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) [23] and the D1/D2 domains of the LSU rRNA gene were amplified using the pair primers forward NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and reverse NL4 (5′ -GGTCCGTGTTTCAAGACGG-3′) [24]. Each 50 µl PCR mixture included 21 µl PCR-grade water, 1 µl DNA template, 1.5 µM of each primer and 1 µl PCR Master Mix (2×; 0.05 u μl^{-1} de Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP; Sangon Biotech). PCR reactions were carried out in an S1000 thermal cycler (Bio-Rad Laboratories). The amplified products were purified with a QIAquick purification kit (Sangon Biotech) according to the manufacturer's instructions. Both DNA strands were sequenced using the ABI BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an ABI 3730 automated DNA sequencer according to the manufacturer's instructions.

Comparisons with sequences from the GenBank database (www.ncbi.nlm.nih.gov/) were done using a BLASTN search [25]. The sequences of related strains retrieved from GenBank were initially aligned using the multiple alignment program CLUSTAL_X 1.83 [26]. The phylogenetic trees were reconstructed based on the D1/D2 domains of the LSU rRNA gene sequences with MEGA software version 5.0 [27]. The evolutionary distances were calculated by using Kimura's two-parameter model [28] for the neighbour-joining and the maximum likelihood analyses, respectively [27, 29]. *Saccharomyces cerevisiae* NRRL Y-12632N^T was used as an outgroup. Bootstrap analyses were performed based on 1000 random resamplings [30], and only values above 50 % were recorded on the resulting trees. Reference sequences were retrieved from GenBank or from the CBS database under the accession numbers indicated in the tree.

Table 1. Physiological characteristics differentiating the novel *Wickerhamomyces menglaensis* f.a., sp. nov. species from closely related species

Species: 1, *Wickerhamomyces menglaensis* f.a., sp. nov.; 2, *Wickerhamomyces xylosivorus* NBRC 111553^T (data from [18]). +, Positive; –, negative; w, weak.

Characteristics	Yeast species	
	1	2
Assimilation of:		
L-Arabinose	w	–
Trehalose	–	w
Raffinose	–	w
Inulin	w	–
Soluble starch	w	–
D-Mannitol	+	–
5-Keto-D-gluconate	–	w
D-Gluconate	–	+
Citrate	+	–
Ethanol	–	+
Ethylamine	–	+
Cadaverine	–	+
Growth at:		
35 °C	+	–
Formation of:		
Raffinose	w	–

SPECIES DELINEATION, CLASSIFICATION AND ECOLOGY

The five investigated strains (NYNU 16637, NYNU 16645, NYNU 1673, NYNU 1680 and NYNU 1689) shared identical sequences in both the D1/D2 domains of the LSU rRNA gene and ITS regions. Sequence analysis showed that the five yeast strains isolated from rotting wood represent a novel species belonging to the genus *Wickerhamomyces* [1, 2]. In terms of pairwise sequence divergence, the closest relative of the novel strains was the type strains of *Wickerhamomyces xylosivorus* [18]. The D1/D2 domains of the LSU rRNA gene sequences of these strains differed by 3.4 % sequence divergence (14 substitutions and six gaps out of 584 bp) from *W. xylosivorus* NBRC 111553^T. The the D1/D2 domains of the LSU rRNA gene sequences of other species in the clade were found to be more divergent. In addition, the ITS sequences were obtained for all strains of the proposed novel species. The nucleotide differences in the ITS regions between the five new isolates and the most similar species, *W. xylosivorus* NBRC 111553^T, was 28 substitutions and 24 gaps in 543 bp (9.6 %). According to the results of DNA barcoding analysis of yeast species by Vu *et al.* [31], this extent of divergence is sufficient to justify the five new

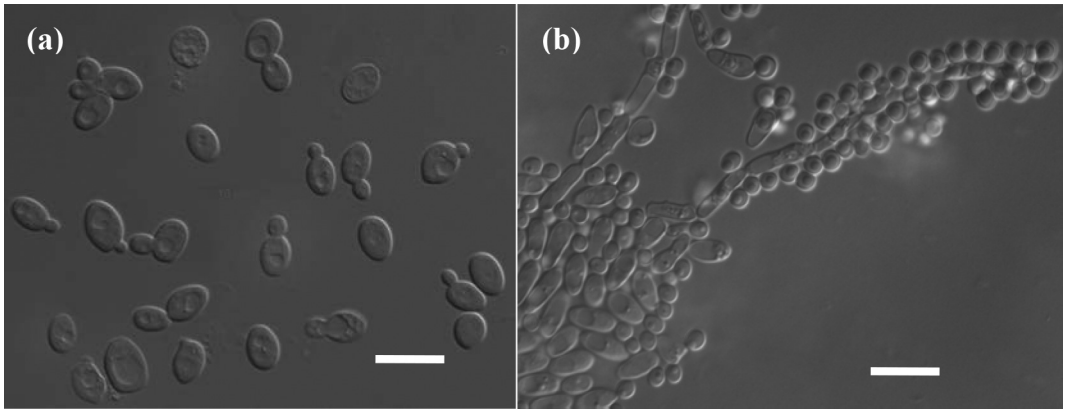

Fig. 2. Photomicrographs of *Wickerhamomyces menglaensis* f.a., sp. nov. NYNU 1673. (a) Budding cells grown on YM broth for 3 days at 25 °C. (b) Pseudohyphae grown on YCBS agar for 13 days at 25 °C. Bar, 10 µm.

isolates are members of a single undescribed yeast species that is different from all known ascomycetous yeasts.

To determine the phylogenetic position of these five novel strains, a neighbour-joining phylogenetic tree was reconstructed based on the D1/D2 domains of the LSU rRNA gene sequences of the novel strains, their closest relatives and members of the *Wickerhamomyces* clade as defined by de Kobayashi *et al.* [18]. The novel species form a subclade together with *W. xylosivorus* NBRC 111553^T. The bootstrap support for this subclade was 51 % (Fig. 1). The phylogenetic relationships of the novel species with related species in the subclade were also supported by the maximum-likelihood analysis (Fig. S1, available in the online version of this article).

The five strains (NYNU 16637, NYNU 16645, NYNU 1673, NYNU 1680 and NYNU 1689) displayed phenotypic and biochemical properties typical of members of the genus *Wickerhamomyces*, characterized by multilateral budding and developed pseudohyphae. However, ascospores were not observed in pure or mixed cultures of the five isolates in sporulation media after 4 weeks at 15 or 25 °C. Our putative new species is physiologically differentiated from its closest described species, *W. xylosivorus* NBRC 111553^T [18]. Physiologically, the five strains could be able to ferment raffinose, the ability to assimilate L-arabinose (weak), inulin (weak), soluble starch, D-mannitol and citrate, and unable to assimilate trehalose, raffinose, 5-keto-D-gluconate, D-gluconate, ethanol, ethylamine and cadaverine. Growth was observed at 35 °C. The differences of the physiological properties between the new species and its closest relative are shown in Table 1.

On the basis of morphological and physiological characteristics and sequence analysis of the D1/D2 domains of the LSU rRNA gene and ITS regions, we conclude that the five investigated strains represent a novel species, which differ from currently recognized ones. The name *Wickerhamomyces menglaensis* f.a., sp. nov. is proposed for the five strains which were isolated from rotting wood in China.

Members of the genus *Wickerhamomyces* have been isolated from various substrates, including soil, sea and ocean waters, plant material, phylloplane, insect tunnels, guts of wood-boring insect larvae, migratory birds, brined vegetables, and jams [1, 3–18]. One species, *Wickerhamomyces anomalus*, was isolated as an opportunistic pathogen of humans and animals [1]. In the present study, five strains of *W. menglaensis* f.a., sp. nov. were isolated from rotten wood from two different localities, thereby suggesting that this substrate is ecological niche of the novel species. Therefore, rotten wood is a source for further investigations of yeasts in the *Wickerhamomyces* clade.

DESCRIPTION OF *WICKERHAMOMYCES MENGLAENSIS* HUI & HUANG SP. NOV.

Wickerhamomyces menglaensis (meng.la.en′sis. N.L. masc. adj. *menglaensis* of or belonging to the city of Mengla, Yunnan Province, PR China, the collection locality of the type strain of the species).

In YM broth after 3 days at 25 °C, the cells are ellipsoidal to elongate (3.0–6.7×5.9–8.2 µm) and occur singly or in pairs. Budding is multilateral (Fig. 2a). On YM agar after 3 days at 25 °C, the streak culture is butyrous, cream and convex with a smooth surface and has an entire margin. On Dalmau plates after 13 days on YCBS agar at 25 °C, pseudohyphae are formed (Fig. 2b), but true hyphae are not formed. Ascospores are not observed on YM, 5 % malt extract, cornmeal and YCBAS agar in pure and mixed cultures at 17 and 25 °C for up to 4 weeks. Glucose and raffinose (weak) are fermented. No fermentation of the following carbohydrates: galactose, sucrose, maltose, lactose, trehalose, D-xylose, cellobiose and melezitose. Assimilation of carbon compounds is as follows: D-glucose, D-xylose (weak), L-arabinose

(weak), L-rhamnose (weak), cellobiose, salicin, arbutin, glycerol (weak), starch (weak), inulin (weak), D-glucitol, D-mannitol, D-glucono-1, 5-lactone, DL-lactate, succinate and citrate. Carbon sources not assimilated are as follows: D-galactose, L-sorbose, D-glucosamine, D-ribose, D-arabinose, sucrose, maltose, trehalose, melibiose, lactose, raffinose, melezitose, erythritol, ribitol, xylitol, L-arabinitol, galactitol, *myo*-inositol, 2-keto-D-gluconate, methanol, D-gluconate, D-glucuronate, D-galacturonate, 5-keto-D-gluconate and ethanol. Assimilation of nitrogen compounds are as follows: nitrate, nitrite, L-lysine (weak), glucosamine (weak) and D-tryptophan (weak). Cadaverine, creatine, creatinine, ethylamine and imidazole are not assimilated. Cell growth is observed in vitamin-free medium. Growth is observed at 35 °C. No growth on 50 % (w/v) glucose or 5 % (w/v) glucose/sodium chloride (10 %). Urea hydrolysis and diazonium blue B reactions are negative.

The holotype, NYNU 1673, was isolated in August 2016, from rotting wood collected in Xishuangbanna Tropical Rainforest in Yunnan Province, PR China. It is preserved in a metabolically inactive state at Microbiology Lab, Nanyang Normal University, Henan, PR China. Ex-type cultures are deposited at the China Center of Industrial Culture Collection (CICC), Beijing, PR China, as strain CICC 31159, and at the Yeast Collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, as strain CBS 14689. The MycoBank number is MB 829372.

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

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