Wickerhamomyces mori sp. nov., an anamorphic yeast species found in the guts of wood-boring insect larvae

Feng-Li Hui, Liang Chen, Xue-Ying Chu, Qiu-Hong Niu and Tao Ke

A novel anamorphic yeast species is described to accommodate three isolates recovered from the guts of three different wood-boring insect larvae collected in Henan, central China. On the basis of sequence analyses of the D1/D2 domains of the large-subunit rRNA gene and the internal transcribed spacer regions, the three strains are assigned to a novel species of the genus Wickerhamomyces, although the formation of ascospores was not observed. These strains also exhibited a number of distinct morphological and physiological characteristics that clearly differentiated them from Wickerhamomyces mucosus, Candida odintsovae and Wickerhamomyces rabaulensis, the most closely related species. In view of the phenotypic differences and unique rRNA gene sequences, we consider that these three isolates represent a novel species of the genus Wickerhamomyces, Wickerhamomyces mori sp. nov. The type strain is NYNU 1216T (=CICC 1983T = CBS 12678T).

During a study of yeasts vectored by insects, we have obtained 66 yeast strains from the guts of insect larvae collected in Henan province, central China. Based on DNA sequence comparisons and phenotypic characteristics, 60 isolates of these yeasts were identified to represent 12 described ascomycetous yeast species, Candida agrestis, Candida akabanensis, Candida dosseyi, Candida gotoi, Candida membranifaciens, Candida melibiosica, Candida ponderosae, Meyerozyma guilliermondii, Pichia salicaria, Pichia exigua, Pichia manshurica and Saturnispora zaruensis. The remaining strains were found to represent two undescribed species. Three of these undescribed yeasts have already been described as representing the novel species Candida ficus (Hui et al., 2012). In the present study, we investigated the phylogenetic position and phenotypic characteristics of three additional strains, NYNU 1204, NYNU 1215 and NYNU 1216T, and identified them as representing a novel species belonging to the genus Wickerhamomyces.

Strains NYNU 1204, NYNU 1215 and NYNU 1216T were isolated from the guts of three different wood-boring insect larvae in February 2012. The samples were collected from trunks of the trees Salix babylonica and Morus alba cultivated on the campus of Nanyang Normal University located in Henan province, central China. Strains NYNU 1204 and NYNU 1215 were isolated from two individuals of insect larvae collected from Salix babylonica, whereas strain NYNU 1216T was found from a sample of an insect larva collected from Morus alba.

Methods for yeast isolation were detailed by Nguyen et al. (2006) and Suh et al. (2004). The insects were usually placed in Petri dishes for 1–3 days without food prior to dissection. Withholding food helps to eliminate some contaminating organisms that might be isolated from the gut. Surface disinfection was performed by submersion in 95% ethanol for 1–2 min. The alcohol wash was followed by a 0.7%
saline rinse. The insect gut was removed aseptically under a dissecting microscope, and gut segments were streaked on acidified YM agar plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% plain agar, adjusted to pH 3.5 with HCl). The plates were incubated at 25 °C, and single colonies were streaked for purification. Purified yeast strains were grown on YM agar at 25 °C for 3 days, followed by preservation at −70 °C and/or on YM agar at 4 °C.

Morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). All assimilation tests were carried out twice, and results were recorded after 5 and 21 days of incubation. Mating and ascus formation were assessed on YM, 5% malt extract, cornmeal, V8 and diluted V8, yeast carbon base supplemented with 0.01% ammonium sulphate and Gorodkowa agars individually or by mixing strains in pairs and incubating for up to 4 weeks at 15 and 25 °C. Ubiquinones were extracted and purified by the method of Yamada & Kondo (1973) with slight modifications and determined by HPLC as described previously (Nguyen Thanh et al., 2003).

Genomic DNA was extracted from yeast cells as described previously (Ramos et al., 2001). The D1/D2 domains of the large-subunit (LSU) rRNA gene and internal transcribed spacer (ITS) regions were amplified using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (Scorzetti et al., 2002), respectively, with a Biometra thermal cycler (TGradient 96; Labrepco). Successful amplification was confirmed by agarose gel electrophoresis. Sequencing of the fragments was performed using an automated DNA sequencer (3730 DNA analysis system; Applied Biosystems). Both DNA strands were sequenced and reactions were carried out using a dye terminator cycle sequencing kit (Applied Biosystems). Comparisons with sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/) were done using BLASTN search. The sequences of related strains retrieved from GenBank were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson et al., 1997) included in the DNAMAN software package, version 5.1.5 (Lynnon BioSoft). The phylogenetic tree was reconstructed from evolutionary distance data calculated from Kimura’s two-parameter model (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987). Zygosaccharomyces bailii NRRL Y-2277T was used as an outgroup. Bootstrap analyses were performed based on 1000 random resamplings (Felsenstein, 1985). Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree or from the CBS database.

**Sequence comparison and species delineation**

Isolates NYNU 1204, NYNU 1215 and NYNU 1216T shared identical D1/D2 sequences. Sequence analysis of the D1/D2 region of these sequences from the GenBank database was used as an outgroup. Bootstrap values above 50% based on 1000 replications are given at nodes. Sequences were retrieved from the GenBank and CBS (*) databases and represent ITS/LSU rRNA D1/D2 sequences. Bar, 5% sequence difference.

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**Fig. 1.** Phylogenetic tree derived from neighbour-joining analysis of concatenated ITS and LSU rRNA gene D1/D2 domain sequences showing the position of strains of *Wickerhamomyces mori* sp. nov. *Zygosaccharomyces bailii* NRRL Y-2277T was used as an outgroup. Bootstrap values above 50% based on 1000 replications are given at nodes. Sequences were retrieved from the GenBank and CBS (*) databases and represent ITS/LSU rRNA D1/D2 sequences. Bar, 5% sequence difference.
domains of the LSU rRNA gene revealed that these novel strains were closely related to the species of the Wickerhamomyces clade (Kurtzman et al., 2008; Kurtzman, 2011). In terms of pairwise sequence divergence, the closest relatives of the novel strains were the type strains of Wickerhamomyces mucosus, Candida odintsovae and Wickerhamomyces rabaulensis. The D1/D2 sequences of these strains differed by 26 substitutions (4.5%) from W. mucosus, by 27 substitutions (4.7%) from C. odintsovae and by 28 substitutions (4.8%) from W. rabaulensis. The D1/D2 region sequences of other species in the clade were found to be more divergent. ITS sequences were also obtained for all strains of the proposed novel species. In the ITS regions, strain NYNU 1216T differed by one substitution from strain NYNU 1204 and by five substitutions from strain NYNU 1215, and the latter two strains differed from each other by four substitutions. The ITS sequences of the three new isolates exhibited 53–58 substitutions (9.0–9.9%) from W. mucosus and W. rabaulensis, but pairwise sequence analysis with W. mucosus could not be performed because the ITS sequences of the type strain of W. mucosus is not currently available from either the GenBank database or the CBS database. According to the discussion of yeast species recognition from molecular data in Kurtzman & Robnett (1998) and Sugita et al. (1999), isolates NYNU 1204, NYNU 1215 and NYNU 1216T are members of a single undescribed yeast species that is different from all known ascomycetous yeasts.

To determine the phylogenetic position of the novel strains, a neighbour-joining phylogenetic tree was reconstructed based on concatenated ITS and LSU rRNA gene D1/D2 domain sequences of the novel strains, their closest relatives and members of the Cyberlindnera clade as defined by de Garcia et al. (2010) (Fig. 1). All three strains of the novel species were at the same position and formed a distinct and separate lineage in the Wickerhamomyces clade (Fig. 1). These results confirmed our provisional determination of the strains as representing a novel species of the genus Wickerhamomyces.

Strains NYNU 1204, NYNU 1215 and NYNU 1216T displayed phenotypic and biochemical properties typical of members of the genus Wickerhamomyces, characterized by multilateral budding, the ability to ferment glucose, the inability to assimilate methanol, a negative diazonium blue B reaction and the presence of Q-7 as the major ubiquinone. However, a sexual state was not observed in pure or mixed cultures of the three isolates in sporulation media after 4 weeks at 15 or 25 °C. Physiologically, strains NYNU 1204, NYNU 1215 and NYNU 1216T differed remarkably from the closely related species W. mucosus, C. odintsovae and W. rabaulensis by their inability to assimilate D-xylose, trehalose, cellobiose and D-sorbitol (Table 1). Additionally, the three novel strains could be distinguished from W. mucosus with respect to growth in vitamin-free medium, ability to assimilate D-glucuronate and DL-lactate and to ferment sucrose and inability to assimilate 2-keto-D-glucuronate. They also differed from C. odintsovae by positive assimilation of D-arabinose and the inability to ferment raffinose and to assimilate L-arabinose, L-rhamnose, raffinose and 2-keto-D-glucuronate. These isolates were well separated from W. rabaulensis by a positive response for growth in the absence of vitamins, ability to assimilate L-sorbose and D-arabinose and failure to assimilate L-arabinose, raffinose and ribitol and to ferment trehalose and raffinose.

On the basis of phenotypic characteristics and sequence analyses of the D1/D2 domains of the LSU rRNA gene and ITS regions, we conclude that strains NYNU 1204, NYNU 1215 and NYNU 1216T represent a novel species of the genus Wickerhamomyces, for which the name Wickerhamomyces mori sp. nov. is proposed.

At present, there are many yeast species in the Wickerhamomyces clade. The species in this clade have been isolated from a wide variety of substrates, such as soil, seawater, plant substrates and animal-associated samples (Limtong et al., 2009, 2012; Groenewald et al., 2011; Kurtzman, 2011). Some species of the clade were isolated from insects and insect frass; for example, a strain of C. mycetangii was obtained from an insect, while C. ponderosae, C. silvicultrix, C. ulmi, Wickerhamomyces bisporus, Wickerhamomyces canadensis, Wickerhamomyces hamshirensis and Wickerhamomyces sydowiourum were isolated from insect frass (Kurtzman, 2011; Lachance et al., 2011). The strains of the novel species reported in this study were isolated from the guts of three

Table 1. Physiological characteristics of Wickerhamomyces mori sp. nov. and closely related species

<table>
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<tr>
<th>Characteristic</th>
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<td>Sucrose</td>
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<td>Trehalose</td>
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<td>Raffinose</td>
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<td>Assimilation of:</td>
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<td>L-Sorbose</td>
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<td>D-Arabinose</td>
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<td>L-Rhamnose</td>
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<td>Trehalose</td>
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<td>Cellobiose</td>
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<td>DL-Lactate</td>
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<td>Growth in vitamin-free medium</td>
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Species: 1, W. mori sp. nov.; 2, W. mucosus; 3, C. odintsovae; 4, W. rabaulensis. Data for type strains of W. mucosus and W. rabaulensis were taken from Kurtzman (2011) and data for the type strain of C. odintsovae were from Lachance et al. (2011). +, Positive; –, negative; d, delayed; w, weak; v, variable.
different wood-boring insect larvae. Therefore, the gut of insect larvae seems to be an additional habitat of yeast species in the Wickerhamomyces clade.

**Description of Wickerhamomyces mori** Hui, Chen, Chu, Niu & Ke sp. nov.

Wickerhamomyces mori (mo’ri. L. gen. n. morus -i of Morus, in reference to the white mulberry, *Morus alba*, from which the host of the type strain was collected).

In YM broth after 3 days at 25 °C, cells are ovoid (2–5 × 2.5–7 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. 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 7 days on cornmeal agar at 25 °C, pseudohyphae are formed (Fig. 2b), but true hyphae are not formed. Ascospores are not formed in common sporulation media. D-Glucose (delayed) and sucrose (weak, delayed) are fermented, but not D-galactose, maltose, trehalose, melibiose, lactose, raffinose, inulin, soluble starch, erythritol, ribitol, D-sorbitol, galactitol, myo-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-glucuronate and methanol, are not assimilated. Ethylamine, L-lysine and D-tryptophan (weak) are assimilated, but nitrate, nitrite, cadaverine, creatine, creatinine, glucosamine and imidazole are not assimilated. Growth in vitamin-free medium is positive. Growth is absent in the presence of 10 % NaCl plus 5 % glucose and 0.01 % cycloheximide, as is growth on 50 % glucose agar. Growth occurs on YM agar at 35 °C, but not at 37 °C. Urea hydrolysis and diazonium blue B reactions are negative. The major ubiquinone is Q-7. The Mycobank deposit number is MB 801751.

The type strain NYNU 1216T was isolated from the gut of a wood-boring insect larva collected from the trunk of a white mulberry tree, *Morus alba*, at Nanyan, Henan province, China. A living culture from the type has been deposited at the China Center of Industrial Culture Collection (CICC), Beijing, China, as CICC 1983T and at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as CBS 12678T.

**Acknowledgements**

This work was supported by a grant from the Research Planning Project of Basic and Advanced Technology of Henan Province, China (no. 122300410032).

**References**


