Candida funiuensis sp. nov., a cellobiose-fermenting yeast species isolated from rotten wood

Yun Wang,† Yong-Cheng Ren,† Zheng-Tian Zhang, Fu-Hua Wu, Tao Ke and Feng-Li Hui

School of Life Science and Technology, Nanyang Normal University, Nanyang 473061, PR China

Two strains of an asexual cellobiose-fermenting yeast species were isolated from Funiu Mountain samples collected in Henan Province, central China. Molecular phylogenetic analysis that included the nearly complete small subunit (SSU), the internal transcribed spacer (ITS) region and the D1/D2 domains of the large subunit (LSU) rDNA showed that these strains belonged to the Candida kruisii clade, with Candida kruisii and Candida cretensis as their closest phylogenetic neighbours. The nucleotide differences between the novel strains and the type strains of C. kruisii and C. cretensis were 30 and 36 substitutions, respectively, in the D1/D2 LSU rDNA, 40 and 44 substitutions, respectively, in the ITS region and 19 and 23 substitutions, respectively, in the SSU rDNA. The novel strains can also be distinguished from their closest described species, C. kruisii and C. cretensis, by a number of physiological characteristics, and represent a novel species of the genus Candida, for which the name Candida funiuensis sp. nov. is proposed. The type strain is NYNU 14625T (=CICC 33050T=CBS 13911T). The Mycobank number is MB 811503.

Candida kruisii, first described as Torulopsis kruisii, was isolated from a mushroom (Kocková-Kratochvílová & Ondrušová, 1971). Later Yarrow & Meyer (1978) transferred the species to the genus Candida. Kurtzman & Robnett (1998) placed C. kruisii as a basal member of the Hyphopichia clade on the basis of sequence analysis of the D1/D2 domains of the large subunit (LSU) rDNA. Meanwhile, an analysis of the small subunit (SSU) rDNA sequence placed C. kruisii as a sister of Candida sake and Candida tanzawaensis (Sugita & Nakase, 1999). Then, the C. kruisii clade was expanded by Suh et al. (2006) with the description of nine novel species, namely, Candida pallodes, Candida tritomae, Candida panamensis, Candida lycoperdinae, Candida atbi, Candida barrocoloradensis, Candida aglyptinia, Candida stri and Candida gatunensis. Subsequently, another novel species in the asexual clade, Candida cretensis, was described (Middelhoven & Kurtzman, 2007). Phylogenetic analyses using several methods with extended taxon sampling from a combined gene sequence of SSU and LSU rDNA consistently placed the C. kruisii clade to be the sister group of C. sake, although their relationship was not well-supported by bootstrap analysis (Suh et al., 2006).

During an extensive study of yeasts associated with rotting wood in central China ecosystems, two isolates of an asexual cellobiose-fermenting yeast species were obtained. Sequence analyses of SSU rDNA, ITS region and D1/D2 LSU rDNA indicated that these strains represent a novel species belonging to the C. kruisii clade, most species of which have been associated with basidioma-feeding beetles (Suh et al., 2006). In the present study, we describe the taxonomic characterisation of this novel species.

Two strains (NYNU 14623 and NYNU 14625T) were isolated from two rotten wood samples collected in Funiu Mountain. Yeast strains were isolated from decayed wood samples in accordance with the method described by Santos et al. (2011). Each sample (1 g) was added to 20 ml sterile cellobiose medium (0.67 % yeast nitrogen base, 0.5 % cellobiose and 0.02 % chloramphenicol) in a 150 ml Erlenmeyer flask and then incubated at 25 °C for 3 days on a rotary shaker. The enrichment culture was spread on cellobiose agar media. Each yeast morphotype was purified by using the conventional streaking technique on plates of yeast extract-malt extract (YM) agar (1 % glucose, 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 2 % agar) supplemented with 0.02 % chloramphenicol. Purified yeast strains were grown on YM agar at 25 °C for 3 days and then preserved at −70 °C and/or on YM agar at 4 °C.

The morphological observations and metabolic tests that constitute the standard yeast description were conducted as

†These authors contributed equally to this work.

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

The GenBank/EMBL/DOJB accession number for the SSU rDNA, ITS region and D1/D2 LSU rDNA sequence of strain NYNU14625T are KM201407, KM201406 and KM201405, respectively.
previously described (Barnett et al., 2000; Kurtzman et al., 2011). Assimilation tests for carbon and nitrogen sources were conducted in liquid media. Starved inocula were used in nitrogen and vitamin assimilation tests. Strains were examined for ascosporation on the following agar media incubated at 17 °C and 25 °C: YM, 1 % malt extract, 5 % malt extract, corn meal and yeast carbon base supplemented with 0.01 % ammonium sulphate (YCBS) agar (1.1 % yeast carbon base, 0.01 % ammonium sulphate and 1.8 % agar).

Genomic DNA was extracted by using a Dr GenTLE (from Yeast) High Recovery kit (Takara Bio) in accordance with the manufacturer’s protocol. The D1/D2 LSU rDNA and the ITS region were amplified by PCR and sequenced using primers NL1 and NL4 (Kurtzman & Robnett, 1998), and ITS1 and ITS4 (White et al., 1990), respectively. The nearly complete SSU rDNA sequence was determined in accordance with the method described by Kurtzman & Robnett (2003). PCR conditions recommended in the references for each primer pair were used. The DNA strands were sequenced and the reactions were conducted by using a Dye Terminator Cycle Sequencing kit (Applied Biosystems).

The sequences were compared pairwise by using BLAST search (Altschul et al., 1997) and were aligned with the sequences of related species retrieved from the GenBank database using the multiple alignment program CLUSTAL X version 1.81 (Thompson et al., 1997). A phylogenetic tree based on the combined sequences of SSU rDNA, ITS region and D1/D2 LSU rDNA was reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA 5.0 software package (Tamura et al., 2011). The evolutionary distance data were calculated from the two-parameter model of Kimura (1980) in the neighbour-joining analyses. All sites containing gaps in the alignment were excluded. Candida blankii NRRL Y-17068T was used as an outgroup. The confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985); only values greater than 50 % were recorded on the resulting tree. Reference sequences were retrieved from the GenBank database under the accession numbers indicated on the trees.

**Species delineation, classification and ecology**

Strains NYNU 14623 and NYNU 14625T had identical SSU rDNA, ITS region and D1/D2 LSU rDNA sequences, indicating their conspecificity. In the phylogenetic tree reconstructed from the combined sequences of SSU rDNA, ITS region and D1/D2 LSU rDNA, the new strains occupied a basal position with respect to *C. kruisii* and *C. cretensis*, suggesting that they represent a species that is distinct from the latter two species (Fig. 1). Stronger support for distinct species status comes from the degree of sequence divergence between the new isolates and their closest relatives. The two strains of the novel species differed from the type strains of *C. kruisii* and *C. cretensis* by 30 (5.7 %) and 36 (6.8 %) substitutions, respectively, in the D1/D2 LSU rDNA and by 40 (11.2 %) and 44 (11.3 %) substitutions, respectively, in the ITS region. The SSU rDNA sequences of the novel strains showed a 1.1 % nucleotide divergence (19 substitutions, 7 gaps) and a 1.3 % nucleotide divergence (23 substitutions, 6 gaps) from those of *C. kruisii* NRRL Y-17087T and *C. cretensis* NRRL Y-27777T, respectively. These results clearly indicated that isolates NYNU 14623 and NYNU 14625T represent a novel species closely related to *C. kruisii* and *C. cretensis*.

Cells of strains NYNU 14623 and NYNU 14625T were ovoid to ellipsoidal (Fig. 2a) and proliferated by multilateral budding. Pseudohyphae and true hyphae were present (Fig. 2b). However, ascospores were not produced by either of the strains individually or when paired on common sporulation medium for 4 weeks at 15 °C and 25 °C. Physiologically, the two novel strains gave the same reactions on standard growth tests. Strains NYNU 14623 and NYNU 14625T could be differentiated from their closest described species, *C. kruisii* and *C. cretensis*, by their ability to ferment methyl α-D-glucoside and grow at 35 °C and by their inability to ferment galactose and grow in galactose, sorbose, melezitose, xylitol, D-glucurate, 10 % NaCl plus 5 % glucose, and 0.01 % cycloheximide (Table 1). Analysis of the DNA sequences and phenotypic characteristics revealed that these strains represent a novel species in the *C. kruisii* clade, for which the name *Candida funiuensis* sp. nov. is proposed, with the type strain NYNU 14625T (=CICC 33050T = CBS 13911T).

At present, the clade *C. kruisii* consists of 11 recognized species. These species are associated with basidiomata substrates or basidioma-feeding beetles that occupy this ecological niche. Most species of the asexual clade, such as *C. pallodes*, *C. trifolae*, *C. panamensis*, *C. lycopterinae*, *C. atbi*, *C. burrocoloredensis*, *C. aglyptina*, *C. strii* and *C. gatunensis*, were often observed in the digestive tracts of beetles, primarily nitidulids, which had been feeding on agarics. Meanwhile, *C. kruisii* and *C. cretensis*, the two species closest to *Candida funiuensis* sp. nov., were isolated from mushroom and rotten polypore, respectively (Kocková-Kratchoviloá & Ondrušová, 1971; Suh et al., 2006; Middelhoven & Kurtzman, 2007). In the present study, the presence of novel species from rotting wood may be a consequence of yeasts being carried to rotten wood by visiting basidioma-feeding beetles. Therefore, rotten wood is a source for further investigation of yeasts in this clade. Given its capacity to ferment cellobiose efficiently, *Candida funiuensis* sp. nov. could provide a new source of genes, enzymes and/or sugar transporters to engineer industrial strains for the efficient production of bioethanol from renewable biomass (Galazka et al., 2010; Li et al., 2010).

**Description of Candida funiuensis**

*Description of Candida funiuensis* Hui, Wang, Ren, Zhang, Wu & Ke sp. nov.

*Candida funiuensis* (fu.niu, en’sis. N.L. fem. adj. funiuensis of or belonging to Funiu Mountain, Henan Province, central China, where the two strains were isolated).
In YM broth after 3 days at 25 °C, cells are ovoid to ellipsoidal, variable in size (2–6 x 2.5–6.5 μm) and occur singly or in pairs. Budding is multilateral (Fig. 2a). After 1 month at 25 °C, pellicle and sediment formation occurs. On YM agar after 6 days at room temperature, colonies are flat with a raised centre, white and smooth with an entire edge. After 2 weeks in Dalmau plate culture on 5 % malt extract agar at 25 °C, pseudohyphae and septated hyphae with blastoconidia are present (Fig. 2b). Aerobic growth is white, shiny and smooth with filamentous margin. No asci or signs of conjugation appear after growth on the most common sporulation media, alone or mixed in pairs, at 17 °C and 25 °C for 4 weeks. Glucose, methyl α-D-glucoside (weak), trehalose and cellobiose are fermented, but not galactose, maltose, sucrose, melibiose, lactose, melezitose, raffinose, inulin or xylene. Carbon compounds, such as glucose, D-glucosamine, D-xylene, sucrose, maltose, trehalose, methyl α-D-glucoside, cellobiose, salicin, arbutin, glycerol, ribitol, glucitol, mannitol, 2-keto-D-gluconate, succinate, citrate and ethanol, are assimilated. No growth occurs in galactose, D-sorbose, D-ribose, L-arabinose, D-arabinose, L-rhamnose, melibiose, lactose, raffinose, melezitose, inulin, soluble starch, erythritol, xylitol, L-arabinobiose, galactosil, myo-inositol, 5-keto-D-gluconate, D-gluconurate, D-galacturonate, D-lactate and methanol. For the assimilation of nitrogen compounds, nitrate is weak; ethylamine, L-lysine, cadaverine and D-tryptophan are positive; and nitrite, creatine, creatinine, glucosamine and imidazole are negative. Growth is observed at 35 °C but not at 37 °C. Growth is not observed in the presence of 10 % NaCl plus 5 % glucose, 0.01 % cycloheximide or 1 % acetic acid. Starch-like compounds are not produced. Urease activity and Diazonium Blue B reactions are negative.

The type strain NYNU 14625^T was isolated from rotten wood collected in Funiu Mountain Nature Reserve in Henan Province, central China. The living culture from the
Table 1. Physiological characteristics differentiating Candida funiuensis sp. nov. from closely related species

<table>
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<th>Characteristic</th>
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<td>Fermentation of:</td>
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<td>Galactose</td>
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<td>Methyl-x-D-glucoside</td>
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<td>Assimilation of:</td>
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<td>Galactose</td>
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<td>Sorbose</td>
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<td>D-Arabinose</td>
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<td>Melezitose</td>
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<td>Xylitol</td>
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<td>D-Gluconate</td>
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<td>DL-Lactate</td>
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<td>Growth at/with:</td>
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<td>35 °C</td>
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<td>0.01% Cycloheximide</td>
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<td>10% NaCl/5%glucose</td>
<td>–</td>
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Species: 1, Candida funiuensis sp. nov. (data from this study); 2, C. kruisii (Suh et al., 2006); 3, C. cretensis (Middelhoven & Kurtzman, 2007). +, Positive; –, negative; D, delayed; W, weak.

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**References**


