Candida laoshanensis sp. nov. and *Candida qingdaonensis* sp. nov., anamorphic, ascomycetous yeast species isolated from decayed wood

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During a study of newly isolated yeast strains utilizing D-xylose as sole carbon source, eight strains, isolated from decayed wood, were found to represent two novel anamorphic, ascomycetous yeast species based on sequence analysis of the 26S rDNA D1/D2 domain and internal transcribed spacer region, and phenotypic characterization. The names *Candida laoshanensis* sp. nov. (type strain MLRW 6-2^T=AS 2.4030^T=CBS 11389^T) and *Candida qingdaonensis* sp. nov. (type strain MLRW 7-1^T=AS 2.4031^T=CBS 11390^T) are proposed for these two novel species; the closest relatives of the two novel species are *Candida pomicola* and *Candida marilandica*, respectively.

D-Xylose constitutes 10–40% of the total carbohydrate of lignocellulose (Ladisch *et al.*, 1983) and, therefore, complete utilization of the sugar is important economically for bioethanol production by yeasts. However, only a limited number of yeast species have been found that convert D-xylose to ethanol (Barnett *et al.*, 2000; Hahn-Hägerdal *et al.*, 2007). Nevertheless, many novel yeast species, including xylose-fermenting taxa, have been discovered in beetle gut in recent years and it is believed that nature might reveal many more yeasts with unusual capacities for xylose, xylan and lignin degradation (Suh *et al.*, 2003, 2004, 2005). As part of studies aimed at exploring xylose-fermenting yeasts from nature, yeasts were isolated from the gut of insects, decayed wood and leaves of plants in Lao Mountain, Shandong Province, East China, in Autumn 2008. Strains able to utilize D-xylose as the sole carbon source were first enriched and isolated; fermentation tests were then performed. Among the strains able to grow on D-xylose but not ferment it, eight were found to represent two novel species based on sequence analysis of the 26S rDNA D1/D2 domain and internal transcribed spacer (ITS) region. Complete descriptions of the two species are given in this report.

Yeast strains inhabiting the decayed wood were isolated by using an enrichment method. Pieces of decayed wood were

placed into yeast minimal medium (YMM) broth containing yeast nitrogen base without amino acids (6.7 g l⁻¹; Difco), 2% (w/v) D-xylose, 0.5% (w/v) (NH₄)₂SO₄ and 200 µg chloramphenicol ml⁻¹. After cultivation for 7–14 days, aliquots (100 µl) of 10⁻¹–10⁻⁴ diluted cultures were spread on YMM agar plates. After incubation for 2 days at room temperature (about 25 °C), colonies with different morphological characters were transferred to YPX slants, which contained 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 2% (w/v) D-xylose and 2% (w/v) agar, for further purification and examination.

Morphological, physiological and biochemical characteristics were examined according to the standard methods commonly used in yeast taxonomy (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase & Suzuki, 1986).

Nuclear DNA was extracted by the method of Makimura *et al.* (1994). The large-subunit rDNA D1/D2 domain was amplified by using primer pairs NL1 (5'-GCATATCGG-TAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGT-TTCAAGACGG-3'). The DNA fragment covering the ITS region (including 5.8S rDNA) was amplified by using primers ITS1 (5'-GTCGTAACAAGGTTTCCGTAGGTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification conditions for the two DNA domains were: 4 min initial denaturation at 94 °C, 30 cycles each of 30 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C, and a final extension period of 8 min at 72 °C. Molecular phylogenetic analysis was performed by the methods described by Bai *et al.* (2002). Reference sequences were retrieved from GenBank (accession numbers are indicated in Fig. 1).

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS region and 26S rDNA D1/D2 domain sequences of strain MLRW 6-2^T are FJ613523 and FJ613526, respectively, and those for strain MLRW 7-1^T are FJ613524 and FJ613527, respectively.

Fig. 1. Phylogenetic tree drawn from neighbour-joining analysis of the 26S rDNA D1/D2 domain, depicting the relationships of the two novel species with closely related taxa. Bootstrap values (%) over 50 % from 1000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated.

Two groups of four strains (MLRW 6-1, MLRW 6-2^T, MLRW 6-3 and MLRW 6-4; and MLRW 7-1^T, MLRW 7-2, MLRW 7-3 and MLRW 7-4) were recognized. Members of each group had identical D1/D2 domain and ITS sequences, but characteristics of the two groups differed from one another indicating that the strains in each of the two groups were conspecific. MLRW 6-2^T and MLRW 7-1^T were selected as representatives of the two groups for further comparison. The sizes of the D1/D2 domains of strains MLRW 6-2^T and MLRW 7-1^T were determined as 532 and 530 bp, respectively. The phylogenetic positions of the two strains are depicted in the tree constructed from D1/D2 sequences (Fig. 1). Strain MLRW 6-2^T differed from the type strains of the closely related species *Candida pomicola* and *Candida peltata* by 15 (2.8 %) and 22 (4.1 %) substitutions in the D1/D2 domain, respectively. Strain MLRW 7-1^T was closely related to the type strain of *Candida marilandica*; these two strains differed by 6 (1.1 %) substitutions in the D1/D2 domain. MLRW 7-1^T differed from the type strains of its close relatives *Sugiyamaella chiloensis* and *Sugiyamaella smithiae* by more than 30 substitutions (>5 %) in the D1/D2 domain. According to previous studies, yeast strains that show nucleotide substitutions greater than 1 % in the D1/D2 domain are likely to represent different species (Fell *et al.*, 2000; Kurtzman & Robnett, 1998).

The distinction of the two species from their close relatives was further supported by ITS sequence comparison. Strain MLRW 6-2^T differed from the type strains of both *C. pomicola* and *C. peltata* by more than 15 % mismatches in the ITS region. Although strain MLRW 7-1^T and the type strain of *C. marilandica* differed by only 1.1 % in the D1/D2 domain, they showed 10.6 % mismatches (49 substitutions and 10 gaps) in the ITS region; all other related strains differed from MLRW 7-1^T by more than 15 % mismatches in the ITS region.

Strains MLRW 6-1 and MLRW 6-2^T and strains MLRW 6-3 and MLRW 6-4 were isolated from two different pieces of an unidentified decayed wood block, respectively. Strains MLRW 7-1^T and MLRW 7-2 and strains MLRW 7-3 and MLRW 7-4 were isolated from two different pieces of a decayed wood block of *Pinus tabulaeformis* Carr., respectively. Within each group, all four strains exhibited similar morphological and physiological characters. In addition to sequence data, phenotypic characters could also be used to differentiate them from closely related taxa, especially in assimilation reactions of carbon compounds (Table 1). For clearer comparisons of close relatives, the species compared were classified into two groups based on their phylogenetic relationships (Fig. 1). Sporulation tests were done in all eight strains, but sexual states were not observed on the sporulation media of Wang *et al.* (2008). Also, mating

Table 1. Physiological characteristics that differentiate the two novel species from closely related taxa

Species: 1, *C. laoshanensis* sp. nov. (this study); 2, *C. pomicola* (Kurtzman *et al.*, 2001); 3, *C. peltata* (Barnett *et al.*, 2000); 4, *C. qingdaonensis* sp. nov. (this study); 5, *C. marilandica* (Kurtzman, 2007). Abbreviations: +, positive; –, negative; D, delayed positive; w, weak; s, slow; v, variable; and NT, not tested. Results that differ between strains of the same species are shown (separated by solidi).

Characteristic	Group I			Group II	
	1	2	3	4	5
Fermentation of:					
Glucose	+	+	+	w	–
Galactose	+	–	D	–	–
Assimilation of:					
L-Sorbose	–	v	+	+	+
Melezitose	D	+	+	+	+
D-Ribose	+	+	+	–/+	+
L-Rhamnose	+	+	+	D	–
D-Glucosamine	+/D	+/D	D/–	+	+
Ethanol	+	+	+/–	–/+	+
Erythritol	–	+/w	+/D	+	+
Galactitol	w/–	–	+/D	D/+	–
Methyl α -D-glucoside	D	+/D	+	+	+
DL-Lactic acid	+	–	+/–	–	–
Succinic acid	+/D	+	+	+/w/s	–
Nitrate	–	+	–	+	+
Nitrite	–	+	–	+	NT

types were not found in mating tests between the isolates in each group.

The molecular and phenotypic data showed that strains MLRW 6-2^T and MLRW 7-1^T represent two novel anamorphic, ascomycetous yeast species. According to the current taxonomy of ascomycetous yeasts (Kurtzman, 1998; Meyer *et al.*, 1998), the two species could only be assigned to the anamorphic genus *Candida*. The names *Candida laoshanensis* sp. nov. (type strain MLRW 6-2^T) and *Candida qingdaonensis* sp. nov. (type strain MLRW 7-1^T) are proposed.

In a previous study, more than 70 % of the 670 described yeast species were capable of utilizing D-xylose, but only approximately 1 % of them were able to ferment the sugar (Barnett *et al.*, 2000; Hahn-Hägerdal *et al.*, 2007). Among the xylose-fermenting yeast species, *Pichia stipitis*, *Pichia segobiensis*, *Candida shehatae* and *Enteroramus dimorphus* clustered in a well-supported clade in the phylogenetic tree constructed from combined LSU and SSU rDNA sequence data, whereas the other five species were located in different clades (Suh *et al.*, 2003, 2004). In our present study, no yeast strains capable of fermenting D-xylose were found among 120 newly isolated xylose-utilizing strains and *Pachysolen tannophilus* is the only xylose-fermenting species among the taxa in the presented phylogenetic tree

(Fig. 1). This implies the rarity of xylose-fermenting yeast species as indicated in previous publications.

Latin diagnosis of *Candida laoshanensis* Li et Wang sp. nov.

In medio liquido YM post 3 dies ad 25 °C, cellulae ellipsoideae, 2.0–5.0 × 2.5–6.5 µm, cellulae singulae, binae et adhaerentes. Per gemmationem multipolarem reproducentes. Post 1 mensem sedimentum formatur. In agarō YM post 1 mensem ad 25 °C, candida vel crenea, glabra. In agarō farinae Zea mays post 7 dies, pseudohyphae eumorphum. Ascomata nulla. Glucosum et galactosum fermentantur at non sucrosum, maltosum, raffinose, lactosum nec D-xylosum. Glucosum, galactosum, sucrosum, maltosum, cellobiosum, trehalosum, melezitosum (lente), amylum solubile, D-xylosum, L-arabinosum (varium), D-arabinosum (varium), D-ribosum, L-rhamnosum, D-glucosaminum (varium), ethanolum, glycerolum, ribitolum, galactitolum (varium), D-mannitolum, D-glucitolum, methyl α -D-glucosidum (lente), salicinum, DL-lacticum, acidum succinicum (varium) et acidum citricum assimilantur at non L-sorbose, lactosum, melibiosum, raffinose, inulinum, methanolum, erythritolum nec inositolum. Ammonium sulfatum, ethylaminum, cadaverinum et L-lysinum assimilantur at non kalium nitricum nec natrum nitrosum. Ad crescentiam vitaminum non necessarium est. Augmentum in 37 °C, at non 40 °C. Materia amyloidea iodophila non formantur. Diazonium caeruleum B non respondens. Ureum non hydrolysat. Typus depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (AS 2.4030^T).

Description of *Candida laoshanensis* Li & Wang sp. nov.

Candida laoshanensis (lao.shan.en'sis. N.L. fem. adj. *laoshanensis* of Lao mountain, referring to the geographical origin of the type strain).

In YM broth, after 3 days at 25 °C, cells are ellipsoidal, 2.0–5.0 × 2.5–6.5 µm, and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. After 1 month at 25 °C, sediment is present. After 1 month on YM agar at 25 °C, the streak culture is white to cream and smooth with an entire margin. Pseudohyphae are not formed in Dalmau plate cultures on corn-meal agar after 7 days at 25 °C. Ascospores are not formed. Glucose and galactose are fermented; sucrose, maltose, raffinose, lactose and xylose are not fermented. Glucose, galactose, sucrose, maltose, cellobiose, trehalose, melezitose (delayed), soluble starch, D-xylose, L-arabinose (variable), D-arabinose (variable), D-ribose, L-rhamnose, D-glucosamine (variable), ethanol, glycerol, ribitol, galactitol (variable), D-mannitol, D-glucitol, methyl α -D-glucoside (delayed), salicin, DL-lactic acid, succinic acid (variable) and citric acid are assimilated; L-sorbose, lactose, melibiose, raffinose, inulin, methanol, erythritol and inositol are not assimilated. Ammonium sulfate, ethylamine hydrochloride, cadaverine hydrochloride

Fig. 2. Budding cells of *Candida laoshanensis* sp. nov. MLRW 6-2^T (a) and *Candida qingdaonensis* sp. nov. MLRW 7-1^T (b) grown in YM broth for 2 days at 25 °C. Bars, 10 µm.

and L-lysine are assimilated; potassium nitrate and sodium nitrite are not assimilated. Growth in vitamin-free medium is negative. Growth at 37 °C is positive; growth at 40 °C is negative. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative.

The type strain is MLRW 6-2^T (=AS 2.4030^T=CBS 11389^T), isolated from a piece of decayed wood from Lao Mountain in Shandong Province in the east of China in September 2008.

Latin diagnosis of *Candida qingdaonensis* Li et Wang sp. nov.

In medio liquido YM post 3 dies ad 25 °C, cellulae ellipsoideae vel elongatae, 2.0–4.0 × 3.5–10.0 µm, cellulae singulae, binae et adhaerentes. Per gemmationem multipolarem reproducentes. Post 1 mensem sedimentum formatur. In agarō YM post 1 mensem ad 25 °C, candida vel crenea, glabra, margo glabro. In agarō farinae Zea mays post 7 dies, pseudohyphae et hyphae eumorphum. Ascomata nulla. Glucosum (infirme) fermentantur at non galactosum, sucrosum, maltosum, raffinolum, lactosum nec D-xylosum. Glucosum, galactosum, L-sorbozum, sucrosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinolum, melezitolum, inulinum (varium), D-xylosum, L-arabinosum, D-arabinosum, D-ribosum (varium), L-rhamnosum (lente), D-glucosaminum, ethanolum (varium), erythritolum, ribitolum, galactitolum (lente), D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum succinicum (varium) et inositolum assimilantur at non lactosum, amyllum solubile, methanolum, glycerolum, DL-lacticum nec acidum citricum. Ammonium sulfatum, ethylaminum, cadaverinum, L-lysinum, kalium nitricum et natrum nitrosum assimilantur. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 37 °C, at non 40 °C. Materia amyloidea iodophila non formantur. Diazonium caeruleum B non respondens. Ureum non hydrolysatur. Typus

depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (AS 2.4031^T).

Description of *Candida qingdaonensis* Li & Wang sp. nov.

Candida qingdaonensis (qing.dao.nen'sis. N.L. fem. adj. *qingdaonensis* of Qingdao, referring to the geographical origin of the type strain).

In YM broth, after 3 days at 25 °C, cells are ellipsoidal to elongate, 2.0–4.0 × 3.5–10.0 µm, and occur singly, in pairs or in groups (Fig. 2b). Budding is multilateral. After 1 month at 25 °C, sediment is present. After 1 month on YM agar at 25 °C, the streak culture is white to cream, smooth and somewhat dull, with an entire margin. Neither pseudohyphae nor true hyphae are formed in Dalmau plate cultures on corn-meal agar after 7 days at 25 °C. Ascospores are not formed. Glucose (weak and slow) is fermented; galactose, sucrose, maltose, raffinose, lactose and xylose are not fermented. Glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, inulin (variable), D-xylose, L-arabinose, D-arabinose, D-ribose (variable), L-rhamnose (delayed), D-glucosamine, ethanol (variable), erythritol, ribitol, galactitol (delayed), D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, succinic acid (variable) and inositol are assimilated; lactose, soluble starch, methanol, glycerol, DL-lactic acid and citric acid are not assimilated. Ammonium sulfate, ethylamine hydrochloride, cadaverine hydrochloride, L-lysine, potassium nitrate and sodium nitrite are assimilated. Growth in vitamin-free medium is positive. Growth at 37 °C is positive; growth at 40 °C is negative. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative.

The type strain is MLRW 7-1^T (=AS 2.4031^T=CBS 11390^T), isolated from a piece of a decayed wood block of *Pinus tabulaeformis* Carr. from Lao Mountain in Shandong Province in the east of China in September 2008.

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