Candida baotianmanensis sp. nov. and Candida pseudoviswanathii sp. nov., two ascosporic yeast species isolated from the gut of beetles

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Four yeast strains were isolated from the gut of beetles collected on Baotianman Mountain and People’s Park of Nanyang in Henan Province, China. These strains produced un conjugated asci with one or two ellipsoidal to elongate ascospores in a persistent ascus. Phylogenetic analysis of the D1/D2 domains of the LSU rRNA gene sequences indicated that the isolates represent two novel sexual species in the Candida/Lodderomyces clade. Candida baotianmanensis sp. nov. was located in a statistically well-supported branch together with Candida maltosa. Candida pseudoviswanathii sp. nov. formed a subclade with its closest relative Candida viswanathii supported by a strong bootstrap value. The two novel species were distinguished from their most closely related described species, Candida maltosa and Candida viswanathii, in the D1/D2 LSU rRNA gene and internal transcribed spacer (ITS) sequences and in phenotypic traits. The type strain of Candida baotianmanensis sp. nov. is NYNU 14719T (=CBS 13915T=CICC 33052T), and the type strain of Candida pseudoviswanathii sp. nov. is NYNU 14772T (=CBS 13916T=CICC 33053T). The MycoBank numbers for Candida baotianmanensis sp. nov. and Candida pseudoviswanathii sp. nov. are MB 812621 and MB 812622.

The Candida/Lodderomyces clade is a monophyletic group closely related to the Spathaspora and Scheffersomyces clades on the basis of gene sequences from the D1/D2 domains of the LSU and the nearly complete SSU rRNA (Kurtzman & Suzuki, 2010; Lachance et al., 2011; Urbina et al., 2013). The number of species belonging to the Candida/Lodderomyces clade has increased rapidly in recent years (Lachance et al., 2011; Nitiyon et al., 2011; Chang et al., 2012; Hui et al., 2013). At the time of description, the Candida/Lodderomyces clade consists of one sexual species, Lodderomyces elongisporus and 30 anamorphic species assigned to genus Candida (Daniel et al., 2014; Kurtzman, 2011; Lachance et al., 2011). Although members of the clade have been isolated from various sources, many of them such as Candida chauliodes, Candida corydali, Candida maltosa, Candida parapsilosis and Candida tropicalis were obtained from plant-associated insects (Li et al., 2009; Lachance et al., 2011; Nguyen et al., 2007; Suh et al., 2008). Recently, several undescribed species in the Candida/Lodderomyces clade, that are closely related to C. tropicalis and C. parapsilosis, were found in the gut of Guatemalan passalids (Urbina et al., 2013). These findings suggested that members of the clade are common in the gut of lignicolous insects, especially beetles.

In a study on species diversity of yeasts associated with insects in Central China’s natural ecosystems, four yeast strains clustered in the Candida/Lodderomyces clade, that are closely related to Spathaspora tropicalis and Spathaspora parapsilosis, were found in the gut of Guatemalan passalids (Urbina et al., 2013). These findings suggested that members of the clade are common in the gut of lignicolous insects, especially beetles.

The yeast strains studied are listed in Table 1. The beetles were collected on Baotianman Mountain (33° 27’ N 111° 48’ E) and People’s Park of Nanyang (32° 58’ N 112° 29’ E) in Henan Province, Central China, in July and September 2014. The two collection localities were separated from one another by a distance of 150.6 km. The methods used to isolate the yeasts from the gut of insects have been described previously (Nguyen et al., 2007; Suh et al., 2008). The insects were usually placed in Petri dishes for 1–3 days without food prior to dissection. Withholding food helps to eliminate some contaminating yeasts.
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 yeast extract–malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose, 2 % plain agar, adjusted to pH 3.5 with HCl) plates and then incubated at 25 °C for 3–4 days. The different yeast morphotypes were purified at least twice and then stored on YM agar slants at 4 °C and in 15 % glycerol at –80 °C.

The morphological observations and metabolic tests that constitute the standard yeast description were performed according to established methods (Kurtzman et al., 2011). All assimilation tests were performed twice in liquid media, and the results were read after 5 and 21 days of incubation. Starved inocula were used in the nitrogen assimilation tests. Sporulation tests were performed on YM agar, 5 % malt extract agar, corn meal agar and yeast carbon base supplemented with 0.01 % ammonium sulphate (YCBSAS) agar (1.1 % yeast carbon base, 0.01 % ammonium sulphate and 1.8 % agar) in pure and mixed cultures at 25 °C for 4 weeks.

Genomic DNA was extracted using an Ezup Column Yeast Genomic DNA Purification kit according to the manufacturer’s protocol (Sangon Biotech). The D1/D2 domains of the LSU rRNA gene and ITS regions were amplified by PCR and then sequenced using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (White et al., 1990), respectively. Both DNA strands were sequenced, and the reactions were carried out using a Dye Terminator cycle sequencing kit (Applied Biosystems).

The sequences were compared pairwise using BLAST search (Altschul et al., 1997) and aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL X version 1.81 (Thompson et al., 1997). Phylogenetic trees based on D1/D2 LSU rRNA gene sequences were reconstructed using MEGA software version 5.0 (Tamura et al., 2011). The evolutionary distance data were calculated using Kimura’s two-parameter model (Kimura, 1980) in the neighbour-joining analyses. The close-neighbour-interchange method with the maximum composite likelihood model was used in the minimum-evolution analyses. Saccharomyces cerevisiae NRRL Y-12632T was used as the outgroup. Confidence limits were estimated from bootstrap analysis (1000 replicates) (Felsenstein 1985), and only values above 50 % were recorded on the resulting trees. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

### Sequence comparison and species delineation

Isolation of the gut contents of a single beetle usually produced approximately 50 yeast colonies on a YM agar plate. By comparison of D1/D2 LSU rRNA gene sequences for all isolates for rapid identification, 163 isolates present in the samples were identified as representing 12 known species, wherein Candida chauiodes, Candida maltosa, Candida melibiosica, Candida palmioleophila, Candida parapsilosis, Candida tropicalis, Cryptococcus magnus, Debaryomyces carsonii, Lachancea thermotolerans, Pichia scutulata, Sporobolomyces beijingerensis and Wickerhamomyces anomalus. Four of the yeast isolates, including NYNU 14719T, NYNU 14924, NYNU 14772T and NYNU 14926, were distinct from any previously described species and were selected for further analyses on the basis of their D1/D2 LSU rRNA gene and ITS sequences.

Molecular phylogenetic analyses of D1/D2 LSU rRNA gene sequences placed the four novel strains in the Candidal Lodderomyces clade (Fig. 1). Strains NYNU 14719T and NYNU 14924, which had identical nucleotide sequences in both the D1/D2 domain and ITS region, were located in a statistically well-supported branch together with Candida maltosa NRRL Y-17677T. These strains differed from the type strain of Candida maltosa by 1.7 % sequence divergence (9 substitutions and one gap) in the D1/D2 LSU rRNA gene and 9 % sequence divergence (18 substitutions and 21 gaps) in the ITS region. A phylogenetic tree based on D1/D2 LSU rRNA gene sequences was also reconstructed by the minimum-evolution method. No differences were detected between the neighbour-joining and minimum-evolution methods, particularly regarding the positions of strains NYNU 14719T and NYNU 14924.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Location</th>
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<tbody>
<tr>
<td><strong>C. baotianmanensis</strong></td>
<td>NYNU 14719T (CICC 33052T=CBS 13915T)</td>
<td>Baotianman Mountain, Nanyang, Henan, China</td>
</tr>
<tr>
<td></td>
<td>NYNU 14924</td>
<td>Baotianman Mountain, Nanyang, Henan, China</td>
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<tr>
<td><strong>C. pseudoviswanathii</strong></td>
<td>NYNU 14772T (CICC 33053T=CBS 13916T)</td>
<td>People’s Park, Nanyang, Henan, China</td>
</tr>
<tr>
<td></td>
<td>NYNU 14926</td>
<td>Baotianman Mountain, Nanyang, Henan, China</td>
</tr>
</tbody>
</table>

CBS, Centraalbureau voor Schimmelcultures; CICC, China Centre of Industrial Culture Collection, Beijing, China; NYNU, Microbiology lab, Nanyang Normal University, Nanyang, Henan, China.

**Fig. 1.** Neighbour-joining tree based on D1/D2 LSU rRNA gene sequences showing the placements of Candida baotianmanensis sp. nov. and Candida pseudoviswanathii sp. nov. among related species in the Lodderomyces clade. Saccharomyces cerevisiae NRRL Y-12632^T was used as an outgroup. Bootstrap values of above 50 % based on 1000 replications are given at nodes. Bar, 2 % sequence difference.
strains of Candida viswanathii (and its synonym Candida aquaetextoris) in the GenBank database by 3–5 substitutions in the D1/D2 LSU rRNA gene. The ITS regions of strains NYNU 14772T and NYNU 14926 were sequenced and compared further. In this region, the sequences of strains NYNU 14772T and NYNU 14926 were also identical and differed from those of the latter strains, which are their closest relatives, by approximately 5% sequence divergence (15–16 substitutions and 7–8 gaps). The phylogenetic relationships of the novel strains with described species in the subclade were also supported by minimum-evolution analyses (Fig. S1).

Clear physiological differences were found between the two novel species and the closely related taxa Candida maltosa and Candida viswanathii. Specifically, strains NYNU 14719T and NYNU 14924 could be differentiated from their close relative Candida maltosa by their ability to assimilate ribose, inulin, soluble starch and galactitol, as well as by their inability to ferment sucrose and grow at 37 °C. Strains NYNU 14772T and NYNU 14926 could be differentiated from their phylogenetic neighbour Candida viswanathii by their positive assimilation reactions for L-sorbose, D-ribose, D-arabinose and inulin (Table 2).

The morphological characteristics of the two novel species fitted well the description of the genus Lodderomyces. Cells of the four strains were ovoid to elongate, proliferated by multilateral budding (Fig. 2a, d) and formed pseudohyphae but not hyphae (Fig. 2b, e). In common sporulation media, including YM agar, 5% malt extract agar, corn meal agar and YCBS agar, all of the four strains formed one or two ellipsoidal to elongate ascospores in a persistent ascus (Kurtzman, 2011) (Figs. 2c, f), even though these isolates had spent 6 months in storage at 4 °C before the sporulation tests were set up.

Phylogeny and phenotypic comparisons made above indicated that the four strains from the gut of beetles represent two novel sexual species that are distinct from Candida maltosa and Candida viswanathii, respectively. Therefore, the four strains should be classified as representatives of two novel species of the genus Candida but not the genus Lodderomyces, named Candida baotianmanensis sp. nov. and Candida pseudoviswanathii sp. nov., because the same clade includes the type species of the genus Candida, a name that has priority over Lodderomyces according to the International Code of Nomenclature (Daniel et al., 2014; Hawksworth, 2011).

The beetles examined were collected in summer from their natural habitats located in Nanyang, belonging to a warm, temperate zone, with monsoon-influenced, semi-humid, continental climate in Central China. The adults of these beetles generally feed on leaves, flowers, tree saps and ripe fruits, and their larvae eat roots or rotting plant materials. Candida baotianmanensis sp. nov. was isolated from the gut of two different insect species collected on one mountain. Strain NYNU 14719T was found from a sample of Nitidula carnaria and the other strain, NYNU 14924, from one Polyphaga planciyi beetle (Table 1). Candida pseudoviswanathii sp. nov. was found in two individuals of insects collected at two different localities. Strain NYNU 14772T was directly recovered from the gut of Dorcus curvidens, while the additional strain NYNU 14926 was obtained from the gut of beetle larvae. Meanwhile, the body surfaces of the beetles collected were also isolated and identified. Unfortunately, none of the strains belonging to the novel species was found. These results suggested that the two novel species may occur in beetle guts and similar substrates.

Table 2. Physiological characteristics differentiating the two novel Candida species from closely related taxa

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Fermentation of sucrose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>v</td>
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<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
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<tr>
<td>L-Sorbose</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Ribose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
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<td>D-Arabinose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Inulin</td>
<td>+</td>
<td>–</td>
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<td>+</td>
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<td>Soluble starch</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Galactitol</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Growth at 37 °C</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Species: 1, Candida baotianmanensis sp. nov.; 2, Candida maltosa (data from Lachance et al., 2011); 3, Candida pseudoviswanathii sp. nov.; 4, Candida viswanathii (Lachance et al., 2011). +, Positive; –, negative; v, variable; w, weakly positive.

Description of Candida baotianmanensis Hui, Ren, Xu & Zhang sp. nov.

Candida baotianmanensis (bao.tian.man.esis. N.L. fem. adj. baotianmanensis of or belonging to Baotianman Mountain, where the type strain of the species was isolated).

In YM broth after 3 days at 25 °C, cells are ovoid (3–8.5 × 3–9 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. Sediment is formed after 1 month, but a pellicle is not observed. On YM agar after 7 days at 25 °C, colonies are white, butyrous and shiny with an entire margin. In Dalmau plate culture on corn meal agar after 12 days at 25 °C, hyphae are not produced, but pseudohyphae are present (Fig. 2b). Sporulation occurs on YM, 5% malt extract, corn meal and YCBS agars at 25 °C after 8 days. Unconjugated asc are formed from single cells with one or two ellipsoidal to elongate ascospores (Fig. 2c). Asc are persistent. Glucose, galactose and trehalose are fermented; sucrose, maltose, lactose and raffinose are not fermented. Assimilation of carbon compounds: glucose, inulin, sucrose, galactose, trehalose, melezitose, methyl α-D-glucoside (weak), soluble starch, L-sorbose, D-xylose, L-arabininitol, D-ribose, ethanol, glycerol, ribitol, galactitol (weak), D-mannitol, D-glucitol, succinate, citrate, D-gluconate, D-glucosamine, xylitol, 2-keto-D-gluconate and 5-keto-D-gluconate. No growth occurs with raffinose, melezitose, lactose, maltose, cellobiose, salicin, L-rhamnose, L-arabinose, D-arabinose,
methanol, erythritol, myo-inositol, DL-lactate, D-glucuronate or arbutin. Assimilation of nitrogen compounds: positive for ethylamine, L-lysine, cadaverine and D-tryptophan, and negative for nitrate, nitrite, creatine, creatinine, glucosamine and imidazole. Maximum temperature for growth is 35 °C. Growth in the presence of 0.1 % cycloheximide is positive. Growth in 10 % NaCl plus 5 % glucose medium and in the presence of 1 % acetic acid is negative. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative.

The type strain, NYNU 14719T, was isolated from the gut of Nitidula carnaria collected from the Baotianman Mountain in Nanyang, Henan Province, China. The living culture from the type strain is preserved by lyophilization as strain CBS 13915T in the Yeast Division of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands and as strain CICC 33052T in the China Centre of Industrial Culture Collection, Beijing, China. The MycoBank number of the type strain is MB 812621.

**Description of Candida pseudoviswanathii Hui, Ren, Xu & Zhang sp. nov.**

*Candida pseudoviswanathii* [pseu.do.vis.wa.na’thi.i. Gr. adj. *pseudes* false; N.L. gen. n. *viswanathii* a specific epithet; N.L. gen. n. *pseudoviswanathii* the false *viswanathii* (the epithet is chosen because of the close relationship of the species to *Candida viswanathii*)].

In YM broth after 3 days at 25 °C, cells are ovoid to elongate (2–7 × 3–8 μm) and occur singly or in pairs (Fig. 2d). Budding is multilateral. Sediment is formed after a month, but a pellicle is not observed. On YM agar after 7 days at 25 °C, colonies are white, butyrous and shiny with an entire margin. In Dalmau plate culture on corn meal agar after 12 days at 25 °C, hyphae are not
produced, but pseudohyphae are present (Fig. 2e). Sporulation occurs on YM, 5 % malt extract, corn meal and YCBAS agars at 25 °C after 7 days. Unconjugated asci are formed from single cells with one or two ellipsoidal to elongate ascospores (Fig. 2f). Asci are persistent. Glucose, galactose, maltose (weak) and trehalose (weak) are fermented; sucrose, lactose and raffinose are not fermented. Assimilation of carbon compounds: glucose, inulin, sucrose, galactose, trehalose, maltose, melezitose, methyl D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, D-xyllose, D-arabinose, D-ribose, ethanol, glycerol, ribitol, D-mannitol, D-glucitol, succinate, citrate, D-gluconate, D-glucosamine, 2-keto-D-gluconate, 5-keto-D-gluconate and arbutin. No growth occurs on raffinose, melezitose, lactose, L-rhamnose, L-arabinose, methanol, erythritol, galactitol, myo-inositol, D1-lactate, xylitol, D-glucuronate and L-arabinitol. Assimilation of nitrogen compounds: positive for ethylamine, L-lysin and cadaverine, and negative for nitrate, nitrite, creatine, creatinine, glucosamine, imidazole and D-tryptophan. Maximum temperature for growth is 37 °C. Growth in 16 % NaCl plus 5 % glucose medium and in the presence of 0.1 % cycloheximide is positive. Growth in the presence of 1 % acetic acid is negative. Starch-like compounds are not produced. Dianzium blue B colour and urease reactions are negative.

The type strain, NYNU 14772T, was isolated from the gut of Dorcus curvidens, a beetle larva collected from the People’s Park of Nanyang in Henan Province, China. The living culture from the type strain is preserved by lyophilization as strain CBS 13916T in the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands and as strain CICC 33053T in the China Centre of Industrial Culture Collection, Beijing, China. The MycoBank number of the type strain is MB 812622.

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References


