Spathaspora allomyrinae sp. nov., a D-xylose-fermenting yeast species isolated from a scarabeid beetle Allomyrina dichotoma

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During an investigation of yeasts associated with insects, three strains of a D-xylose-fermenting yeast species were isolated from the gut of the host beetles Allomyrina dichotoma (Coleoptera: Scarabaeidae) collected on the Baotianman National Nature Reserve, Nanyan, Henan Province, China. These strains formed two elongated ascospores, which were tapered and curved at the ends in persistent asci. Sequence analyses of the D1/D2 domains of the large subunit (LSU) and small subunit (SSU) rRNA genes showed that these new strains represent a phylogenetically distinct species in the Spathaspora clade. This novel species differed from the closest species, Candida lyxosophila NRRL Y-17539\(^{\text{T}}\), by a 6.7 % sequence divergence (31 substitutions and 7 gaps) in the D1/D2 LSU rRNA gene and a 1.2 % divergence (17 substitutions, 4 gaps) in the SSU rRNA gene. The novel species can also be distinguished from C. lyxosophila NRRL Y-17539\(^{\text{T}}\) in terms of the ability to assimilate myo-inositol and to grow in the presence of 0.1 % cycloheximide, as well as the inability to assimilate citrate. The name Spathaspora allomyrinae sp. nov. is proposed for this species. The type strain is NYNU 1495\(^{\text{T}}\) (\(=\)CICC 33057\(^{\text{T}}\)=CBS 13924\(^{\text{T}}\)). The MycoBank number is MB 815071.

The genus Spathaspora, which belongs to the family Debaromyoscaceae, was proposed by Nguyen et al. (2006) to accommodate the single species, Spathaspora passalidarum, which produces elongated ascospores with curved ends. Species described later in the genus Spathaspora include Spathaspora arborariae (Cadete et al., 2009), Spathaspora brasilienis, Spathaspora roxainianensis, Spathaspora suhii and Spathaspora xylofermentans (Cadete et al., 2013). Five anamorphic Candida species, namely Candida jeffriesii, Candida lyxosophila, Candida materiae, Candida subhashii and Candida xylanilytica, are also related to this genus as indicated by phylogenetic analyses of the D1/D2 domains of the large subunit (LSU) rRNA gene (Boonmak et al., 2011; Cadete et al., 2013). Species of the Spathaspora clade are associated with rotting wood or insects linked with this substrate (Boonmak et al., 2011; Cadete et al., 2009, 2013; Nguyen et al., 2011). Most species of the clade, such as C. jeffriesii, C. lyxosophila, Spathaspora arborariae, Spathaspora brasiliensis, Spathaspora passalidarum, Spathaspora roxainianensis, Spathaspora suhii and Spathaspora xylofermentans, are known for their ability to ferment D-xylose. These xylose-fermenting species can be used directly for ethanol production, or may provide a source of genes, enzymes and/or sugar transporters to engineer industrial strains for the efficient production of bioethanol from renewable biomass (Hahn-Hägerdal et al., 2007; Wohlbach et al., 2011).

During an investigation of yeasts associated with insects in the Baotianman National Nature Reserve in China, we isolated three strains of a D-xylose-fermenting yeast species belonging to the Spathaspora clade. The strains formed ascii without conjugation and asci containing two elongated ascospores. Sequence analyses of the D1/D2 LSU and small subunit (SSU) rRNA genes showed that these strains represented a single species closely related to C. lyxosophila.

Sixteen adult insects of Allomyrina dichotoma were collected from the Baotianman National Nature Reserve near Nanyang (approximate GPS coordinates: 33° 27’ N 111° 48’ E), which experiences a typical transitional climate from the northern subtropical zone to the warm temperate zone in central China. The strains belonging to the novel species in the current study, namely NYNU 1495\(^{\text{T}}\), NYNU 1497 and NYNU 15835, were isolated from the gut of three individuals of Allomyrina dichotoma in September 2014 and August 2015. The methods for yeast isolation were detailed by Nguyen et al. (2006) and Urbina et al. (2013). The insects were placed in...
Petri dishes for 1–3 days without food prior to dissection; withholding food helps eliminate some contaminating organisms that may be isolated from the gut. Each insect individual was surface disinfected by washing in 70 % ethanol (5 min), 5 % bleach (5 min) and sterile water (10 min) prior to dissection. The aseptically removed gut contents and saline wash solution were plated separately on acidified yeast extract-malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose and 2 % agar; adjusted to pH 3.5 with HCl) and incubated at 25 °C for 3–4 days. 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.

Morphological observations and metabolic tests representing the standard yeast description were conducted as previously reported (Kurtzman et al., 2011). Assimilation tests for carbon and nitrogen sources were performed in liquid media. Starved inocula were used in nitrogen assimilation tests. Strains were examined for ascosporulation on the following agar media: YM, 1 % malt extract, 5 % malt extract, corn meal and yeast base supplemented with 0.01 % ammonium sulphate (YCBS; 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 Dr GenTLE (from Yeast) High Recovery (Takara Bio) in accordance with the manufacturer’s protocol. The SSU, ITS and D1/D2 LSU rRNA genes were amplified by PCR and sequenced using the primer pairs P1 and NS8 (Suzuki & Nakase, 2002), ITS1 and ITS4 (White et al., 1990), and NL1 and NL4 (Kurtzman & Robnett, 1998), respectively. PCR conditions recommended in the references for each primer pair were adopted. Both DNA strands were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems).

The obtained sequences were compared pairwise via BLAST search (Altschul et al., 1997) and aligned with the sequences of related species retrieved from the GenBank database by using the multiple alignment program CLUSTAL X version 1.81 (Thompson et al., 1997). A phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene sequences was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA 5.0 software package (Tamura et al., 2011). The evolutionary distances were calculated from the two-parameter model of Kimura (1980) for the neighbour-joining analyses. Saccharomyces cerevisiae NRRL Y-12632T was used as an outgroup. The confidence levels of neighbour-joining analyses from the two-parameter model of Kimura (1980) for the D1/D2 LSU rRNA genes, indicating that these strains are conspecific. A BLAST search of the GenBank database with D1/D2 sequences showed that these new strains belong to the Spathaspora clade, and C. lyxosophila was identified as their closest relative. The D1/D2 sequences of the three isolates differed from those of C. Lyxosophila NRRL Y-17539 by a 6.7 % sequence divergence (31 substitutions and 7 gaps). The novel yeasts also differed from other close species in the Spathaspora clade by more than 7.5 % sequence divergences (17 substitutions and 4 gaps) in the D1/D2 LSU rRNA gene. The SSU rRNA gene sequences of these strains showed a 1.2 % divergence (17 substitutions, 4 gaps) from those of C. Lyxosophila NRRL Y-17539. Unfortunately, the ITS sequences of the three strains could not be successfully aligned with the type strain of C. lyxosophila, which contained ITS sequences not currently available from either the NCBI GenBank database or the CBS database. DNA sequence comparisons indicated that the three strains represent a novel species of the Spathaspora clade.

A phylogenetic tree was reconstructed by the neighbour-joining method based on the D1/D2 LSU rRNA gene sequences as defined by Cadete et al. (2013). The results showed that the genus Spathaspora is not monophyletic, but includes at least two phylogenetically distinct suclades on the tree (Fig. 1). The novel species formed an independent lineage in the Spathaspora roraimanensis suclade. The position of this species within the Spathaspora clade was unclear because of the low bootstrap value. A further study on multi-locus phylogenies with an extended taxon in the vicinity is needed in order to clarify the phylogenetic position of these isolates.

All strains of the novel species comprised globose cells, which proliferated by multilateral budding (Fig. 2a) and formed pseudohyphae (Fig. 2b). These cells also produced elongated ascospores with tapered and curved ends in persistent asci (Fig. 2c), and furthermore, the cells fermented D-xylene effectively in Durham tubes but did not assimilate nitrate. These phenotypic characteristics are known from Spathaspora passalidarum, the type species of the genus Spathaspora (Nguyen et al., 2006, 2011). However, there are a few morphological differences between the novel species and other species of the genus Spathaspora, such as Spathaspora arborariae, Spathaspora brasiliensis, Spathaspora passalidarum, Spathaspora roraimanensis and Spathaspora suhii. The novel species formed two ascospores surrounded by a rather circular membrane enclosing the two spores in an ascus (Fig. 2c), whereas other species of the genus Spathaspora produced one ascospore with a membrane running around the long axis of the spore (Cadete et al., 2009, 2013; Nguyen et al., 2006). Furthermore, the size of the ascospores of the novel species look quite smaller (or shorter) than those of other species of the genus Spathaspora. The discovery of this novel species showed that the size, number and membrane-shape of ascospores in an ascus formed by species of the genus Spathaspora may be different. On the basis of the aforementioned data, we concluded that the three strains

Species delineation, classification and ecology

The three strains, NYNU 1495T, NYNU 1497 and NYNU 15835, contained identical sequences in the SSU, ITS and D1/D2 LSU rRNA genes, indicating that these strains
Fig. 1. Phylogenetic tree derived from neighbour-joining analysis based on the D1/D2 domains of the LSU rRNA gene sequences, showing the placement of Spathaspora allomyrinae sp. nov. and other relevant species. Saccharomyces cerevisiae NRRL Y-12632T was used as an outgroup. Numbers at nodes are bootstrap values based on 1000 replications; only values >50% are shown. Bar, 2% sequence difference.
represents a single novel species of the genus *Spathaspora*, for which the name *Spathaspora allomyrinae* sp. nov. is proposed, with isolate NYNU 1495^T^ as the type strain. *Spathaspora allomyrinae* sp. nov. can be differentiated from the closest described species, *C. lyxosophila* (Lachance et al., 2011), with respect to the ability to assimilate myo-inositol and to grow in the presence of 0.1 % cycloheximide, as well as the inability to assimilate citrate. The differentiation of *Spathaspora allomyrinae* sp. nov. from *C. lyxosophila* based on phenotypic characteristics alone is difficult to some extent as these species exhibit nearly identical growth profiles. Therefore, sequencing of the D1/D2 domains of the LSU rRNA gene is recommended to differentiate these species.

The rhinoceros beetle *Allomyrina dichotoma*, which is known for its single powerful horn, is a common large beetle in the Baotianman National Nature Reserve. The adults of this beetle generally feed on tree saps and ripe fruits, and their larva eat roots or rotting plant materials. Several yeast species, such as *Candida maltosa*, *Candida boleti*, *Trichosporon moniliforme* and *Wickerhamiella allomyrinae*, have been isolated from the gut of rhinoceros beetles (Ren et al., 2014). Of the 78 yeast strains isolated in this study, *Ambrosiozyma angenhorae*, *C. maltosa*, *Candida cetoniae*, *Kluyveromyces dobzhanski*, *Lachancea thermotolerans* and *Saccharomyces cerevisiae* were the most frequently isolated species from rhinoceros beetles. By comparison, only three isolates of *Spathaspora allomyrinae* sp. nov. were found from sixteen samples of the rhinoceros beetle, suggesting *Spathaspora allomyrinae* sp. nov. is not a common associate of *A. dichotoma*. The more usual habitat of these yeasts may be the beetle environment (i.e. gallery walls or decayed wood) rather than the gut from which they could be ingested occasionally. More collecting effort should be directed towards finding the yeasts in decayed wood.

**Description of Spathaspora allomyrinae Hui, Wang, Ren, Zhang & Ke sp. nov.**

*Spathaspora allomyrinae* (al.o.my.r'i.nae. N.L. fem. gen. n. allomyrinae referring to the genus of the host beetle, *Allomyrina dichotoma*).

In YM broth after 3 days at 25 °C, cells are globose with variable size (3–7 × 3–7 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 25 °C, colonies are raised, white and smooth with an entire edge. After 2 weeks in Dalmau plate culture on corn meal agar at 25 °C, pseudohyphae are formed but true hyphae are not formed. (Fig. 2b). Aerobic growth is white, shiny and smooth with filamentous margin. Sporulation occurs on corn meal and YCBAS agars after 7 days at 25 °C. Unconjugated asci are formed from single cells with two elongated ascospores (1.5–3 × 7–11.5 μm) which are tapered and curved at the ends (Fig. 2c). Asci are not dehiscent. Glucose, galactose, maltose, cellubiose and xylose are weakly fermented, but not sucrose, lactose, raffinose, trehalose, methyl-α-D-glucoside, melibiose, melezitose, inulin or soluble starch. Glucose, sucrose, galactose, trehalose, maltose, melezitose, methyl-α-D-glucoside, soluble starch, cellubiose, salicin, D-xylene, ethanol, glycerol, ribitol, mannitol, glucitol, myo-inositol, D-lactate, succinate, D-glucosamine, arbutin, 2-keto-D-gluconate and 5-keto-D-gluconate are assimilated. No growth occurs in inulin, raffinose, melibiose, lactose, L-sorbose, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methyl, erythritol, galactitol, citrate, D-glucuronate, xylitol, L-arabinol, D-glucuronate or D-glucono-1,5-lactone. In tests for the assimilation of nitrogen compounds, ethylamine, L-lysine, cadaverine and D-tryptophan are positive, whereas nitrate, nitrite, creatine, creatinine, glucosamine and imidazole are negative. Growth is observed at 35 °C but not at 37 °C. Growth in the presence of 0.1 % cycloheximide is positive, but growth in the presence of 10 % NaCl plus 5 % glucose.

![Photomicrographs of *Spathaspora allomyrinae* sp. nov. NYNU 1495T.](image-url)


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References


and in 1 % acetic acid is negative. Starch-like compounds are not produced. Urease activity and diazonium blue B reactions are also negative.

The type strain, NYNU 1495T (=CICC 33057T=CBS 13924T), was isolated from the gut of Allomyrina dichotoma collected in the Baotianman National Nature Reserve in Nanyang, Henan Province, China. The living culture from this type strain is maintained in lyophilized state in the China Center of Industrial Culture Collection (CICC), Beijing, China and in the yeast collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. The MycoBank number is MB 815071.